

Structure-Based Discovery of Novel Chemotypes for Adenosine A_{2A} Receptor Antagonists

Vsevolod Katritch,^{*,†,‡,§} Veli-Pekka Jaakola,^{†,||} J. Robert Lane,[⊥] Judy Lin,[⊥] Adriaan P. IJzerman,[⊥] Mark Yeager,^{‡,#} Irina Kufareva,^{†,§} Raymond C. Stevens,^{*,†} and Ruben Abagyan^{*,†,§}

[†]Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, [‡]Department of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, [§]Current address: University of California, San Diego Skaggs School of Pharmacy and Pharmaceutical Sciences 9500 Gilman Drive, La Jolla, California 92093, ^{||}Current address: Biocenter Oulu and Department of Biochemistry, University of Oulu, PO Box 3000, FIN-90014 Oulu, Finland, [⊥]Division of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research, PO Box 9502, 2300RA Leiden, The Netherlands, and [#]Department of Molecular Physiology and Biological Physics, University of Virginia Health System, PO Box 800736, Charlottesville, Virginia 22908-0736

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The recent progress in crystallography of G-protein coupled receptors opens an unprecedented venue for structure-based GPCR drug discovery. To test efficiency of the structure-based approach, we performed molecular docking and virtual ligand screening (VLS) of more than 4 million commercially available “drug-like” and “lead-like” compounds against the A_{2A}AR 2.6 Å resolution crystal structure. Out of 56 high ranking compounds tested in A_{2A}AR binding assays, 23 showed affinities under 10 μM, 11 of those had sub-μM affinities and two compounds had affinities under 60 nM. The identified hits represent at least 9 different chemical scaffolds and are characterized by very high ligand efficiency (0.3–0.5 kcal/mol per heavy atom). Significant A_{2A}AR antagonist activities were confirmed for 10 out of 13 ligands tested in functional assays. High success rate, novelty, and diversity of the chemical scaffolds and strong ligand efficiency of the A_{2A}AR antagonists identified in this study suggest practical applicability of receptor-based VLS in GPCR drug discovery.

Introduction

G-Protein coupled receptor (GPCR^a) signaling is a key to molecular pathways involved in the normal function and pathologies of nervous, cardiovascular, immune, and other major systems in human body. Among about 400 nonolfactory receptors of the GPCR superfamily, more than 50 represent molecular targets for prescription drugs,^{1,2} and the range of clinically validated GPCRs and investigational drugs is rapidly expanding. One of the major spotlights is on the adenosine receptor (AR) subfamily,³ all four subtypes of which (A₁, A_{2A}, A_{2B}, and A₃) have been considered as potential therapies for neurodegenerative,^{4,5} cardiac,^{6,7} immune, and inflammatory disorders^{8,9} and cancer.¹⁰ Despite some obstacles in clinical development of early drug candidates for ARs,^{11,12} the year 2008 has been marked by successful FDA

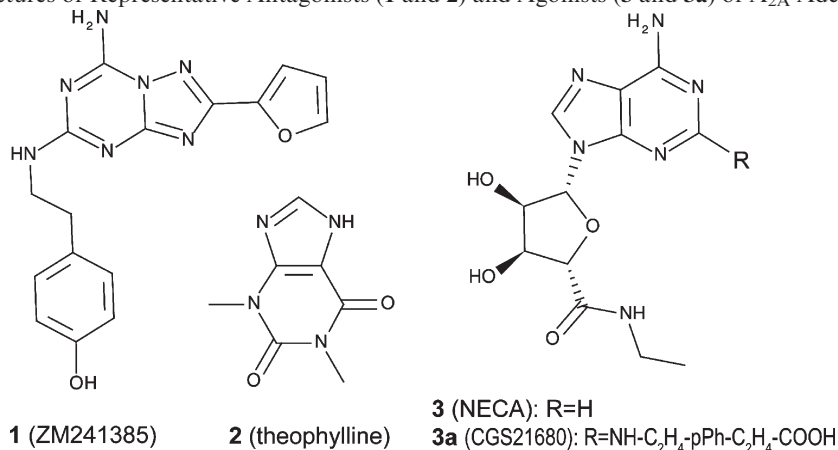
approval of the new generation A_{2A}AR selective agonist regadenoson as a coronary vasodilator for use in myocardial perfusion imaging.¹³ This breakthrough, along with other advances in preclinical and clinical studies³ boosts interest in development of a new generation of bioavailable and safe agonists and antagonists for adenosine receptors.

Historically, GPCR drug discovery relied on known natural ligands or screening assay hits as starting points for optimization of affinity, subtype selectivity, and pharmacokinetic properties.^{14–16} The most useful scaffolds for design of AR ligands have been provided by adenosine^{17,18} and xanthine^{19,20} chemotypes. Thus, adenosine derivatives with various substitutions in position 2 or N⁶ of the adenine ring and 3', 4', or 5' position of the ribose ring¹² have been developed as selective agonists for all four AR subtypes; only a few other chemotypes²¹ have been found with agonist activity. Since the early discovery of caffeine and theophylline as nonselective AR antagonists,^{19,20,22} derivatization of the xanthine scaffold yielded a number of high affinity subtype selective antagonists.¹² Several other chemotypes for AR antagonists have been discovered over the past decade^{23–30} using a combination of experimental screening and ligand-based methods. The ligand-based approaches, however, require preexisting knowledge of ligand structure–activity relationships (SAR) and are largely limited to relatively close analogues of known ligands (Chart 1).

The breakthroughs in GPCR crystallography, including determination of high resolution structures of β-adrenergic receptors (human β₂AR^{31–33} and turkey β₁AR³⁴), and most recently of human A_{2A} adenosine receptor³⁵ (A_{2A}AR) in complex with antagonist **1** (ZM241385³⁶), open an opportunity

*To whom correspondence should be addressed. For V.K.: phone, (858) 822-3639; Fax, (858) 822-5591; E-mail: vkatritch@ucsd.edu; address, University of California, San Diego, Skaggs School of Pharmacy and Pharmaceutical Sciences, 9500 Gilman Drive, MC 0747, La Jolla, California 92093-0747. For R.C.S.: phone: (858) 784-9417; fax, (858) 784-9483; E-mail: stevens@scripps.edu; address, Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, GAC-1200, La Jolla, CA 92037. for R.A.: phone, (858) 822-3639; fax, (858) 822-5591; E-mail: rabagyan@ucsd.edu; address, University of California, San Diego, Skaggs School of Pharmacy and Pharmaceutical Sciences, 9500 Gilman Drive, MC 0747, La Jolla, California 92093-0747.

