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$_1$AR) 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. 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 ($A_1$AR) and the $A_2B$AR. In the meantime, Otsuka Pharmaceuticals described a series of 4-amino-6-aryl-5-cyano-2-thiopyrimidines as selective agonists for the $A_2B$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. A 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. 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.

In this Article, we report the synthesis and evaluation of 4-amino-6-aryl-5-cyano-2-thiopyrimidines as (selective) $A_1$AR 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$_1$AR, 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)
the formation of the thiopyrimidine by the three-component reaction between malononitrile, thiourea, and various substituted benzaldehydes in the presence of K$_2$CO$_3$ at the reflux of ethanol,\textsuperscript{9} and (ii) the alkylation of the obtained thiol with various alkyl chlorides in the presence of Na$_2$CO$_3$ at 50° Ci n DMF (Scheme 1).\textsuperscript{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,\textsuperscript{10} or ethanol.\textsuperscript{11} Noncommercially available amides were synthesized from the corresponding acyl chlorides by treatment with aqueous ammonia;\textsuperscript{12} similarly, thioamides were obtained by treatment of the corresponding nitrile upon reaction with ammonium sulfide in pyridine/Et$_3$N.\textsuperscript{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.\textsuperscript{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).\textsuperscript{15}

Scheme 2. Synthesis of Alkylating Agents 93--112\textsuperscript{a}

\begin{align*}
\text{Scheme 1. Synthetic Route toward S-Substituted 4-Amino-6-aryl-5-cyano-2-thiopyrimidines 24--54}\textsuperscript{a}
\end{align*}

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\text{Reagents and conditions: (a) } \text{K}_2\text{CO}_3, \text{EtOH, reflux, 1--42%}; (b) R^1\text{CH}_2\text{Cl, Na}_2\text{CO}_3, \text{DMF, 50 }^\circ\text{C, 7--61%}.\n```

Biology. All of the compounds were initially tested in a single-point radioligand binding assay in the presence of 2.5 nM \(^{113}\text{[H]}\text{DPCPX}\) (Figure 2),\textsuperscript{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$_1$AR ($K_i < 100$ nM). Subsequently, compounds with high A$_1$AR affinity were screened in a so-called dual-point competition association assay in the presence of \(^{113}\text{[H]}\text{DPCPX}\) (RT = 4.8 min), which we recently developed in our lab for fast and high-throughput kinetics screening.\textsuperscript{17} In brief, we obtained specific 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 relatively 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...
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 A1AR 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 hA1AR and negligible radioligand displacement at other adenosine receptor subtypes (10% of $[^3H]ZM241385$20 at hA2AAR, 3.5% of $[^3H]PSB603$21 at hA2BAR, and 3% of $[^3H]PSB11$22 at hA3AR at 1 μM (Figure 2)), thus showing high selectivity for hA1AR. 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 A1AR affinity (25, 14 nM), while selectivity over the hA2AAR decreased somewhat (39% at 1 μM). Interestingly, this finding, taken together with the fact that the Otsuka compounds showed selectivity at the hA2AAR,4 indicates that the acetamide group on the phenyl ring is responsible for the affinity at the hA2AAR, whereas selectivity for the hA2AAR over hA1AR is achieved through the substituent at the sulfur atom (Figure 1). Removing the $R_1$ substituent did not change the affinity at the A1AR (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 A1AR affinity (27, 66% displacement at 1 μM; 29, 50% displacement at 1 μM).
Table 1. Affinity and Kinetic Parameters of Compounds 24–33 on the Human A1AR

