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Agonists for the Adenosine A_1 Receptor with Tunable Residence Time. A Case for Nonribose 4-Amino-6-aryl-5-cyano-2-thiopyrimidines

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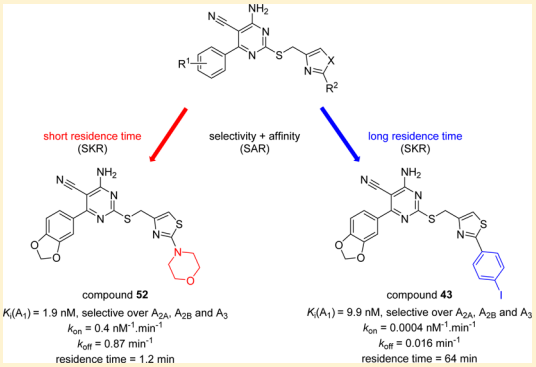
Supporting Information

ABSTRACT: We report the synthesis and evaluation of previously unreported 4-amino-6-aryl-5-cyano-2-thiopyrimidines as selective human adenosine A_1 receptor (hA_1AR) agonists with tunable binding kinetics, this without affecting their nanomolar affinity for the target receptor. They show a very diverse range of kinetic profiles (from 1 min (compound **52**) to 1 h (compound **43**)), and their structure–affinity relationships (SAR) and structure–kinetics relationships (SKR) were established. When put in perspective with the increasing importance of binding kinetics in drug discovery, these results bring new evidence of the consequences of affinity-only driven selection of drug candidates, that is, the potential elimination of slightly less active compounds that may display preferable binding kinetics.



INTRODUCTION

The adenosine receptors are members of the G protein-coupled receptor superfamily of cell membrane-bound proteins (GPCRs). The physiological roles of these receptors have been extensively reviewed.[1] They can be subdivided into four subtypes (A_1, A_{2A}, A_{2B}, and A_3), all of which accept adenosine as an endogenous ligand. Over the past two decades, many synthetic ligands have been developed that selectively bind to a determined number of receptor subtypes along with different functional profiles: partial or full agonists and antagonists/inverse agonists. Until the early 2000s, all agonists included a ribose sugar, and any attempt to remove this moiety yielded antagonists at best. Since then, research from Bayer and our group has unveiled the existence of 2-aminopyridine-3,5-dicarbonitrile derivatives such as Capadenoson and **1** (LUF5834) as nonribose agonists for the A_1 adenosine receptor (A_1AR) and the $A_{2B}AR$.[2,3] In the meantime, Otsuka Pharmaceuticals described a series of 4-amino-6-aryl-5-cyano-2-thiopyrimidines as selective agonists for the $A_{2A}AR$ (Figure 1).[4]

Despite the plethora of ligands active in vitro that have been synthesized for the adenosine receptors, very few of them have reached the market yet because most failed to pass the clinical trials, either because they show severe side-effects or because of their lack of in vivo efficacy. This latter issue seems to arise from the fact that all of the drug candidates have been selected for their in vitro affinity and/or potency. Over the years, it has been shown, however, that these parameters do not necessarily correlate with in vivo efficacy.[5] Another parameter has been recently proposed to be a possibly more accurate representation of in vivo behavior, which has been coined residence time (RT).[6] It is defined as the reciprocal of the ligand's dissociation rate constant ($RT = 1/k_{off}$) and represents the lifetime of the ligand–receptor complex.[7] Its relevance to in vivo conditions was shown by retrospectively analyzing many marketed GPCR drugs that have improved in vivo efficacies, exemplified by several long-acting antagonists, tiotropium on the muscarinic M_3 receptor or candesartan on the angiotensin II AT_1 receptor.[8]

In this Article, we report the synthesis and evaluation of 4-amino-6-aryl-5-cyano-2-thiopyrimidines as (selective) A_1AR agonists. These compounds are hybrids between the pyrimidine scaffold present in the Otsuka compounds and the methylene-heterocyclic substituent present in the Bayer/LUF compounds. They were evaluated in a radioligand displacement binding assay and a competition association assay. The compounds are selective over the other AR subtypes and show high affinities and a very diverse range of kinetic profiles at the hA_1AR, and their structure–kinetics relationships (SKR) were established. Additionally, they were characterized in a functional assay as A_1 receptor agonists.

RESULTS AND DISCUSSION

Design and Synthesis. The S-substituted thiopyrimidines were all synthesized in a two-step procedure consisting of (i)

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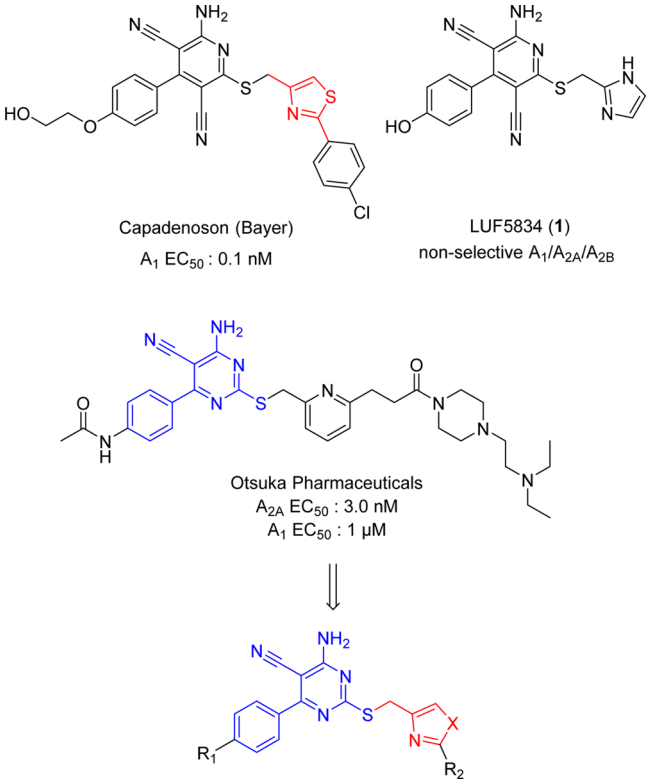
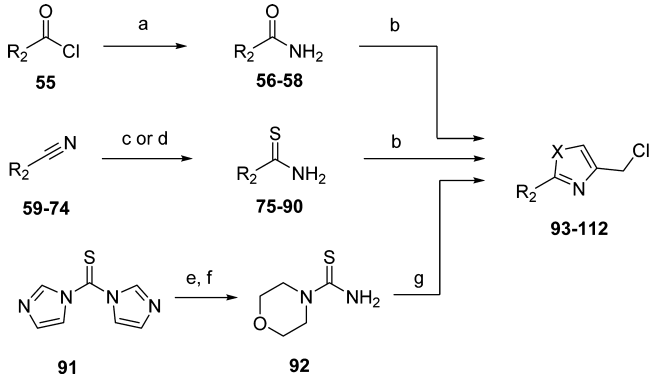


Figure 1. Examples of selective nonribose agonists for A_1AR (top left), A_1AR/A_{2A}AR/A_{2B}AR (top right), A_{2A}AR (middle), and chosen pharmacophore for this study (bottom).

the formation of the thiopyrimidine by the three-component reaction between malononitrile, thiourea, and various substituted benzaldehydes in the presence of K_2CO_3 at the reflux of ethanol,[9] and (ii) the alkylation of the obtained thiol with various alkyl chlorides in the presence of Na_2CO_3 at 50 °C in DMF (Scheme 1).[3a]