^a Abbreviations: GPCR, G protein-coupled receptor; AR, adenosine receptor; A_{2A}AR, subtype A_{2A}; β₂AR, β₂ adrenergic receptor; TM, transmembrane; EL2, extracellular loop 2; VLS, virtual ligands screening; PDB protein databank; rmsd, root-mean-square deviation; LE, ligand efficiency; ROC, receiver operating characteristic; AUC, area under ROC curve; NSQ_AUC, normalized square root AUC; BPMC, biased probability Monte Carlo method.

Chart 1. Chemical Structures of Representative Antagonists (**1** and **2**) and Agonists (**3** and **3a**) of A_{2A} Adenosine Receptor

for alternative, receptor-based approaches to finding new GPCR ligand chemotypes.³⁷ Indeed, the β_2 AR crystal structure-based models have already proved to be efficient in virtual screening for antagonists/inverse agonists and, with some modifications,³⁸ also for full and partial agonists of the receptor.^{39,40} Recently, virtual ligand screening for the β_2 AR allowed identification of new nanomolar and submicromolar inverse agonists for this receptor.⁴¹

In this study, we assessed performance of the human A_{2A}AR structure³⁵ (PDB code: 3EML) in virtual screening, which resulted in identification of several novel ligand chemotypes for ARs. The initial benchmarking with known antagonists showed enrichment factors for the A_{2A}AR on par with previously published assessments for the β_2 AR.^{39,40} Performance of the screening model was further improved by retaining several highly structured water molecules in the binding site and refining side chains in the binding pocket. The optimized model was used for virtual screening of more than 4 million commercially available lead-like and drug-like compounds. Out of 56 high scoring compounds, which were subsequently tested in radioligand binding assays, 23 compounds were identified as A_{2A}AR ligands with affinity $K_i < 10 \mu\text{M}$ (41% hit rate), of which 11 had sub- μM affinity and two compounds showed K_i under $0.06 \mu\text{M}$. Functional assays confirmed significant A_{2A}AR antagonist activities for 10 out of 13 newly identified ligands. The novel ligands represent at least nine novel chemotypes and include low molecular weight compounds with high ligand binding efficiency ($LE > 0.3$ kcal/mol per heavy atom), potentially suitable as leads for drug discovery. Overall, a very good success rate of VLS, high ligand efficiency of the hits, as well as diversity and novelty of the identified chemical scaffolds, suggest applicability of the receptor-based screening to discovery of new candidate drugs to adenosine receptors and potentially other GPCRs.

Results

High-Performance A_{2A}AR Screening Model Based on the Crystal Structure. The A_{2A}AR crystal structure³⁵ (PDB code: 3EML) and ligand-refined models of the A_{2A}AR were evaluated for their ability to select known A_{2A}AR specific antagonists from random decoy compounds in a docking and virtual ligand screening (VLS) benchmark test. As illustrated in Figure 1 examples, the predicted binding poses for majority of the known A_{2A}AR antagonists in the diverse benchmark set display key similarities with the binding mode of antagonist **1** in the crystal structure. This common binding

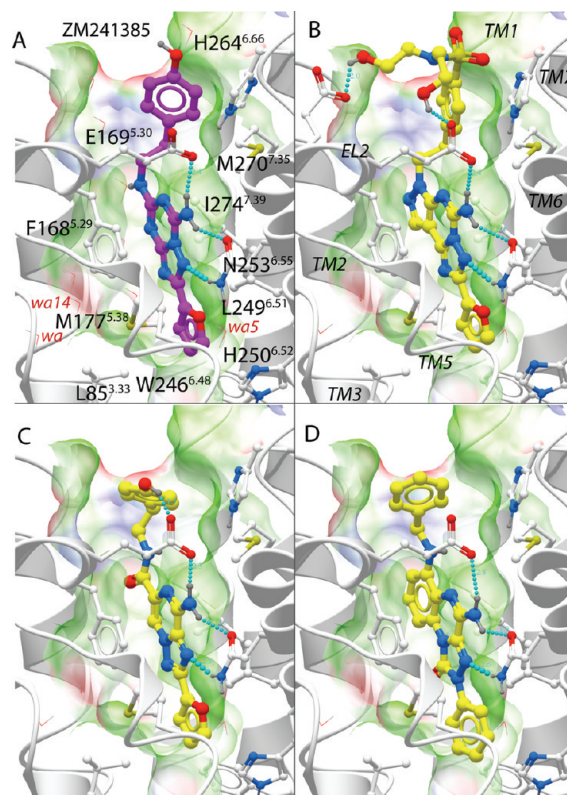


Figure 1. Binding of known antagonists into human adenosine A_{2A} receptor models. (A) Co-crystal structure with compounds **1** as in PDB entry 3EML,³⁵ ligand carbons are shown in magenta color. (B–D) Optimized model 3EML_{W3_opt} with three top scoring compounds from the benchmark set (B) mantri(n),²⁷ (C) mantri(f),²⁷ (D) mantri(k).²⁷ For all ligands, the A_{2A} binding pocket is shown as transparent skin colored by properties (green, hydrophobic; red, acceptor; blue, donor of H-bond). Water molecules are shown by thin red lines and with labels for the three structured waters with lowest *B* factors. Hydrogen bonds are shown by cyan spheres.

motif involves stacking between aromatic moieties of the ligands and the conserved Phe168(5.29) side chain of the receptor, as well as polar interactions with conserved Asn253(6.55) side chain (Residue numbers in parentheses are based on Ballesteros–Weinstein nomenclature⁴²). Compounds shown in Figure 1 also form a hydrogen bond between the unsubstituted exocyclic amine group and the Glu-169(5.30) side chain. In addition to these core interactions,