<table>
<thead>
<tr>
<th>R¹</th>
<th>Kᵢ (nM) ± SEM or disp. at 1 μM (%)ᵃ</th>
<th>Kᵢᵇ</th>
<th>Kᵣ (M⁻¹ min⁻¹)ᵇ</th>
<th>kᵣᵢ (min⁻¹)ᵇ (RT (min))ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>4-O(CH₂)₂OH</td>
<td>4.8 ± 0.1</td>
<td>1.18 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>4-NHAc</td>
<td>14 ± 0.5</td>
<td>0.97 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>H</td>
<td>8.9 ± 2.1</td>
<td>1.24 (1.39, 1.09)</td>
<td>(2.0 ± 1.2) × 10³ 0.031 ± 0.017 (32.3)</td>
</tr>
<tr>
<td>27</td>
<td>4-OMe</td>
<td>73% (80, 66)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>4-OH</td>
<td>15 ± 2.4</td>
<td>0.99 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>4-OMe</td>
<td>49% (50, 48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>4-OCF₃</td>
<td>185 ± 66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>4-NMe₂</td>
<td>233 (235, 232)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>4-Me</td>
<td>44% (46, 43)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>3,4-OCH₂O</td>
<td>14 ± 6</td>
<td>1.64 (1.75, 1.53)</td>
<td>(3.0 ± 1.3) × 10³ 0.019 ± 0.004 (51.4)</td>
</tr>
</tbody>
</table>

ᵃKᵢ ± SEM (n = 3) or % displacement (n₁, n₂) (n = 2), obtained from radioligand binding assays with 113 on CHO cell membranes stably expressing human adenosine A₁ receptors. bKᵢᵇ ± SEM (n = 3) or Kᵢᵇ (n₁, n₂) (n = 2), obtained from dual-point competition association assays with 113 on CHO cell membranes stably expressing human adenosine A₁ receptors. cNot determined. dKᵣᵇ ± SEM (n = 3), obtained from competition association assays with 113 on CHO cell membranes stably expressing human adenosine A₁ receptors. eKᵣᵢ (min⁻¹)ᵇ (RT (min))ᵇ. fRT = 1/kᵣᵢ₄

Table 2. SAR and SKR of Compounds 34–54

<table>
<thead>
<tr>
<th>R²</th>
<th>X</th>
<th>Kᵢ (nM) ± SEM or disp. at 1 μM (%)ᵃ</th>
<th>Kᵢᵇ</th>
<th>Kᵣ (M⁻¹ min⁻¹)ᵇ</th>
<th>kᵣᵢ (min⁻¹)ᵇ (RT (min))ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>4-Cl-Ph</td>
<td>O</td>
<td>15 ± 2</td>
<td>1.17 (1.20, 1.14)</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>4-F-Ph</td>
<td>O</td>
<td>20 ± 8.6</td>
<td>1.01 (1.10, 0.92)</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>3-F-Ph</td>
<td>O</td>
<td>15 ± 4</td>
<td>1.24 (0.94, 1.54)</td>
<td>(6.1 ± 2.2) × 10⁶ 0.328 ± 0.050 (3.05)</td>
</tr>
<tr>
<td>37</td>
<td>4-F-Ph</td>
<td>S</td>
<td>6.7 ± 1.4</td>
<td>1.22 (1.33, 1.11)</td>
<td>(1.7 ± 0.30) × 10⁶ 0.029 ± 0.005 (34.5)</td>
</tr>
<tr>
<td>38</td>
<td>3-F-Ph</td>
<td>S</td>
<td>14 ± 1</td>
<td>1.24 (1.11, 1.37)</td>
<td>(5.1 ± 1.3) × 10⁶ 0.030 ± 0.008 (33.0)</td>
</tr>
<tr>
<td>39</td>
<td>4-Me-Ph</td>
<td>S</td>
<td>3.1 ± 0.7</td>
<td>1.15 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>4-OMe-Ph</td>
<td>S</td>
<td>1.8 ± 0.2</td>
<td>1.00 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>3,4-OCH₂O-Ph</td>
<td>S</td>
<td>2.5 ± 0.1</td>
<td>0.93 (0.96, 0.90)</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>4-Br-Ph</td>
<td>S</td>
<td>5.2 ± 0.3</td>
<td>1.67 ± 0.20</td>
<td>(3.3 ± 1.2) × 10⁵ 0.017 ± 0.003 (59.0)</td>
</tr>
<tr>
<td>43</td>
<td>4-Ph</td>
<td>S</td>
<td>9.9 ± 1.0</td>
<td>1.40 ± 0.17</td>
<td>(4.3 ± 0.87) × 10⁶ 0.016 ± 0.004 (63.8)</td>
</tr>
<tr>
<td>44</td>
<td>3-Cl-Ph</td>
<td>S</td>
<td>42 ± 10</td>
<td>1.24 ± 0.05</td>
<td>(1.8 ± 0.50) × 10⁶ 0.034 ± 0.007 (30.0)</td>
</tr>
<tr>
<td>45</td>
<td>3,4-dCl-Ph</td>
<td>S</td>
<td>23% (25, 20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>2-Cl-Ph</td>
<td>S</td>
<td>75 ± 4</td>
<td>1.29 (1.25, 1.33)</td>
<td>(2.6 ± 0.7) × 10⁵ 0.028 ± 0.010 (35.3)</td>
</tr>
<tr>
<td>47</td>
<td>2-F-Ph</td>
<td>S</td>
<td>12 ± 2</td>
<td>1.46 (1.76, 1.16)</td>
<td>(8.2 ± 0.4) × 10⁶ 0.044 ± 0.001 (22.5)</td>
</tr>
<tr>
<td>48</td>
<td>4-Cl,Ph</td>
<td>S</td>
<td>18 ± 2.3</td>
<td>1.20 (1.07, 1.33)</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>4-NO₂-Ph</td>
<td>S</td>
<td>11 ± 2.7</td>
<td>0.95 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>Ph</td>
<td>S</td>
<td>2.4 ± 0.7</td>
<td>1.10 (1.04, 1.16)</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>cHex</td>
<td>S</td>
<td>6.3 ± 1.2</td>
<td>1.17 (1.30, 1.04)</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>morpholino</td>
<td>S</td>
<td>1.9 ± 0.4</td>
<td>0.81 (0.64, 0.98)</td>
<td>(3.9 ± 1.2) × 10⁸ 0.870 ± 0.060 (1.2)</td>
</tr>
<tr>
<td>53</td>
<td>H</td>
<td>S</td>
<td>9.7 ± 1.7</td>
<td>0.93 (1.11, 0.75)</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>NH</td>
<td>S</td>
<td>83 ± 10</td>
<td>1.15 (0.96, 1.34)</td>
<td></td>
</tr>
</tbody>
</table>