The alkyl chlorides that were not commercially available were synthesized in three different ways, albeit with a similar final step. Oxazole-containing compounds were obtained from primary amides, thiazole-containing compounds from primary thioamides, in both cases reacted with 1,3-dichloroacetone at the reflux of toluene,[10] or ethanol.[11] Noncommercially available amides were synthesized from the corresponding acyl chlorides by treatment with aqueous ammonia;[12] similarly, thioamides were obtained by treatment of the corresponding nitrile upon reaction with ammonium sulfide in pyridine/Et_3N.[13] In the case of a 4-nitro-substituted benzonitrile, the desired product was not obtained, and another procedure was thus used, with phosphorus pentasulfide in ethanol.[14] The morpholine-containing amide was obtained in a one-pot, two-step procedure from thiocarbonyldiimidazole, which was treated successively with morpholine in THF and ammonia (Scheme 2).[15]

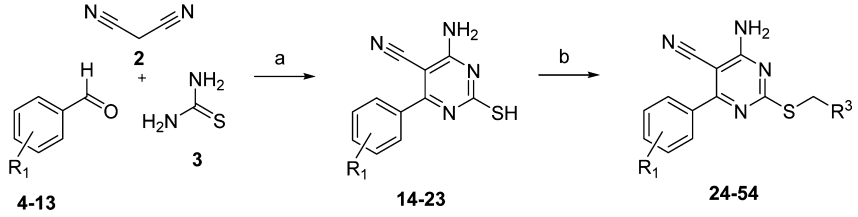
Scheme 2. Synthesis of Alkylating Agents 93−112[a]



[a]Reagents and conditions: (a) $NH_{3(aq)}$, EtOAC, 0 °C, 81%; (b) 1,3-dichloroacetone, toluene, reflux, 16−97%; (c) $(NH_4)_2S$, Et_3N, pyridine, 50 °C, 57−100%; (d) P_4S_{10}, EtOH, rt to reflux, 19%; (e) morpholine, THF, rt to 55 °C; (f) NH_3, THF, rt to 55 °C, 65% (two steps); (g) 1,3-dichloroacetone, EtOH, reflux, 44%.

Biology. All of the compounds were initially tested in a single-point radioligand binding assay in the presence of 2.5 nM **113** ([^{3}H]DPCPX) (Figure 2),[16] on the adenosine A_1 receptor. Because we were interested in long residence time compounds with high A_1 receptor affinity, we only selected those derivatives displacing more than 80% of the total radioligand binding at 1 μM (yielding an estimated K_i value of at least 300 nM), which were subsequently evaluated in full-range concentration-dependent displacement assays (Figure 3). All compounds, except **27**, **29−32**, and **45**, displayed a high affinity for the human A_1AR (K_i < 100 nM). Subsequently, compounds with high A_1AR affinity were screened in a so-called dual-point competition association assay in the presence of **113** (RT = 4.8 min), which we recently developed in our lab for fast and high-throughput kinetics screening.[17] In brief, we obtained specific **113** binding in the presence of an unlabeled compound of interest at two time points and calculated their ratio at the earlier and later time points, respectively, to yield the kinetic rate index (KRI); a KRI value above 1.0 indicates a relative slow dissociation from the target, while a value below 1.0 predicts a relatively fast dissociation rate as compared to the dissociation rate of the radioligand. Such a measure enables a quick qualitative estimation of the binding kinetics of unlabeled ligands screened. In the present study, those compounds with a KRI value above 1.2 were then subjected to a follow-up full competition association assay (Figure 4) for quantitative

Scheme 1. Synthetic Route toward S-Substituted 4-Amino-6-aryl-5-cyano-2-thiopyrimidines 24−54[a]



[a]Reagents and conditions: (a) K_2CO_3, EtOH, reflux, 1−42%; (b) R^3CH_2Cl, Na_2CO_3, DMF, 50 °C, 7−61%.

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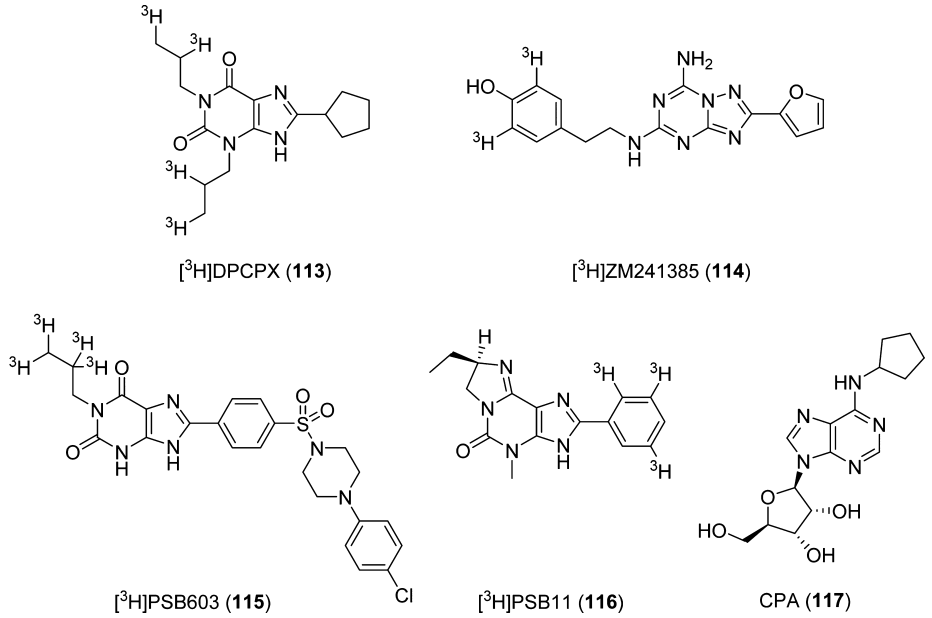

Figure 2. Structures of the radioligands and reference compounds used in this study.

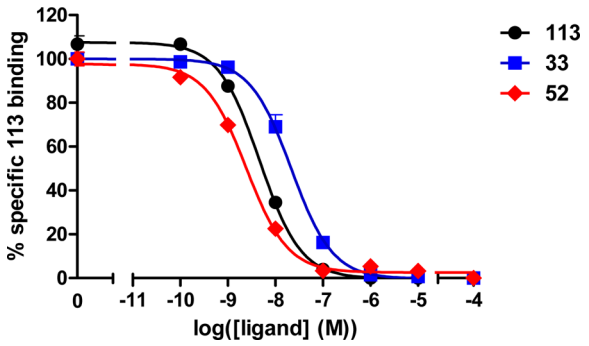

Figure 3. 113 displacement curves on the hA_1AR for unlabeled **113**, **33**, and **52** in CHO cell membranes stably expressing human adenosine A_1 receptors. The curves were obtained by coincubation of 2.5 nM **113** and different concentrations of the unlabeled ligands.