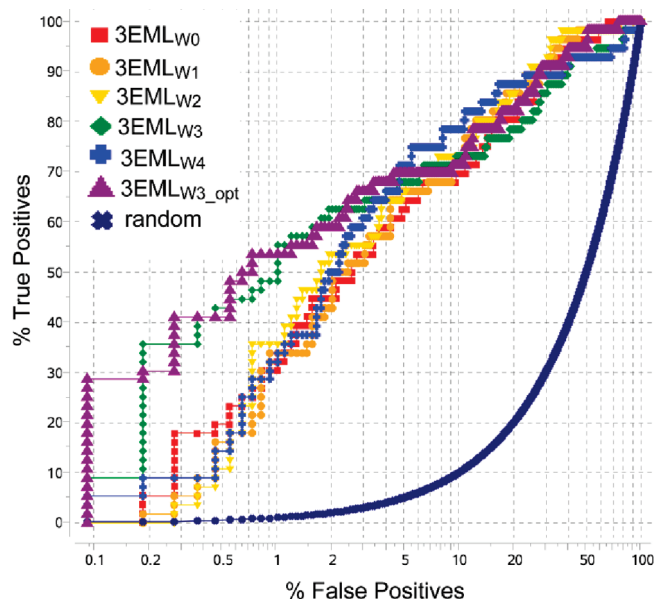


Figure 2. Performance of $A_{2A}AR$ screening models with different number of structured water molecules (W0 to W4) and with conformational optimization (W3_opt). Receiver operating characteristic (ROC) curves are shown in semilogarithmic scale.

most high affinity $A_{2A}AR$ antagonists have an aromatic group extending deeper into the binding pocket and/or flexible extensions toward the extracellular opening of the pocket.

The results in Figure 2 assess overall performance of several screening models, which were generated from the crystal structure (3EML) by optimizing polar hydrogens and structured water configuration. The receiver operating characteristic (ROC) curves, in semilogarithmic scale to emphasize initial enrichment, suggest very good overall screening performance for all six models, with the area under curve (AUC) values within 89–91% range. However, the model with three structured water molecules in the binding pocket, 3EML_{W3}, achieved a significantly higher initial enrichment factor, $EF(1\%) = 70\%$, as compared to the 3EML_{W0} model without water, $EF(1\%) = 43\%$, or any of the other 13 water configurations tested. Analysis of the crystal structure shows that the three waters selected for the 3EML_{W3} model (*wa*, *wa14*, and *wa5* in the 3EML PDB entry) have the lowest *B* factor values and form an extended hydrogen bonding network with the binding pocket residues, suggesting their highly structured nature. While the presence of water in the models does not contribute significantly to the predicted binding scores of the known ligands, the selected structured water molecules occupy highly polar subpockets in the $A_{2A}AR$ and apparently prevent adverse binding of some decoy compounds into these subpockets.

Further modest improvement of initial recognition performance ($EF(1\%) = 78$) was achieved for 3EML_{W3_opt} model by ligand-guided optimization of side chains in the binding site of the $A_{2A}AR$ structure, as described in Methods. More than 200 candidate models were obtained by docking representative antagonists into the all-atom model of the $A_{2A}AR$ with fully flexible side chains of the binding pocket. The best performing model was selected based on the same VLS benchmark test as described above, using a normalized square root AUC (*NSQ_AUC*) as a metric for model selection (see Methods for *NSQ_AUC* definition).

Note that the conformational changes between the crystal structure and the 3EML_{W3_opt} model are minor, with rmsd about 0.27 Å for the 12 side chains of the binding pocket.

Identification of Candidate $A_{2A}AR$ Antagonists by Virtual Compound Screening. The optimized model of the $A_{2A}AR$, 3EML_{W3_opt}, was then used to screen for candidate binders from about 4.3 million commercially available drug- and lead-like compounds collected in the Molsoft ScreenPub nonredundant screening database. About 1800 of the library compounds docked into the $A_{2A}AR$ model with the ICM binding score better than the standard threshold (-32 kJ/mol). These candidate hits were clustered into 340 groups for chemical similarity (Tanimoto distance cutoff > 0.3); the cluster size ranged from 1 to 277 compounds. In each cluster, we selected from 1 to 10 different compounds according to their predicted binding score and predicted ligand efficiency, LE_{pred} . The LE_{pred} , which is defined as binding score per heavy atom of the compound, may correlate with compound suitability as a lead for chemical optimization. Other criteria for the compound selection included their predicted log *P* (octanol/water partition) and log *S* (aqueous solubility) values, as well as immediate availability of compounds from major chemical vendors. Compounds with less than 0.3 Tanimoto distance to known AR ligands in GLIDA database⁴³ were removed to maintain novelty of the compounds in the testing library. This resulted in a final set of 56 diverse drug-like or lead-like compounds that were ordered and screened in experimental assays (see Supporting Information Table SII for the full list of tested compounds).

Figure 3 illustrates predicted $A_{2A}AR$ binding poses of selected candidate compounds representing 16 different chemical scaffolds. All candidate chemotypes except the **L** cluster were predicted to share some key features with antagonist (**1**) binding in the $A_{2A}AR$ crystal structure, including stacking interaction with Phe168(5.29) and hydrogen bonds between exocyclic amine donor and acceptors in Asn253(6.55) and Glu169(5.30) side chains. Most compounds have an additional acceptor, which forms a hydrogen bond to Asn253(6.55) donor in our models.

Apart from these core interactions, many predicted compounds in Figure 3 (clusters **A**, **D**, **F**, **G**, **H**, **I**, **K**, **P**) have moieties extending deep in the binding pocket toward Trp246(6.48) and His250(6.52) and making additional, mostly hydrophobic contacts. Many compounds also have extensions toward the extracellular opening of the pocket (clusters **A**, **B**, **D**, **E**, **F**, **G**, **J**, **K**, **M**, **N**, **O**) and/or toward helix TM1 (clusters **B**, **E**, **H**, **N**, **O**), which may play a role in their subtype selectivity.

Results of $A_{2A}AR$ Binding Assay in Insect Cell Membranes.

Experimental testing of the 56 selected compounds resulted in identification of 23 new $A_{2A}AR$ ligand with K_i better than $10 \mu M$ (41% hit rate) and 11 ligands with K_i better than $1 \mu M$. The most efficient $A_{2A}AR$ binders are presented in Table 1; this includes compounds **9** ($K_i = 0.032 \mu M$) and **15** ($K_i = 0.06 \mu M$) and other compounds with sub- μM affinity or exceptional ligand efficiency. For the results of the binding test for all 56 compounds, see Supporting Information, Table SII.