ᵃKᵢ ± SEM (n = 3), % displacement (n₁, n₂) (n = 2), obtained from radioligand binding assays with 113 on CHO cell membranes stably expressing human adenosine A₁ receptors. bKᵢᵇ ± SEM (n = 3) or Kᵢᵇ (n₁, n₂) (n = 2), obtained from dual-point competition association assay with 113 on CHO cell membranes stably expressing human adenosine A₁ receptors. cNot determined. dKᵣᵇ ± SEM (n = 3), obtained from competition association assay with 113 on CHO cell membranes stably expressing human adenosine A₁ receptors. eKᵣᵢ (min⁻¹)ᵇ (RT (min))ᵇ. fRT = 1/kᵣᵢ₄
Increasing the electronegativity and lipophilicity of the methoxy group by using a trifluoromethoxy substituent resulted in a better A1AR 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 Kᵢ value below 100 nM, only compounds 26 and 33 had a Kᵢ value above 1.20 (1.24 and 1.64, respectively) with associated A₁AR 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 (kᵣ = 2.0 × 10⁶ vs. 3.0 × 10⁷ M⁻¹ min⁻¹). These results highlight the crucial role of the methylenedioxy substituent because it is essential for both obtaining high A₁AR affinity and achieving faster association and slower dissociation kinetics. The remaining three compounds (24, 25, and 28) showed low Kᵢ values, which could be due to their pronounced hydrophobic/lipidophilicity (−0.8 < π < −0.6). 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 Kᵢ 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 A₁AR 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 R₃ position, we learned that 50 displayed a high A₁AR 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 A₁AR in our study, and was selective over the other three receptor subtypes (21% at hA₂₅AR, 12% at hA₂₆AR, and 18% at hA₃AR).