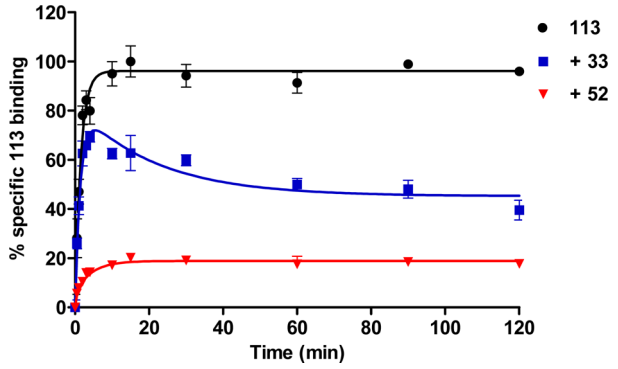

Figure 4. Curves of competition association assay of **33**, **52** and association curve of **113** in CHO cell membranes stably expressing human adenosine A_1 receptors. The curves were obtained by coincubation of 2.5 nM **113** and a 10 × K_i concentration of the unlabeled ligands.

measurement of their kinetic parameters (k_{on}, k_{off}) and thus deduce their association and dissociation characteristics and residence time (RT) (see ref 17 and Experimental Section). We decided to focus on compounds with a longer residence time because phase IIa clinical trials for Capadenoson for the treatment of atrial fibrillation lacked efficacy.[18] Logically, aiming for compounds with slower dissociation could favor in vivo efficacy. Capadenoson, for reference, had a KRI value of 1.42 (RT = 28 min).[19] To establish a complete structure–kinetic relationship study of this series of compounds, we also specifically selected one representative compound with a high affinity but the lowest KRI value (**52**, KRI = 0.81) and subjected it to a competition association assay too.

Structure–Affinity Relationships and Structure–Kinetics Relationships. *1. Aromatic Substituent.* Keeping the 4-chlorophenylthiazole substituent present in Capadenoson, the influence of the substituent on the phenyl ring adjacent to the pyrimidine was studied. Binding and kinetic data at the A_1AR are gathered in Table 1, and selectivity data are in Table 3.

1.1. Structure–Affinity Relationships. The compound selected as a starting point was **24** (R^1 = $O(CH_2)_2OH$, R^3 = 4-Cl-Ph), similar to Capadenoson. This compound displayed an affinity of 4.8 nM at the hA_1AR and negligible radioligand displacement at other adenosine receptor subtypes (10% of **114** ([^{3}H]ZM241385)[20] at $hA_{2A}AR$, 3.5% of **115** ([^{3}H]PSB603)[21] at $hA_{2B}AR$, and 3% of **116** ([^{3}H]PSB11)[22] at hA_3AR at 1 μM (Figure 2)), thus showing high selectivity for hA_1AR. Replacing the R_1 moiety of **24** with a 4-acetamido group, which mimics the substitution in the Otsuka compound at the same position, offered a good A_1AR affinity (**25**, 14 nM), while selectivity over the $hA_{2A}AR$ decreased somewhat (39% at 1 μM). Interestingly, this finding, taken together with the fact that the Otsuka compounds showed selectivity at the $hA_{2A}AR$,[4] indicates that the acetamide group on the phenyl ring is responsible for the affinity at the $hA_{2A}AR$, whereas selectivity for the $hA_{2A}AR$ over hA_1AR is achieved through the substituent at the sulfur atom (Figure 1). Removing the R_1 substituent did not change the affinity at the A_1AR (**26**, 8.9 nM), similar to the effect of cleaving the ethanol moiety of **24** (4-OH, **28**, 15 nM).[23] In comparison, introducing a 4-methoxy group (**27**) or 4-ethoxy group (**29**) led to a great decrease of A_1AR affinity (**27**, 66% displacement at 1 μM; **29**, 50% displacement at 1 μM).

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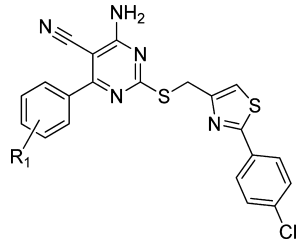
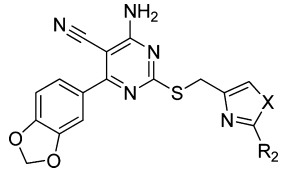
Table 1. Affinity and Kinetic Parameters of Compounds 24−33 on the Human A_1AR



	R^1	K_i (nM) ± SEM or disp. at 1 μM (%)[a]	KRI[b]	k_{on} (M^{-1} min^{-1})[d]	k_{off} (min^{-1})[e] (RT (min))[f]
24	4-O$(CH_2)_2$OH	4.8 ± 0.1	1.18 ± 0.09		
25	4-NHAc	14 ± 0.5	0.97 ± 0.09		
26	H	8.9 ± 2.1	1.24 (1.39, 1.09)	(2.0 ± 1.2) × 10^5	0.031 ± 0.017 (32.3)
27	4-OMe	73% (80, 66)	–[c]		
28	4-OH	15 ± 2.4	0.99 ± 0.10		
29	4-OEt	49% (50, 48)			
30	4-OCF_3	185 ± 66			
31	4-NMe_2	233 (235, 232)			
32	4-Me	44% (46, 43)			
33	3,4-OCH_2O	14 ± 6	1.64 (1.75, 1.53)	(3.0 ± 1.3) × 10^6	0.019 ± 0.004 (51.4)

[a]K_i ± SEM (n = 3) or % displacement (n_1, n_2) (n = 2), obtained from radioligand binding assays with **113** on CHO cell membranes stably expressing human adenosine A_1 receptors. [b]KRI ± SEM (n = 3) or KRI (n_1, n_2) (n = 2), obtained from dual-point competition association assays with **113** on CHO cell membranes stably expressing human adenosine A_1 receptors. [c]Not determined. [d]k_{on} ± SEM (n = 3), obtained from competition association assays with **113** on CHO cell membranes stably expressing human adenosine A_1 receptors. [e]k_{off} ± SEM (n = 3), obtained from competition association assays with **113** on CHO cell membranes stably expressing human adenosine A_1 receptors. [f]RT = $1/k_{off}$.

Table 2. SAR and SKR of Compounds 34−54



	R^2	X	K_i (nM) ± SEM or disp. at 1 μM (%)[a]	KRI[b]	k_{on} (M^{-1} min^{-1})[d]	k_{off} (min^{-1})[e] (RT (min))[f]
34	4-Cl-Ph	O	15 ± 2	1.17 (1.20, 1.14)		
35	4-F-Ph	O	20 ± 8.6	1.01 (1.10, 0.92)		
36	3-F-Ph	O	15 ± 4	1.24 (0.94, 1.54)	(6.1 ± 2.2) × 10^6	0.328 ± 0.050 (3.05)
37	4-F-Ph	S	6.7 ± 1.4	1.22 (1.33, 1.11)	(1.7 ± 0.30) × 10^6	0.029 ± 0.005 (34.5)
38	3-F-Ph	S	14 ± 1	1.24 (1.11, 1.37)	(5.1 ± 1.3) × 10^5	0.030 ± 0.008 (33.0)
39	4-Me-Ph	S	3.1 ± 0.7	1.15 ± 0.07		
40	4-OMe-Ph	S	1.8 ± 0.2	1.00 ± 0.06		
41	3,4-OCH_2O-Ph	S	2.5 ± 0.1	0.93 (0.96, 0.90)		
42	4-Br-Ph	S	5.2 ± 0.3	1.67 ± 0.20	(3.3 ± 1.2) × 10^5	0.017 ± 0.003 (59.0)
43	4-I-Ph	S	9.9 ± 1.0	1.40 ± 0.17	(4.3 ± 0.87) × 10^5	0.016 ± 0.004 (63.8)
44	3-Cl-Ph	S	42 ± 10	1.24 ± 0.05	(1.8 ± 0.30) × 10^5	0.034 ± 0.007 (30.0)
45	3,4-diCl-Ph	S	23% (25, 20)	–[c]		
46	2-Cl-Ph	S	75 ± 4	1.29 (1.25, 1.33)	(2.6 ± 0.7) × 10^5	0.028 ± 0.010 (35.3)
47	2-F-Ph	S	12 ± 2	1.46 (1.76, 1.16)	(8.2 ± 0.4) × 10^5	0.044 ± 0.001 (22.5)
48	4-CF_3-Ph	S	18 ± 2.3	1.20 (1.07, 1.33)		
49	4-NO_2-Ph	S	11 ± 2.7	0.95 ± 0.09		
50	Ph	S	2.4 ± 0.7	1.10 (1.04, 1.16)		
51	*c*Hex	S	6.3 ± 1.2	1.17 (1.30, 1.04)		
52	morpholino	S	1.9 ± 0.4	0.81 (0.64, 0.98)	(3.9 ± 1.2) × 10^8	0.870 ± 0.060 (1.2)
53	H	S	9.7 ± 1.7	0.93 (1.11, 0.75)		
54	H	NH	83 ± 10	1.15 (0.96, 1.34)		