Diversity of the identified $A_{2A}AR$ ligands is supported by the fact that compounds in Table 1 belong to six different clusters (**A**, **B**, **C**, **D**, **E**, **I** in Figure 3) and an additional three clusters (**F**, **G**, **H**) contain other binders with K_i better than $10 \mu M$. The *novelty* of the ligands was also assessed by searches in GLIDA database that comprises 795 specific binders of ARs used in clinical studies and research. We confirmed that all

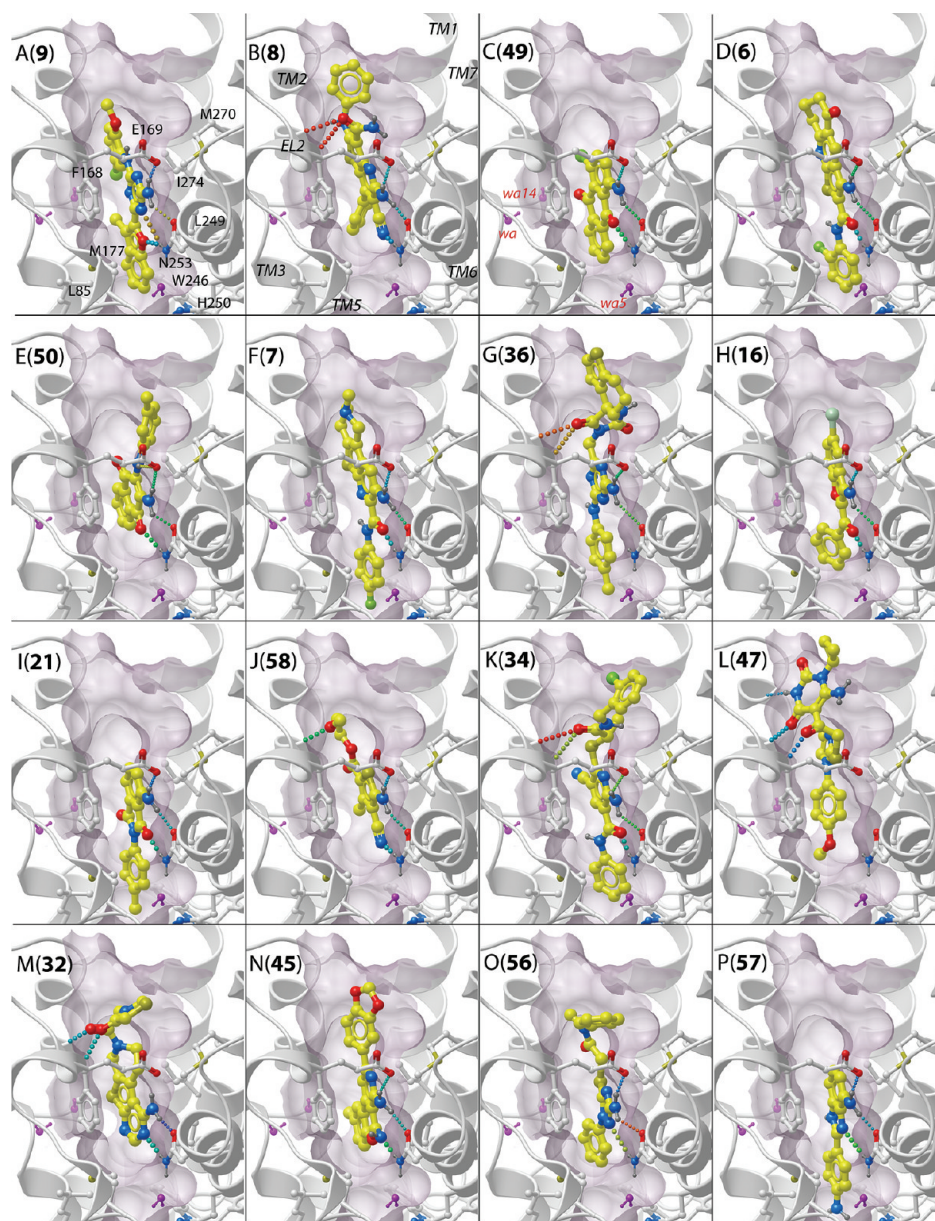


Figure 3. Examples of binding poses in the 3EML_{W3_opt} receptor model for predicted A_{2A}AR candidate ligands representing 16 different chemical clusters. The letters (A–P) show cluster names, and the numbers in brackets indicate compound IDs. Labels for the (i) contact side chains, (ii) TM domains, and (iii) structured waters of the receptor model are shown in (A–C). Side chains of the receptor are shown by sticks with white carbon atoms; in ligands the carbon atoms are colored yellow. Structured water molecules in the model are shown by purple sticks. Hydrogen bonds are colored according to their predicted strength, from blue (strongest) to red (weakest). The binding pocket surface is shown by purple skin, which is clipped in the foreground for better view.

compounds in Table 1 have Tanimoto distances exceeding 0.3 to their closest analogues in the GLIDA database.⁴³

Figure 4 illustrates the binding curves for some of the compounds in Table 1. Antagonists **1** and **2** (theophylline), as well as agonist NECA⁴⁴ (**3**), were used as the reference compounds, and their observed K_i values (0.0006, 0.140, and 4.4 μM , respectively) correlated well with the K_i values published previously.¹² The K_i value and the binding curve for compound **9** are shown for concentrations below 2 μM ; at this concentration, the compound displaces up to 80% of the hot ligand. In contrast, at higher concentrations of this compound (10–1000 μM), displacement of the hot ligand drops to lower levels (see Supporting Information Figure SI1). This unusual behavior of compound **9**, confirmed in 6 runs of the binding assay, can be attributed

to cooperative aggregation, as also suggested by visible sedimentation of the compound at concentrations above 10 μM .