The substitution pattern of the phenyl ring was then studied. An increase in 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 hA₁AR selectivity), and neither did an increase in electron-withdrawing character (CF₃; 48, NO₂; 49). Moving the halogen substituent along the ring led to two distinct cases: for a chloro- substituent, the Kᵢ 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. Dichlorination on the meta and para positions led to a significant loss of affinity at the hA₁AR (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 R² = 4-Me (39), 1.8 nM for R² = 4-OMe (40), 2.5 nM for R² = 3,4-OCH₂O (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 A₁AR affinities. As evidence, changing the thiazole into an oxazole did not lead to a significant change in affinity, with 34, 35, and 36 having Kᵢ 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 hA₂₅AR, while being selective over the two other subtypes. Exchanging the 4-thiazole substituent for a 4-imidazolyl substituent gave a Kᵢ value of 83 nM (54) at hA₁AR while maintaining selectivity over the hA₂₅AR. 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 hA₁AR. 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.
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 A1AR 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 (\(\pi\)) as well as lipophilicity (\(\pi\)) 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 \(k_{\text{on}}\) 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 hA1AR similar to or even better than the halogen-substituted ones.

Notably, in contrast to the rather unaffected A1AR 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 \(\pi\)-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 \(\pi\)–\(\pi\) stacking interaction with residue Tyr271 at the hA2AR,\(^{28}\) which is also present in the hA1AR. 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 (\(k_{\text{on}} = 3.9 \times 10^8 \text{M}^{-1} \text{min}^{-1}\)), 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 hA2AR, 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 hA2AR, and a binding pose was determined.\(^{29}\) More specifically, the central heterocyclic scaffold is engaged in a \(\pi\)–\(\pi\) 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-dihydroxytetrahydrofuran-2-yl)-N-(3-(3-(1-(pyridin-2-yl)-piperidin-4-yl)ureido)ethyl)-1H-purine-2-carboxamide (UK432097) in hA3AR).\(^{30}\) 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 \(\times \) K) to assess an increase in \[^{35}\text{S}\]GTP\(\gamma\)S binding as a measure for a compound's intrinsic activity, using 117 (CPA) (Figure 2) as a reference full agonist (maximum response \(E_{\text{max}}\) set to 100%), at a concentration of 100 \(\mu\)M.\(^{32}\) Partial agonists are expected to give an \(E_{\text{max}}\) 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, \(E_{\text{max}}\) values of 99%, 112%, and 104%, respectively) (Figure 4, Figure 5, Table 3, Supporting Information). Partial A1AR 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,\(^{33}\) which is believed to be responsible for their side-effects such as AV-block.\(^{34}\) As such, there does not seem to be any correlation between \(E_{\text{max}}\) and residence time/\(k_{\text{off}}\) (\(r^2 = 0.2389, E_{\text{max}} \text{ vs RT determined for compounds 26, 33, 36, 37, 38, 42, 43, 44, 46, 47, and 52},\)) 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 hA2AR agonists,\(^{34}\) and by Sykes et al.\(^{35}\) on M1 receptor agonists. Indeed, in these two studies, slower dissociating compounds had a higher efficacy. We do not have an obvious
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_{1}AR.

- **CONCLUSION**

In this study, we describe a series of new cyano-amino-pyrimidines that display high affinity and high selectivity at the A_{1}AR. 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_{1}AR, most as partial agonists, whereas three behaved as full agonists (24, 25, and 40). However, on the A_{1}AR 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_{2}O, as was used in all cases unless stated otherwise (i.e., brine). H and ^{13}C NMR spectra were recorded on a Bruker AV 400 liquid spectrometer (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 CDC1_{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/McCN; 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 = 66000 at m/z 400 (mass range m/z = 150–2000) and calibrated for dioctylphthalate (m/z = 901.2848)). Analytical purity of the final compounds was determined by high pressure liquid chromatography (HPLC) with a Phenomenex 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_{3}CN/H_{2}O/tBuOH and eluted from the column within 15 min at a flow rate of 1 mL/min, with a three-component system of H_{2}O/CH_{3}CN/1% TFA in H_{2}O. The elution method was set up as follows: 1–4 min isocratic system of H_{2}O/CH_{3}CN/1% TFA in H_{2}O, 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 Davasil 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 for each below. 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_{2}CO_{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 deposits 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. δ 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_{4}OH (50 mL of 33% solution in H_{2}O) 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_{2}O, two times with brine, dried with MgSO_{4} filtered, and concentrated in vacuo. Yield: 81%. δ 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 sulfd 20 wt % solution in water (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-Chlorobenzothioamide 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. A solution of P₅S₁₀ (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 washed and dried with brine (75 mL), dried over MgSO₄, filtered, and the volatiles were removed in vacuo, affording 90% in 19% yield. "H NMR (CDCl₃, δ 8.10 (d, J = 8.8 Hz, 2H), 8.05 (d, J = 8.8 Hz, 2H) ppm.