[a]K_i ± SEM (n = 3), % displacement (n_1, n_2) (n = 2), obtained from radioligand binding assays with **113** on CHO cell membranes stably expressing human adenosine A_1 receptors. [b]KRI ± SEM (n = 3) or KRI (n_1, n_2) (n = 2), obtained from dual-point competition association assay with **113** on CHO cell membranes stably expressing human adenosine A_1 receptors. [c]Not determined. [d]k_{on} ± SEM (n = 3), obtained from competition association assay with **113** on CHO cell membranes stably expressing human adenosine A_1 receptors. [e]k_{off} ± SEM (n = 3), obtained from competition association assay with **113** on CHO cell membranes stably expressing human adenosine A_1 receptors. [f]RT = $1/k_{off}$.

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Table 3. Selectivity Data and Functional Profile of Selected Compounds.

	displ. at A_{2A}AR at 1 μM (%)[a]	displ. at A_{2B}AR at 1 μM (%)[c]	displ. at A_3AR at 1 μM (%)[d]	E_{max} (%)[f]	EC_{50} (nM)[h]
24	10 (15, 5)	3.5 (2.0, 5.0)	3.0 (0.0, 6.0)	99 ± 3.7	12 ± 0.5
25	44 ± 5.6[b]	2.5 (4.0, 1.0)	5.5 (−1.0, 10)	112 ± 2.9	3.9 ± 0.2
33	4.5 (3.3, 5.7)	0.0 (0.0, 0.0)	4.5 (18, −9.0)	71 ± 3.3	4.9 ± 0.9
40	3.2 (10, −3.8)	3.5 (0.1, 7.0)	11 (7.5, 15)	104 ± 0.45	1.90 ± 0.26
42	2.9 (2.3, 3.5)	1.7 (0.0, 3.4)	−1.8 (−4.0, 0.40)	60 ± 4.5	4.6 ± 0.6
43	2.5 (4.0, 1.0)	7.5 (2.0, 13)	9 (8.0, 10)	82 ± 3.0	9.5 ± 1.0
50	−2.0 (−2.0, −2.0)	20 (16, 23)	24 ± 6.2[e]	84 ± 1.8	4.6 ± 0.2
52	21 (19, 24)	12 (3.6, 20)	18 (11, 25)	65 ± 6.9	1.8 ± 0.4
53	31 (31, 31)	42 ± 6.1 nM	35 (28, 41)	27 (20, 34)[g]	–[i]

[a]% displacement at 1 μM concentrations of specific **114** binding on HEK 293 cell membranes stably expressing human adenosine A_{2A} receptors. [b]n = 5. [c]% displacement at 1 μM concentrations of specific **115** binding on CHO-spap cell membranes stably expressing human adenosine A_{2B} receptors or affinity (cpd **53**). [d]% displacement at 1 μM concentrations of specific **116** binding on CHO cell membranes stably expressing human adenosine A_3 receptors. [e]n = 4. [f]Maximum specific binding of [^{35}S]GTPγS in CHO cell membranes expressing human adenosine A_1 receptors, with 0% being the binding in the absence of any ligand (buffer) and 100% being the reference agonist **117** (n = 3). [g]Determined at a concentration of 100 × K_i (n = 2). [h]Concentration causing half-maximal stimulation of specific binding of [^{35}S]GTPγS in CHO cell membranes expressing human adenosine A_1 receptors (n = 3). [i]Not determined.

Increasing the electronegativity and lipophilicity of the methoxy group by using a trifluoromethoxy substituent resulted in a better A_1AR affinity as compared to **27**, albeit still relatively low (**30**, 185 nM). On the other hand, introducing an alkyl substituent led to a near complete loss of affinity (4-Me, **32**, 44% at 1 μM). A strong electron-donating dimethylamino group gave a relatively modest affinity (**31**, 233 nM). Finally, a 3,4-methylenedioxy substituent gave a good affinity (**33**, 14 nM), with a high selectivity over the other receptor subtypes.

1.2. Structure–Kinetics Relationships. Out of the five compounds displaying a K_i value below 100 nM, only compounds **26** and **33** had a KRI value above 1.20 (1.24 and 1.64, respectively) with associated A_1AR residence times of 32.3 and 51.4 min, respectively. However, **26** had an association rate 15 times lower than its 3,4-methylenedioxy analogue **33** (k_{on} = 2.0 × 10^5 vs 3.0 × 10^6 M^{-1} min^{-1}). These results highlight the crucial role of the methylenedioxy substituent because it is essential for both obtaining high A_1AR affinity and achieving faster association and slower dissociation kinetics. The remaining three compounds (**24**, **25**, and **28**) showed low KRI values, which could be due to their pronounced hydrophilicity/low lipophilicity (−0.8 < π < −0.6). [24a] Worthy of note is that the compound with the best affinity (**24**, analogue of Capadenoson) showed a shorter residence time than **26** and **33**, indicated by its KRI value of 1.18, yet in a "traditional" hit or lead selection process, only compound **24** would most likely have been prioritized, thus overlooking compound **33**, which still displays an acceptable affinity but with slower dissociation kinetics.

2. Heterocycle Substituent. Building on the results obtained so far, and keeping the 3,4-methylenedioxy substituent, the influence of the substituent present on the 2-position of the thiazole was then studied. Binding and kinetic data at the A_1AR are gathered in Table 2, and selectivity data are in Table 3.

2.1. Structure–Affinity Relationships. Starting with an unsubstituted phenyl group at the R_2 position, we learned that **50** displayed a high A_1AR affinity (2.4 nM) and selectivity over the other receptor subtypes. Similar results were obtained with replacement of the phenyl ring with cyclohexyl (**51**, 6.3 nM) or morpholine (**52**, 1.9 nM). The latter compound proved to have one of the highest affinities at the A_1AR in our study, and was selective over the other three receptor subtypes (21% at hA_{2A}AR, 12% at hA_{2B}AR, and 18% at hA_3AR).