Note that all A_{2A}AR binders shown in Table 1 have ligand efficiency better than 0.3 kcal/mol per heavy atom, which is considered to be optimal for lead-like compounds.⁴⁵ The highest value LE = 0.5 kcal/mol per heavy atom was detected for compound **49** (18 heavy atoms and MW = 257) and two of its analogues in cluster C. Smaller compounds, like compound **21** in cluster I with LE = 0.4 kcal/mol per heavy atom, may be especially valuable as lead scaffolds for drug discovery because they provide more room for chemical optimization.⁴⁵ Each of the chemical clusters and some of the binding features of the ligands are discussed in more detail in the Discussion section.

Table 1. Representative Hits Clustered by Different Chemotypes, with Their A_{2A}AR Binding Characteristics and Predicted ICM Scores^d

ID Cluster	Chemical Structure	K _i μM	95% confidence interval	Max ^a Inhibition %	LE ^b	ICM Score ^c	Closest Analog in GLIDA (Tanimoto Distance)
9 A		0.032	(0.023-0.043)	80	0.38	-36.3	
8 B		0.63	(0.12-3.3)	54	0.3	-34.3	
15 B		0.06	(0.0082-0.45)	91	0.45	-32.4	
49 C		0.25	(0.21-0.3)	100	0.5	-36.4	
35 C		0.64	(0.43-0.94)	96	0.4	-38.1	
29 C		0.32	(0.25-0.4)	88	0.49	-37.5	
26 C		0.53	(0.29-0.98)	90	0.45	-37.8	
6 D		0.3	(0.15-0.62)	70	0.35	-35	
14 D		1	(0.73-1.4)	78	0.35	-34.8	
10 D		2.7	(1.4-5.3)	92	0.3	-36.6	
50 E		0.8	(0.44-1.5)	78	0.39	-37.8	
17 E		0.56	(0.19-1.6)	80	0.35	-38.4	
21 I		2.9	(1.7-5.1)	66	0.4	-38.5	

^a Maximum Inhibition was achieved at 10 μM concentration for compound 9 and at 32 μM for compound 15. All other compounds showed maximum inhibition at 1 mM. ^b Experimental ligand efficiency, kcal/mol, calculated as ligand binding energy per heavy atom. ^c Predicted ICM binding score, kJ/mol. ^d Clusters are named as in Figure 3. The last column shows compounds in GLIDA database of ~30000 GPCR ligands⁴³ with highest chemical similarity to the identified hits.

Ligand Binding and AR Subtype Selectivity in Mammalian Cell Membranes.

In addition to Sf9 insect cells, binding of

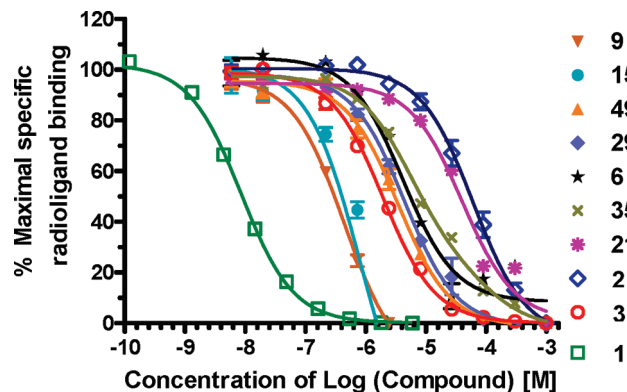


Figure 4. Examples of competition binding curves for the identified A_{2A}AR ligands, as compared for known A_{2A}AR antagonists 1, 2, and agonist 3. Tritiated compound 1 was used as the radioligand.

some A_{2A}AR ligands was also assessed for A₁ and A₃ AR subtypes expressed in mammalian cells (A_{2B} subtype selectivity was not measured here). The left columns in Table 2 show the results of this assay for the A_{2A}AR in HEK293 cell membranes. These results confirm the K_i values under 1 μM in mammalian-based assays for all submicromolar binders shown in Table 1. The observed variations in affinities are expected between Sf9 and HEK-based assay systems, as these assays involve a different pattern of posttranslational modifications, lipid composition, and a different set of experimental conditions. The most significant deviation was found for compound 10, which did not show any substantial binding to the HEK293 expressed A_{2A}AR protein.

Results of binding to the A₁AR subtype in the right columns of Table 2 suggest only a modest decrease in binding affinities for majority of the tested compounds, as compared to A_{2A}AR binding. Substantial (more than 10-fold) selectivity for the A_{2A}AR over the A₁AR was observed for two compounds, 17 and 50, which both belong to the same chemotype (cluster E). One compound, 8, had a pronounced selectivity to the A₁AR subtype (20-fold). Full affinity measurements for A₃AR subtype were not attempted because single point assays suggested that none of the compounds had apparent selectivity to the A₃ subtype while a majority was characterized by the impaired A₃AR binding (see Supporting Information Figure S12).

The Identified Compounds Inhibit A_{2A}AR-Mediated cAMP Production in Functional Assays.

We performed cell-based functional assays to evaluate activity of the newly identified adenosine A_{2A} receptor binders as receptor antagonists. HEK293T cells stably expressing the A_{2A}AR were stimulated with either 10 nM or 10 μM of the A_{2A}AR agonist 3a CGS21680 (K_i = 27 nM),¹² and intracellular cAMP levels were determined using the LANCE cAMP TrFRET kit (Perkin-Elmer), as described in Methods. The ability of the various compounds to block A_{2A} receptor mediated cAMP generation was assessed at a 10 μM concentration of the compounds. The results in Figure 5 show that 11 out of the 14 tested A_{2A}AR binders were able to effectively block more than 75% of cAMP generation at 10 nM concentration of agonist 3a, which strongly supports their A_{2A}AR antagonist activity. Most effective antagonists (compounds 29, 35, 49) inhibited more than 90% of cAMP production for cells stimulated with 10 nM of 3a. Because of the comparatively

Table 2. Competition Binding of the Hit Compounds to Mammalian Expressed Adenosine Receptor Subtypes A_{2A} and A₁