**General Procedures for the Synthesis of 4-Chloromethyl-thiazole 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/petroleum ether (1:99) as an eluent to give 93. Yield: 72%. "H NMR (CDCl₃): δ 2.28 (s, 3H), 3.69 (m, 4H) ppm, identical to the data reported in the literature.

**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 re, 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%. "H NMR (CDCl₃): δ 3.81 (s, 4H), 3.69–3.67 (m, 4H) ppm, identical to the data reported in the literature.

**4-(Chloromethyl)thiazol-2-yl)morpholine 112.** Morpholine-4-carboxylic acid (1.49 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%). "H NMR (CDCl₃): δ 6.13 (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 5-Substituted Thiopyrimidine 54–54.** A mixture of 14–23 (1 equiv, 0.5 mmol), Na₂CO₃ (1 equiv, 0.5 mmol), 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-(benz[d][1,3]dioxol-5-yl)-12-[(4-chlorophenyl)thiazol-4-yl)methyliothio]pyrimidine 5-carbonitrile 33.** Reagents: 14, 93. Yield: 11.2%. "H NMR (DMSO-d₆): 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. 13C NMR (101 MHz, DMSO-d₆): 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). Bis-chloroacetic acid (BCA) and BCA protein assay reagent were obtained from Pierce Chemical Co. (Rockford, IL). Chinese hamster ovary cells stably expressing the hA₂BAR were provided by Prof. Steve Hill (University of Nottingham, UK). HEK 293 cells stably expressing the human adenosine A₃A receptor were a gift from Dr. J. Wang (Biogen, U.S.). Chinese hamster ovary cells stably expressing the hA₂BAR and A₃ 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 A₁ Receptor.** Affinity at the A₁ 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 MgCl₂ 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 A₂A Receptor.** Affinity at the A₂A 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 A₂B Receptor.** Affinity at the A₂B 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]) 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 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 A₃ Receptor.** Affinity at the A₃ 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 MgCl₂, 1 mM EDTA, and 0.10% [w/v] CHAPS) 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 (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.

**Competition Association Assays.** 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.
The binding kinetics of unlabeled ligands was quantified using the competition association assay based on the theoretical framework by Motulsky and Mahan. A concentration of 10-fold of the \( K_d \) 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 mM 113 in the absence or presence of competing ligand (10-fold \( K_d \)). 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 \([^{35}S]GTP^\gamma S\) binding assay, membrane homogenates (CHO-hA1, 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 \([^{35}S]GTP^\gamma S\) (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 \([^{35}S]GTP^\gamma S\) binding assays were performed similarly to the standard accumulation assay; however, unlabeled ligands were only tested at a 100 × \( K_d \) concentration.

Data Analysis. \( K_d \) 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 A1R, A2AR, A3R, and A2R, respectively. The data from the functional assays were generated and normalized to the value of 100 μM 117 (set at 100%).

ASSOCIATED CONTENT

1 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.J., 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.J.

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; cHex, 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 A1 receptor; EDTA, ethylenediaminetetraacetic acid; IC\(_{50}\), maximum response elicited by an unlabeled ligand in a functional assay (relatively to CPA) at membranes of CHO cells stably expressing the adenosine A1 receptor; FBS, fetal bovine serum; G418, geneticin; GDP, guanosine diphosphate; GF, glass filter; GTP\(\gamma\)S, guanosine 5′-O-[(y-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 A1 receptor; \( K_d \), affinity of ligand; \( k_{off} \), dissociation rate constant at the hA1AR; \( k_{on} \), molar association rate constant at the hA1AR; KRI, kinetic rate index; NECA, 5′- (N-ethylcarbazolamido)adenosine; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; RT, residence time; SAR, structure–affinity relationships; SKR, structure relationships; THF, tetrahydrofuran; TLC, thin layer chromatography; Tris, tris(hydroxymethyl)aminomethane

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