The substitution pattern of the phenyl ring was then studied. An increase of lipophilicity and size in the halogen series (F, **37**; Cl, **33**; Br, **42**; I, **43**) did not lead to any significant affinity change (while retaining hA_1AR selectivity), and neither did an increase in electron-withdrawing character (CF_3, **48**; NO_2, **49**). Moving the halogen substituent along the ring led to two distinct cases: for a chloro- substituent, the K_i increased following the para, meta, ortho order (**33**, 14 nM; **44**, 42 nM; **46**, 75 nM); for a fluoro- substituent, the order was para, meta/ortho (**37**, 6.7 nM; **38**, 14 nM; **47**, 12 nM). To explain such a difference, we assumed that in the ortho position, a smaller fluorine atom might have a smaller effect on the conformation of the thiazole–phenyl system, whereas a bigger chlorine atom might lead to a rotation of this system, thereby modifying the affinity.[25] Dichlorination on the meta and para positions led to a significant loss of affinity at the hA_1AR (**45**, 23% displacement at 1 μM). Furthermore, introducing lipophilic and/or electron-donating substituents in the para position led to better affinities (3.1 nM for R^2 = 4-Me (**39**), 1.8 nM for R^2 = 4-OMe (**40**), 2.5 nM for R^2 = 3,4-OCH_2O (**41**)). **40** was actually the most potent compound, next to **52**. Besides the abovementioned chemical modification, we also examined the role of the connecting heterocyclic ring. Clearly, this part is very tolerant upon chemical modifications in terms of their A_1AR affinities. As evidence, changing the thiazole into an oxazole did not lead to a significant change in affinity, with **34**, **35**, and **36** having K_i values of 15, 20, and 15 nM, respectively. This holds true even after removing the substituent on the heterocyclic ring (**53**, 9.7 nM). Remarkably, this ligand displayed an affinity of 41 nM at the hA_{2B}AR, while being selective over the two other subtypes. Exchanging the 4-thiazole substituent for a 4-imidazolyl substituent gave a K_i value of 83 nM (**54**) at hA_1AR while maintaining selectivity over the hA_{2A}R. In the end, in terms of affinity, adding a phenyl substituent on the thiazole (**53** vs **50**) did not increase the affinity much, probably because the aromatic ring further fills the binding pocket in a somewhat loose manner. However, when substituents were added onto this ring, their nature and position significantly impacted the affinity at the hA_1AR. This might be due to electronic or steric interactions with residues inside the binding site or to a modification of the conformation of the ligand, particularly in the case of the ortho chloro substituent.

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2.2. Structure–Kinetics Relationships. Starting with the above-mentioned unsubstituted compounds, it appears that changing from a 4-thiazolyl to a 4-imidazolyl substituent led to a slight increase in residence time (**54**, KRI = 1.15 vs **53**, KRI = 0.93), albeit still similar to that of **113**.

Introduction of a halogen substituent on the phenyl ring had a dramatic effect on the ligand's receptor residence time. Indeed, *para*- fluoro (**37**)-, chloro (**33**)-, bromo (**42**)-, and iodo (**43**)-substituted compounds had KRI values all above 1.20, and their corresponding A_1AR residence times ranged from 34.5 min (**37**) to 63.8 min (**43**), whereas the unsubstituted **30** had a KRI of 1.10. For chloro-substituted analogues, the residence times increased following the meta ≈ ortho < para order (**44**, 30 min; **46**, 35.3 min; and **33**, 51.4 min, respectively). In the case of fluorinated compounds, the order was ortho < meta ≈ para (**47**, 22.5 min; **38**, 33.0 min; and **37**, 34.5 min, respectively). More specifically for the para-substituted compounds, a significantly increased receptor residence time was observed from fluoro to chloro, that is, from 34.5 min (**37**) to 51.4 min (**33**), and a less significant increase through bromo (**42**, 59.0 min) and to iodo (**43**, 63.8 min). To explain this result, we looked into the physicochemical properties of these substituents. It is notable that from fluoro to chloro there is an increase in both electronegativity/polarity (σ) as well as lipophilicity (π) and size,[24] whereas the chloro, bromo, iodo progression only sees an increase in lipophilicity and size. This suggests that, in this case, lipophilicity does not influence residence time as much as polarity/electronegativity does. Polarizability/size could also play a significant role because the increase of these parameters is much more significant from fluoro to chloro than in the rest of the sequence.[26] Also noteworthy is that for similar rates of dissociation, the chloro-substituted compound **33** associates faster than its bulkier and more lipophilic brominated and iodinated counterparts **42** and **43**, which have k_{on} values 10 times lower than the former (Table 2).

All of the other modifications introducing either electron-donating (**39**, **40**, **41**) or -withdrawing (**48**, **49**) groups with varying lipophilicities led to shorter residence times (KRI < 1.20), even though these compounds displayed affinities at the hA_1AR similar to or even better than the halogen-substituted ones.

Notably, in contrast to the rather unaffected A_1AR affinity, a significant decrease in the residence time was observed upon replacement of the thiazole ring with an oxazole (**33**, 51.4 min vs **34**, KRI < 1.20; **38**, 33.0 min vs **36**, KRI < 1.20; and **37**, 34.5 min vs **35**, KRI < 1.20). This is most probably due to the fact that oxazoles have a weaker aromaticity than thiazoles (because of a smaller electron delocalization),[27] thus making the π-interactions that stabilize the receptor–ligand complex weaker. Indeed, we have shown in a previous study that the imidazole substituent (instead of the thiazole/oxazole) of similar compound **1** is involved in a π–π stacking interaction with residue Tyr271 at the hA_{2A}AR,[28] which is also present in the hA_1AR. If we assume a similar binding mode in our case, this interaction might be conserved.

Substitution of the 2-position of 4-thiazolyl-bearing compounds with simple rings was studied, and no effect due to the nature of the substituent was observed, with KRI values between 0.81 and 1.17 (**50**, **51**, **52**, and **53**). Variations within this series could be attributed to size, with a trend of increasing KRI values when the substituent increases in size (cyclohexyl > phenyl > H), leaving the more polar morpholino substituent (**52**) aside. This observation correlates with previous observations reported by Miller et al., which showed that dissociation rates of compounds tend to decrease when molecular weight increases.[29] Also, the best compounds in terms of affinity, **40** and **52** (1.8 and 1.9 nM, respectively), had low KRI values (1.00 and 0.81, respectively). The residence time of **52** was determined to be 1.2 min, much shorter than the radioligand **113**. This compound has a very high association rate (k_{on} = 3.9 × 10^8 M^{-1} min^{-1}), certainly due to the flexibility and polarity of its morpholine substituent, which overthrows its quick dissociation, thus giving it a high affinity. This is a representative example that high affinity does not necessarily correlate with slow dissociation kinetics. Furthermore, this compound, along with **40**, would most likely have been selected in a "traditional" hit and/or lead selection process based solely on affinity.

A rationalization of the ligand–receptor interactions can be hypothesized from previous work conducted by our group on the hA_{2A}AR, the only adenosine receptor subtype for which a crystal structure is available. In this study, a similar compound (**1**) was docked in the crystal structure of the hA_{2A}AR, and a binding pose was determined.[28] More specifically, the central heterocyclic scaffold is engaged in a π–π stacking interaction with a phenylalanine residue (F168) of EL2, the aromatic ring appended to it is pointing downward in the binding pocket, while the imidazole (in the present case thiazole) is facing outside the binding pocket toward the extracellular loops (EL2 and EL3, as inferred from the crystal structure of 6-(2,2-diphenylethylamino)-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-*N*-(2-(3-(1-(pyridin-2-yl)-piperidin-4-yl)ureido)ethyl)-9*H*-purine-2-carboxamide (UK-432097) in hA_{2A}AR).[30] It would then make sense that the nature of the substituent borne by the thiazole plays a crucial role (as we observe here) because it would be on the "front line" for interaction with the extracellular loops during dissociation of the ligand. Recent reports have indeed evidenced the important role played by the extracellular domain in the binding and unbinding of ligands to GPCRs.[31]