ID	Displacement of radioligand at A _{2A} AR-HEK membranes			Displacement of radioligand A ₁ AR-CHO membranes			A _{2A} / A ₁ selectivity fold
	K _i , μM	(95% Confidence Interval), μM	Max Displacement ^a , %	K _i , μM	(95% Confidence Interval), μM	Max Displacement ^a , %	
6	0.06	(0.03-0.12)	48	0.09	(0.04-0.19)	32	1.42
8	1.14	(0.67-1.94)	53	0.06	(0.05-0.08)	100	0.05
9	0.49	(0.20-1.17)	41	1.44	(0.56-3.70)	41	2.94
10	>10	ND	ND	1.12	(0.44-2.88)	28	ND
14	0.06	(0.05-0.09)	54	0.14	(0.04-0.52)	25	2.24
15	0.56	(0.31-0.99)	73	0.48	(0.12-1.96)	27	0.87
17	0.72	(0.55-0.95)	73	>10	ND	ND	>10.
21	3.01	(0.69-13.1)	57	0.39	(0.31-0.50)	86	0.13
26	0.10	(0.08-0.11)	100	0.26	(0.21-0.33)	100	2.74
29	0.11	(0.09-0.14)	100	0.28	(0.23-0.33)	100	2.51
35	0.20	(0.15-0.25)	100	0.15	(0.13-0.17)	100	0.75
49	0.94	(0.78-1.13)	100	0.19	(0.16-0.22)	100	2.01
50	0.15	(0.11-0.21)	50	1.75	(0.45-6.81)	27	11.42

^aMax displacement refers to the maximal displacement of bound radioligand observed using 10 μM of test compound.

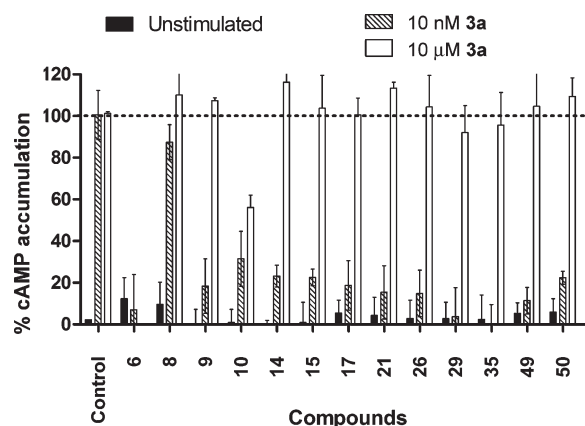


Figure 5. Inhibition of agonist-induced cAMP production by the A_{2A}AR hit compounds. Activity is measured at 10 μM concentration of the compounds, as compared to no compound in the control. Data is normalized where 0% represents the unstimulated condition (black bars) and 100% represents the accumulation of intracellular cAMP observed for stimulation with compound **3a** at 10 nM or 10 μM concentrations (shaded and open bars, respectively).

low affinity of the tested compounds as compared to the agonist **3a**, the majority of compounds were unable to block the effect of this agonist used at a saturating concentration of 10 μM. No A_{2A}AR specific activity was observed for compound **8**. Compounds **6** and **10** blocked cAMP production at both 10 nM and 10 μM concentrations of **3a** to the same extent, suggesting their ability to inhibit cAMP production was not A_{2A}AR-specific. Subsequent experiments revealed that compound **6** inhibited forskolin stimulated cAMP generation, clearly indicating an effect independent of the A_{2A}AR (data not shown).

Discussion

Our results suggest that receptor-based virtual ligand screening is an efficient tool for discovery of novel chemically diverse antagonists of adenosine A_{2A} receptor. A number of A_{2A}AR ligands selected here by VLS not only display sub-micromolar affinity and significant functional activity in mammalian cells but also have small molecular weight and high ligand efficiency (LE > 0.3 kcal/mol per heavy atom) suitable for lead optimization. Below we discuss each of the diverse chemical scaffolds identified and their A_{2A}AR interaction modes as predicted by VLS docking (see Figure 3 and Table 1 above).

Cluster **A** is represented by only one compound **9** based on 1,3,5-triazine-2,4-diamine scaffold, which has the best A_{2A}AR affinity in the set ($K_i = 0.03 \mu\text{M}$ in Sf9-based assay). The functional activity of compound **9** in HEK293 cells is also one of the highest among the tested ligands (see Figure 4). As seen in Figure 3A, compound **9** is predicted to form a hydrogen bond network with Asn253(6.55) and Glu169(5.30). The compound's 3-methyl-benzofuran moiety is extended downward and reaches deeper into the binding pocket than the furan moiety of the antagonist **1** in the crystal structure,³⁵ suggesting that this part of the pocket can be exploited in ligand design. The 5-chloro-2-methoxy-phenyl moiety stretches toward TM2 helix and forms predominantly hydrophobic contacts with Ile66(2.64), Ala63(2.61), and Ile274(7.39), although an alternative extended orientation of this ring has also been observed with only slightly inferior binding scores.

Cluster **B** includes compounds **15** and **8** ($K_i = 0.06$ and $0.63 \mu\text{M}$, respectively in Sf9 assays), which have a common 2-amino-3-cyano-4-phenylpyridine motif, although in a different context of bi- and tricyclic aromatic ring systems. For both compounds, the amine and cyano moieties were

predicted to form a strong hydrogen bonding network with Asn253(6.55) and Glu169(5.30). Interestingly, while compound **1** and most other known antagonists have nearly flat configurations deep in the binding pocket, the phenyl ring of both compounds **15** and **8** is intrinsically out of plane with the core pyridine ring. The nonplanar configuration is apparently accommodated by the pocket as it is in the crystal structure, but this feature may result in different conformational preferences for the A_{2A}AR receptor, potentially leading to unusual binding and/or functional properties of these ligands. Indeed, we found that compound **8** does not have any functional activity in A_{2A}AR cells (see Figure 4); at the same time it is the only compound in our set with significant binding selectivity to A₁AR subtype.

Cluster **C** is the most populous one and represents as many as nine 1-amino-anthraquinone compounds in our test list. Of those, four (**26**, **29**, **35**, **49** in Table 1) have sub- μ M affinity for the A_{2A}AR and another three (**38**, **20**, **28** in Table SII of the Supporting Information) have low μ M affinity. Quite remarkably, all four sub- μ M ligands in this cluster have significant functional activity as antagonists in mammalian cells. Some of the smaller compounds in this group are characterized by an exceptionally high ligand efficiency (LE) that exceeds 0.45 kcal/mol per heavy atom (compounds **49**, **29**, **26**). These compounds have different small groups in anthraquinone position 4, which seems to be rather permissive to substitutions. This site may be useful for optimization of the scaffold affinity and chemical properties, although a few bulky substituents tested in this initial screening (**38**, **20**, **18**, **23**) were apparently suboptimal. Compound **35** ($K_i = 0.64 \mu\text{M}$ in Sf9) has a modified scaffold with an additional conjugated ring, which extends toward TM2 and makes contacts with the binding pocket side chains. Interestingly, anthraquinone-based analogues of Reactive Blue 2 dye have been found that inhibit distantly related purinergic P2Y receptors,⁴⁶ but no AR activity for this chemotype had previously been reported to the best of our knowledge.