Functional Assays. All of the compounds were also tested in a single-point functional assay (at 100 × K_i) to assess an increase in [^{35}S]GTPγS binding as a measure for a compound's intrinsic activity, using **117** (CPA) (Figure 2) as a reference full agonist (maximum response E_{max} set to 100%, at a concentration of 100 μM).[32] Partial agonists are expected to give an E_{max} value in the range 10%–90%, neutral antagonists in the range from −10% to 10%, and inverse agonists below −10%. Most compounds were partial agonists in this scenario, while three of them were full agonists (**24**, **25**, and **40**, E_{max} values of 99%, 112%, and 104%, respectively) (Figure 4, Figure 5, Table 3, Supporting Information). Partial A_1AR agonists seem to be the best choice for cardiovascular therapies, because the response they elicit does not trigger the broad range of effects full agonists do,[23] which is believed to be responsible for their side-effects such as AV-block.[33] As such, there does not seem to be any correlation between E_{max} and residence time/k_{off} (r^2 = 0.2389, E_{max} vs RT determined for compounds **26**, **33**, **36**, **37**, **38**, **42**, **43**, **44**, **46**, **47**, and **52**), meaning that a compound can have a fast or slow dissociation rate from the receptor independently from its functional profile. These results seem to be in contradiction with a previous report from our own group on hA_{2A}AR agonists,[34] and by Sykes et al.[35] on M_3 receptor agonists. Indeed, in these two studies, slower dissociating compounds had a higher efficacy. We do not have an obvious

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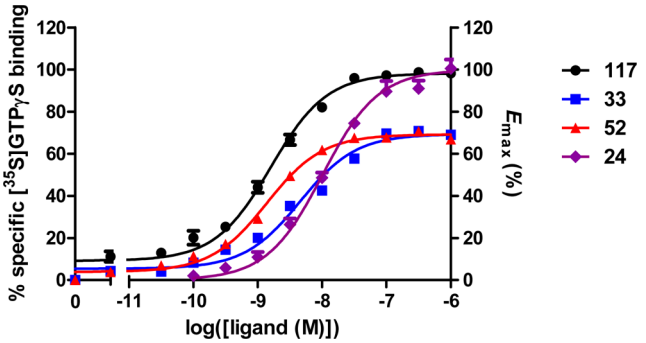

Figure 5. Functional profile of **24**, **33**, **52** and reference agonist **117**, expressed as [^{35}S]GTPγS binding in CHO cell membranes stably expressing human adenosine A_1 receptors. The curves were obtained by coincubating [^{35}S]GTPγS and different concentrations of the ligands. The maximum response of **117** was set at 100%.

explanation for these results, other than trivial ones such as the huge differences between the assays used (GTPγS binding on cell membranes for A_1R, label-free and cAMP experiments on intact cells for A_{2A}R). Further investigations on this question are currently being carried out and will be the subject of future publications.

Seven compounds including those showing the faster (**52**) and slower (**33**, **42**, and **43**) dissociation kinetics were also tested at different concentrations, and their EC_{50} values were determined. The values obtained are all in the nanomolar range (between 1.8 nM for **52** and 12 nM for **24**, Table 3), revealing that the compounds tested are potent agonists at the hA_1AR.

CONCLUSION

In this study, we describe a series of new cyano-amino-pyrimidines that display high affinity and high selectivity at the A_1AR. We showed that kinetic parameters of such compounds, such as target residence time, can be fine-tuned independently from affinity. As a result, the molecules range from very fast associating and dissociating compounds (**52**, residence time of 1.2 min) to much slower dissociating ones (**43**, residence time of 63.8 min), while displaying nanomolar affinities. Structure−kinetics relationships were also elaborated. While no definitive statement could be made about factors favoring rapid dissociation, a global bigger size and locally increased lipophilicity favor slow dissociation. All compounds activated the A_1AR, most as partial agonists, whereas three behaved as full agonists (**24**, **25**, and **40**). However, on the A_1AR there is no apparent correlation between binding kinetics and agonist efficacy, as has been demonstrated for a few other GPCRs. Structure−kinetics relationships, as in the present study, might eventually help in the development of potent and in vivo effective drugs, which remains a challenge for the pharmaceutical industry.

EXPERIMENTAL SECTION

Chemistry. All solvents and reagents were purchased from commercial sources and were of analytical grade. Demineralized water is simply referred to as H_2O, as was used in all cases unless stated otherwise (i.e., brine). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV 400 liquid spectrometer (^{1}H NMR, 400 MHz; ^{13}C NMR, 100 MHz) at ambient temperature. Chemical shifts are reported in parts per million (ppm), are designated by δ, and are downfield to the internal standard tetramethylsilane (TMS) in $CDCl_3$. Coupling constants are reported in Hz and are designated as *J*. High-resolution mass spectrometry was performed by Leiden Institute of Chemistry and recorded by direct injection (2 μL of a 2 μM solution in water/MeCN; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275 °C) with resolution R = 60 000 at m/z 400 (mass range m/z = 150−2000) and calibrated for dioctylphthalate (m/z = 391.28428). Analytical purity of the final compounds was determined by high pressure liquid chromatography (HPLC) with a Phenominex Gemini 3 μm C18 110A column (50 × 4.6 mm, 3 μm), measuring UV absorbance at 254 nm. Sample preparation and HPLC method is, unless stated otherwise, as follows: 0.3−0.8 mg of compound was dissolved in 1 mL of a 1:1:1 mixture of CH_3CN/H_2O/tBuOH and eluted from the column within 15 min at a flow rate of 1 mL/min, with a three-component system of H_2O/CH_3CN/1% TFA in H_2O. The elution method was set up as follows: 1−4 min isocratic system of H_2O/CH_3CN/1% TFA in H_2O, 80:10:10, from the fourth minute a gradient was applied from 80:10:10 to 0:90:10 within 9 min, followed by 1 min of equilibration at 0:90:10 and 1 min at 80:10:10. All compounds show a single peak at the designated retention time and are at least 95% pure. Thin-layer chromatography (TLC) was routinely consulted to monitor the progress of reactions, using aluminum-coated Merck silica gel F_{254} plates. Purification by column chromatography was achieved by use of Grace Davison Davisil silica column material (LC60A 30−200 μm). Solutions were concentrated using a Heidolph laborota W8 2000 efficient rotary evaporation apparatus and by a high vacuum on a Binder APT line vacuum drying oven. The procedure for a series of similar compounds is given as a general procedure for all within that series, annotated by the numbers of the compounds. The procedure for the synthesis of similar compounds is given as a general procedure; deviations therefrom are given below for each compound. None of the procedures were optimized for their yield.

General Procedure for the Synthesis of 4-Amino-6-benzo-[substituted]-5-yl-2-mercapto- pyrimidine-5-carbonitriles 14−23. A mixture of aldehyde (50 mmol), malononitrile (50 mmol), and thiourea (50 mmol) and K_2CO_3 (50 mmol) in EtOH (25 mL) was heated at reflux for 5 h. The reaction was monitored by TLC (EtOAC:petroleum ether 1:2). At the end of the reaction, the precipitate was collected and washed with EtOH. The crude salt was stirred in warm water and filtered. After being cooled, the filtrate was acidified with acetic acid until pH = 1. The deposited precipitate, thus formed, was collected and washed with water and dried in vacuo to afford **14−23** as a solid.

4-Amino-6-benzo[1,3]dioxol-5-yl-2-mercapto-pyrimidine-5-carbonitrile 14. This was obtained from piperonal. Yield 42%; yellow solid. ^{1}H NMR (DMSO-d_6): δ 7.26 (s, 1H), 7.24 (s, 1H), 6.14 (s, 2H) ppm.

4-Fluorobenzamide ***56.*** Benzoyl chloride (31.6 mmol) was dissolved in dry EtOAc (50 mL) and added dropwise to an ice-cold solution of NH_4OH (50 mL of 33% solution in H_2O) in EtOAc (250 mL) over 30 min. After addition, the mixture was stirred on ice for 30 min. The layers were separated, and the organic layer was washed two times with H_2O, two times with brine, dried with $MgSO_4$, filtered, and concentrated in vacuo. Yield: 81%. ^{1}H NMR ($CDCl_3$): δ 7.85−7.82 (m, 2H), 7.15−7.11 (m, 2H), 6.00−5.90 (m, 2H) ppm.

General Procedure for the Synthesis of Thioamides 75−89. An appropriate nitrile (1 equiv, 30 mmol) was dissolved in pyridine (20 mL). Triethylamine (1.1 equiv, 33 mmol) and ammonium sulfide 20 wt % solution in water (1.1 equiv, 33 mmol) were added into the mixture. The reaction mixture was stirred at 50 °C for 3−6 h and monitored by TLC (DCM). After being cooled to room temperature, the mixture was diluted with cold water (50 mL). In case of precipitation, the precipitated solid was filtered off and washed with cold water (procedure a). If there was no precipitation, the product was extracted with ethyl acetate (3 × 50 mL). The organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuum to give the thiobenzamide (procedure b).

4-Chloro-benzothioamide ***75.*** Reagent: 4-chlorobenzonitrile. Reaction time: 12 h. Procedure b, yield 96%. Spectral data were identical to those of the commercially available material.

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4-Nitrobenzothioamide ***90***. A solution of P_4S_{10} (100 mmol) in EtOH (50 mL) was stirred for 1 h. 4-Nitrobenzonitrile (5 mmol) was added, and the resulting solution was heated to reflux for 6.5 h. The solvent was evaporated in vacuo, and water (75 mL) was added. The mixture was extracted with EtOAc (3 × 75 mL), and the organic layers were gathered and washed with brine (75 mL), dried over $MgSO_4$, filtered, and the volatiles were removed in vacuo, affording **90** in 19% yield. ^{1}H NMR ($CDCl_3$ + CD_3OD): δ 8.30 (d, J = 8.8 Hz, 2H), 8.05 (d, J = 8.8 Hz, 2H) ppm.

General Procedures for the Synthesis of 4-Chloromethyl-oxazole and 4-Chloromethyl-thiazole Derivatives 93−111. A mixture of (thio)amide (1 equiv, 15 mmol) and 1,3-dichloroacetone (0.99 equiv, 14.9 mmol) in toluene (15 mL) was refluxed for 2−18 h. After completion of the reaction, monitored by TLC (petroleum ether:ethyl acetate), toluene was evaporated under reduced pressure, and water was added. The mixture was extracted three times with ethyl acetate (50 mL). The organic layer was washed (brine) and dried ($MgSO_4$). The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography using ethyl acetate/petroleum ether (1:99) as an eluent to give **93−111**.

4-Chloromethyl-2-(4-chloro-phenyl)-thiazole ***93***. Reagent: **79**. Yield: 72%. ^{1}H NMR ($CDCl_3$): δ 7.89 (d, J = 8.8 Hz, 2H), 7.42 (d, J = 8.8 Hz, 2H), 7.32 (s, 1H), 4.74 (s, 2H).

Morpholine-4-carbothioamide ***92***. Thiocarbonyldiimidazole (3.96 g, 20 mmol, 1 equiv.) was dissolved in THF (50 mL), and morpholine (1.74 mL, 20 mmol, 1 equiv) was added. After 1 h the mixture was heated to 55 °C, and after 3 h the THF was evaporated in vacuo. Ammonia (40 mL of 2 M solution in EtOH, 80 mmol, 4 equiv) was added to the mixture, and after overnight stirring, the mixture was heated at 55 °C for 1 h. After cooling to room temperature, the precipitate was filtered off. The filtrate was concentrated in vacuo and recrystallized from THF, yielding the product as an off-white powder (1.904 g, 13 mmol). Yield: 65%. ^{1}H NMR (CD_3OD): δ 3.81 (s, 4H), 3.69−3.67 (m, 4H) ppm, identical to the data reported in the literature.

4-(4-(Chloromethyl)thiazol-2-yl)morpholine ***112***. Morpholine-4-carbothioamide **92** (1.9 g, 13 mmol, 1 equiv) and dichloroacetone (1.74 g, 13 mmol, 1 equiv) were dissolved in EtOH (18 mL) and heated to reflux (90 °C). After 1.5 h, the mixture was cooled to room temperature, and the white precipitate in the pink mixture was filtered off. The filtrate was concentrated in vacuo, and the product was recrystallized from EtOH, yielding the product as an off-white powder (1.25 g, 5.7 mmol, 44%). ^{1}H NMR (CD_3OD): δ 7.16 (s, 1H), 4.67 (s, 2H), 3.89−3.87 (m, 4H), 3.71−3.69 (m, 4H) ppm, identical to the data reported in the literature.

General Procedure for the Synthesis of S-Substituted Thiopyrimidines 24−54. A mixture of **14−23** (1 equiv, 0.5 mmol), Na_2CO_3 (1 equiv, 0.5mmol), and alkylating agent **93−112** (1 equiv, 0.5 mmol) in DMF (2 mL) was stirred at 50 °C for 3−4 h. After completion of the reaction, monitored with TLC (DCM:MeOH 9:1), water (10 mL) was added. The precipitate was filtered off and washed with EtOAc. It was then collected and dissolved in DMF (1 mL) and EtOAc (1 mL). Water was added, causing precipitation. After filtration, the pure final compound was obtained as a solid.

4-Amino-6-(benzo[*d*][1,3]dioxol-5-yl-)2-((2-(4-chlorophenyl)thiazol-4-yl)methylthio)pyrimidine-5-carbonitrile 33. Reagents: **14**, **93**. Yield: 11.2%. ^{1}H NMR (DMSO-d_6): δ 7.94 (s, 2H), 7.73 (s, 1H), 7.56 (s, 2H), 7.47 (s, 1H), 7.40 (s, 1H), 7.07 (s, 1H), 6.13 (s, 2H), 4.54 (s, 2H) ppm. ^{13}C NMR (101 MHz, DMSO-d_6): δ 166.8, 163.9, 153.8, 150.3, 147.9, 135.2, 132.2, 130.1, 129.8, 128.2, 124.1, 118.5, 116.9, 109.0, 108.7, 102.3, 102.2, 30.6 ppm. HPLC: 12.27 min.

Biology. *Chemicals and Reagents.* **113** was purchased from ARC Inc. (St. Louis, U.S.). **114** was obtained from Tocris Cookson, Ltd. (UK). **115** and **116** were kind gifts from Prof. C.E. Müller (University of Bonn, Germany). Adenosine deaminase (ADA) was purchased from Boehringer Mannheim (Mannheim, Germany). Bicinchoninic acid (BCA) and BCA protein assay reagent were obtained from Pierce Chemical Co. (Rockford, IL). Chinese hamster ovary cells stably expressing the hA_1AR were provided by Prof. Steve Hill (University of Nottingham, UK). HEK 293 cells stably expressing the human adenosine A_{2A} receptor were a gift from Dr. J. Wang (Biogen, U.S.). Chinese hamster ovary cells stably expressing the $hA_{2B}AR$ and A_3 receptor were obtained from Dr. Steve Rees (GSK, Stevenage, UK) and Dr. K.-N. Klotz (University of Würzburg, Germany), respectively. All other chemicals were of analytical grade and obtained from standard commercial sources.

Binding Assays. Displacements of radioligands in the presence of 1 μM of unlabeled compounds are an average of at least two independent experiments, each consisting of two replicates. Affinity values are an average of at least three independent experiments, each consisting of two replicates.