Cluster **D** comprises a relatively diverse set of compounds with a common thieno[2,3-*b*]pyridin-3-amine core motif and a variety of amide or ketone substituents in position 2; some of the highest affinity compounds in this cluster (**6** ($K_i = 0.3 \mu\text{M}$ in Sf9), **14**, **51**, **10**) are presented in Table 1. In addition to the common set of core interactions, cluster **D** compounds have aromatic groups predicted to extend deep into the binding pocket, although too bulky or highly polar extensions apparently compromise binding (e.g., compound **13** in Table SII of the Supporting Information). Functional assays for compound **6**, however, suggest that while this compound blocks cAMP signaling, the effect does not depend on agonist concentration. Additional experiments (results not shown) confirm that compound **6** might inhibit cAMP production through an A_{2A}AR-independent mechanism.

Cluster **E** includes 2,3-diaminonaphthalene-1,4-dione compounds **50** and **17** with $K_i < 1 \mu\text{M}$. Both compounds have an aryl substitution at one of the amines, which is predicted to extend toward extracellular loops. An interesting feature suggested by docking for both compounds is a polar interaction of both unsubstituted and substituted amine groups with the Glu169(5.30) side chain, as shown in Figure 3E. Both compounds show significant activity in functional assays. Interestingly, compounds **50** and **17** are the only compounds in the set with a pronounced A_{2A} subtype selectivity (> 10 fold). Given their small size and very good ligand efficiency LE ~ 0.4 kcal/mol per heavy atom, they could provide reasonable leads for optimization of A_{2A} selectivity.

Cluster **F** is represented in our set by only one compound **7** with $K_i = 1.9 \mu\text{M}$ (Supporting Information Table SII). It has a pattern of interaction somewhat similar to cluster **D**, although its tricyclic system has a different orientation and extends more toward TM2 helix.

Cluster **G**, which has a 1,3,5-triazine-2,4-diamine moiety similar to cluster **A**, lacks the methyl-benzofuran extension and is likely to have a different binding mode with the phenylamine ring oriented deep into the binding pocket. Seven out of the nine tested compounds of this cluster showed some A_{2A}AR affinity, although only two had K_i under $10 \mu\text{M}$ (compounds **36** and **31** in Supporting Information Table SII).

Cluster **H** includes two compounds (**16** and **46** in Supporting Information Table SII) based on different chemical scaffolds sharing a similar core polar interaction motif, i.e., amine donor and carboxyl acceptor connected to a five-membered ring. Although the compounds have only modest affinity, their small size leaves room for optimization (ligand efficiency LE = 0.38 and 0.32 kcal/mol per heavy atom, respectively).

Cluster **I** has only one representative compound **21**, based on 4-amino-2-isoindole-1,3-dione scaffold, shown in Table 1. Though affinity of this small compound is modest, it has a very high ligand efficiency (LE = 0.38 kcal/mol per heavy atom) and may represent an attractive lead for optimization.

Other clusters (**J–X**) of the set do not have compounds with affinities under $10 \mu\text{M}$, though some of them, e.g., compound **57** in cluster **P** with LE = 0.32 kcal/mol per heavy atom, may still present interesting leads. No active compounds were identified in clusters **Q**, **R**, **T**, **U**, **V**, **W**, and **X**.

Although highly diverse, all high scoring VLS compounds used in experimental testing contained an *exocyclic amine group*. The preference for compounds with exocyclic amines is also noticeable in the evaluation benchmark with the crystal structure (*3EML_{W0}*) or optimized models (*3EML_{W3_opt}*), where other A_{2A}AR ligand types all scored below the default binding score threshold of -32 kJ/mol, with only a few xanthine analogues approaching this threshold. The docking preference for exocyclic amine compounds apparently reflects their exclusively high affinity to the A_{2A}AR (the best compounds have $K_i < 0.1$ nM).¹² At the same time, it may be at least partially attributed to some bias in the crystal structure of the A_{2A}AR complex with ligand **1**. For example, the flexible side chain of Glu169(5.30) in the EL2 is perfectly positioned to form a strong polar interaction with the unsubstituted exocyclic amine of compound **1**. As we have shown previously, binding of other ligand classes, e.g., xanthine analogues lacking an exocyclic amine may be somewhat suboptimal in this conformation on the A_{2A}AR⁴⁷ and thus may require a modified model or multiple models to capture the associated pocket plasticity.⁴⁸

Most of the novel A_{2A}AR antagonists tested for subtype selectivity are characterized by reduced binding to A₃AR subtype (see Supporting Information Figure S12). Such A_{2A}/A₃ selectivity is expected for chemotypes with unsubstituted exocyclic amine forming a hydrogen bond interaction network with Asn253(6.55) and Glu169(5.30) side chains in the A_{2A}AR, because A₃ has a hydrophobic valine in the 5.30 position. No substantial A_{2A}/A₁ selectivity was observed for most compounds in the set, with only two compounds **17** and **50** having 10-fold selectivity to the A_{2A}AR and only one compound **8** with about 20-fold selectivity to A₁ subtype. At the same time, most of the newly discovered scaffolds boast compounds with very high ligand efficiency LE > 0.30 kcal/mol per heavy atom. Predicted models of ligand receptor

interactions can suggest specific functional groups exposed to the extracellular opening of the binding pocket and provide an accurate structural template for rational optimization of selectivity and chemical properties for these ligands.