Adenosine A_1 Receptor.[17] Affinity at the A_1 receptor was determined on membranes from CHO cells stably expressing the human receptors, using **113** as the radioligand. Membrane aliquots containing 5 μg of protein were incubated in a total volume of 100 μL of assay buffer (25 mM Tris-HCl [pH 7.4], supplemented with 5 mM $MgCl_2$ and 0.1% [w/v] CHAPS) at 25 °C for 1 h. Nonspecific binding was determined in the presence of 100 μM **117**. The incubation was terminated by filtration over Whatman GF/B filters under reduced pressure with a Brandell harvester or through 96-well GF/B filter plates using a PerkinElmer Filtermate-harvester. Filters were washed three times with ice-cold buffer and placed in scintillation vials. Emulsifier Safe (3.5 mL) was added, and after 2 h radioactivity was counted in an LKB rack β scintillation counter.

Adenosine A_{2A} Receptor.[36] Affinity at the A_{2A} receptor was determined on membranes from HEK 293 cells stably expressing the human receptors, using **114** as the radioligand. Membrane aliquots containing 25 μg of protein were incubated in a total volume of 100 μL of assay buffer (50 mM Tris-HCl [pH 7.4]) at 25 °C for 2 h. Nonspecific binding was determined in the presence of 100 μM **117**. The incubation was terminated by filtration over Whatman GF/B filters under reduced pressure with a Brandell harvester or through 96-well GF/B filter plates using a PerkinElmer Filtermate-harvester. Filters were washed three times with ice-cold buffer and placed in scintillation vials. Emulsifier Safe (3.5 mL) was added, and after 2 h radioactivity was counted in an LKB rack β scintillation counter.

Adenosine A_{2B} Receptor.[21] Affinity at the A_{2B} receptor was determined on membranes from CHO cells stably expressing the human receptors, using **115** as the radioligand. Membrane aliquots containing 20 μg of protein were incubated in a total volume of 100 μL of assay buffer (50 mM Tris-HCl [pH 7.4] supplemented with 0.1% [w/v] CHAPS) at 25 °C for 2 h. Nonspecific binding was determined in the presence of 10 μM unlabeled **114**. The incubation was terminated by filtration over Whatman GF/B filters under reduced pressure with a Brandell harvester or through 96-well GF/B filter plates using a PerkinElmer Filtermate-harvester. Filters were washed three times with ice-cold buffer (0.1% BSA instead of CHAPS) and placed in scintillation vials. Emulsifier Safe (3.5 mL) was added, and after 2 h radioactivity was counted in an LKB rack β scintillation counter.

Adenosine A_3 Receptor.[22] Affinity at the A_3 receptor was determined on membranes from CHO cells stably expressing the human receptors, using **116** as the radioligand. Membrane aliquots containing 45 μg of protein were incubated in a total volume of 100 μL of assay buffer (50 mM Tris-HCl [pH 8.0] supplemented with 10 mM $MgCl_2$, 1 mM EDTA, and 0.01% [w/v] CHAPS) at 25 °C for 2 h. Nonspecific binding was determined in the presence of 100 μM 5′-(*N*-ethylcarboxamido)adenosine (NECA).[37] The incubation was terminated by filtration over Whatman GF/B filters under reduced pressure with a Brandell harvester or through 96-well GF/B filter plates using a PerkinElmer Filtermate-harvester. Filters were washed three times with ice-cold buffer (without CHAPS) and placed in scintillation vials. Emulsifier Safe (3.5 mL) was added, and after 2 h radioactivity was counted in an LKB rack β scintillation counter.

Competition Association Assays.[17] KRI values are an average of at least two independent experiments, each consisting of two replicates. Kinetic rate constant values are an average of at least three independent experiments, each consisting of two replicates.

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The binding kinetics of unlabeled ligands was quantified using the competition association assay based on the theoretical framework by Motulsky and Mahan.[38] A concentration of 10-fold of the K_i value was used to determine the binding kinetics of unlabeled ligands. The competition association assay was initiated by adding membrane aliquots (5 μg/well) at different time points for a total of 90 min to a total volume of 100 μL of assay buffer at 25 °C with 2.5 nM **113** in the absence or presence of competing ligand (10-fold K_i). Incubations were terminated, and samples were obtained as described under Binding Assays. The dual-point competition association assays were run similarly, with only two time points, at 15 and 120 min, respectively.

Functional Assays. For the [^{35}S]GTPγS binding assay, membrane homogenates (CHO-hA$_1$, 3 μg, saponin, 3 μg) were equilibrated in 80 μL total volume of assay buffer (50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4, 1 mM EDTA, 1 mM DTT, and 0.05% BSA) containing 3 μM GDP and a range of concentrations of ligand at 25 °C for 30 min. After this, 20 μL of [^{35}S]GTPγS (final concentration 0.3 nM) was added, and incubation continued for 90 min at 25 °C. Samples were harvested and counted as described for radioligand binding assay.

Single-point [^{35}S]GTPγS binding assays were performed similarly to the standard accumulation assay; however, unlabeled ligands were only tested at a 100 × K_i concentration.

Data Analysis. K_i values were calculated by use of a nonlinear regression curve-fitting program (GraphPad Prism 5, GraphPad Software Inc., San Diego, CA). K_D values of the radioligands were 1.6, 1.0, 0.4, and 4.9 nM for **113**, **114**, **115**, and **116** on the adenosine A$_1$R, A$_{2A}$R, A$_{2B}$R, and A$_3$R, respectively. The data from the functional assays were generated and normalized to the value of 100 μM **117** (set at 100%).

■ ASSOCIATED CONTENT

Supporting Information

Full experimental procedures for the synthesis of compounds **14**−**54**, **56**, **75**−**90**, and **92**−**112**, including NMR and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

J.L. conceived the study; A.P.IJ., J.B., and L.H.H. supervised the project. The chemical synthesis was designed and supervised by J.L. and performed by M.A., R.K., and L.X. The bioassays were supervised by D.G. and J.L. and performed by J.L., T.A.M.M., T.P.P., and H.de.V. The manuscript was written by J.L., D.G., and A.P.IJ.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ADA, adenosine deaminase; Ac, acetyl; BCA, bicinchoninic acid; BSA, bovine serum albumin; CPA, N^6-cyclopentyladenosine; *c*Hex, cyclohexyl; CHO, Chinese hamster ovary; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; DPCPX, 1,3-dipropyl-8-cyclopentyl-xanthine; DTT, dithiothreitol; EC_{50}, concentration of unlabeled ligand, which elicits 50% of the maximum response (E_{max}) in a functional assay at membranes of CHO cells stably expressing the adenosine A$_1$ receptor; EDTA, ethylenediaminetetraacetic acid; E_{max}, maximum response elicited by an unlabeled ligand in a functional assay (relatively to CPA) at membranes of CHO cells stably expressing the adenosine A$_1$ receptor; FBS, fetal bovine serum; G418, geneticin; GDP, guanosine diphosphate; GF, glass filter; GTPγS, guanosine 5′-*O*-[γ-thio]triphosphate; HPLC, high-pressure liquid chromatography; IC_{50}, concentration of unlabeled ligand, which displaces 50% of [^{3}H]DPCPX binding to membranes of CHO cells stably expressing the adenosine A$_n$ receptor; K_i, affinity of ligand; k_{off}, dissociation rate constant at the hA$_1$AR; k_{on}, molar association rate constant at the hA$_1$AR; KRI, kinetic rate index; NECA, 5′-(*N*-ethylcarboxamido)adenosine; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; RT, residence time; SAR, structure−affinity relationships; SKR, structure kinetics relationships; THF, tetrahydrofuran; TLC, thin layer chromatography; Tris, tris(hydroxymethyl)aminomethane

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