Conclusions

Our results present one of the first examples of virtual ligand screening, which employs a high resolution crystal structure of a GPCR target. We show that for adenosine A_{2A} receptor structure, which has a deep and well-defined binding pocket, virtual ligand screening can predict small molecule binders with success rates of as high as 41%. The 23 ligands selected by VLS from a 4 million compound library and confirmed active by A_{2A}AR binding assays represent at least nine novel chemotypes, supporting high diversity of the hits generated through structure-based virtual screening. Two of the identified compounds had *K_i* values of 0.06 μM and below, and 11 had submicromolar binding affinity. As expected in screening with antagonist-bound model of the receptor, most ligands (10 out of 13 tested) were proved A_{2A}AR antagonists in functional assays, while none showed agonistic activity. The virtual screening in this study did not explicitly target subtype selectivity, yielding hits that bind equally well to both A_{2A} and A₁ adenosine receptor subtypes. Most hit compounds, however, are relatively small (< 400 Da) and have high ligand binding efficiency (LE > 0.3 kcal/mol per heavy atom), suitable for optimization of their subtype selectivity, affinity, and chemical properties. Overall, the results demonstrate that high resolution structures of GPCR, combined with accurate docking and virtual ligand screening methods provide a highly efficient tool for identification of new GPCR antagonist chemotypes as lead candidates for drug discovery.

Experimental Section

1. Conformational Methods. Generation of All-Atom A_{2A}AR Models. The initial all-atom model of the adenosine A_{2A} receptor was prepared from the PDB coordinates (PDB ID: 3EML)³⁵ using molecular conversion procedure,⁴⁹ implemented in ICM molecular modeling software (Molsoft, LLC). The procedure includes the addition of hydrogen atoms to the PDB receptor structure, selection of the energetically favorable conformations of *His*, *Asn*, and *Gln* side chains, and local minimization of polar hydrogens in the internal coordinates space.

For each of the 13 water configuration models tested, hydrogen atoms of the structured water molecules were co-optimized together with A_{2A}AR hydrogens, while coordinates of water oxygen atoms were retained as in the crystal structure. The 3EML_{W1} model included only one water molecule, *wa*, with the lowest *B* factor = 39 (as in the PDB entry), the 3EML_{W2} model comprised *wa* and *wa14* (*B* factor = 46), the 3EML_{W3} model had *wa*, *wa14*, and *wa5* (*B* factor = 62). Other water configurations were obtained by adding water molecules in the binding pocket in the order of their increasing *B* factor.

Virtual Ligand Libraries for Optimization and Validation of A_{2A}AR Models. A diverse set of 23 known high affinity A_{2A}AR antagonists was compiled from 8 A_{2A}AR clinical candidates listed in Table 1 of ref 12 and an additional 15 compounds in Figure 2 of ref 28. The all-atom molecular models of antagonists were built from their published 2D structures. A library of 2000 decoy compounds was randomly selected from Chemdiv discovery collection of drug-like compounds (www.chemdiv.com).

Ligand Docking and Small Scale VLS Benchmark. To use ICM fast docking and VLS procedures, the receptor all-atom models were converted into energy potential maps calculated on a fine 3D grid (0.5 Å cell).⁴⁹ The grid potential maps account for van der Waals, hydrogen-bonding, hydrophobic, and electrostatic interactions between ligand and receptor.^{50,51} The ligand

was represented by an all-atom model and considered fully flexible in the potential field of the receptor. The ligand and decoy compounds were automatically docked into the A_{2A}AR models using the biased probability Monte Carlo (BPMC) global energy minimization procedure⁵² and sorted according to their ICM binding scores. To ensure convergence of the Monte Carlo optimization, three independent runs of the docking procedure were performed, and the best scoring pose per compound was kept. No distance restraints or any other experimentally derived information was used in the ligand docking procedure. The docking procedure takes about 30 s of Intel Xeon 2.8 Ghz CPU time per compound and was performed using a 100 processor Linux cluster.

Metrics for VLS Benchmark Performance. On the basis of the values of ICM binding scores for the docked compounds, we used several complementary metrics to assess VLS performance.⁵³ The *Initial Enrichment Factor at 1% cutoff* was calculated as:

$$EF(1\%) = (Lf / (Lf + Df)) / (Lt / (Lt + Dt))$$

where *Lt* and *Dt* are the total number of known ligands and decoy compounds in the data set, respectively, while *Lf* and *Df* are the number of known ligands and decoys respectively in the top 1% scoring fraction the database. *Receiver Operator Characteristic* (ROC) curves were plotted with *True Positive Rate* (TP = *Lf/Lt*) on the *Y* axis vs *False Positive Rate* (FP = *Df/Dt*) on the *X* axis for different fractions *f* of the data set.

Area under ROC curve (AUC) was calculated along with *Normalized Square Root AUC* (NSQ_AUC).⁴⁷ For NSQ_AUC, the area AUC* is calculated for the ROC curve plotted with *X* coordinate *X* = √FP. The NSQ_AUC value is then calculated as:

$$NSQ_AUC = 100((AUC^* - AUC_{random}^*) / (AUC_{perfect}^* - AUC_{random}^*))$$

The value of NSQ_AUC is more sensitive to initial enrichment than the commonly used linear AUC. The NSQ_AUC measure returns the value of 100 for any perfect separation of signal from noise and values close to 0 for a random subset of noise.

Additional Ligand Guided Receptor Optimization. A set of more than 200 conformational models with 3EML_{W3} water configuration was generated by co-optimization of representative A_{2A}AR antagonists in the binding pocket of the receptor model. The optimization was performed using BPMC method,^{50,52} which allows extensive sampling of the flexible ligand and flexible receptor side chain conformations. The protein backbone and water oxygen atom coordinates were fixed to that of the crystal structure (PDB: 3EML). For each of the 200 resulting conformational models, a small-scale VLS benchmarking was performed as described above and the model with the best NSQ_AUC value, 3EML_{W3_opt} was selected as an optimal model for VLS screening.

VLS of Available Compound Libraries and Selection of Candidate A_{2A}AR Ligands. Virtual screening of a large library of available compounds was performed using the same ICM VLS procedure as in the small-scale benchmark above. The screening library was prepared from the Molsoft ScreenPub database of 4.3 million unique drug-like compounds available from more than 40 vendors by automatically removing compounds with reactive groups and molecular weight exceeding 500 Da. On the basis of docking results, compounds with an ICM binding score better than -32 kJ/mol were selected and clustered by chemical similarity with 0.3 Tanimoto distance cutoff. Predicted values of ligand efficiency were calculated as a ratio of ICM binding score to the number of heavy atoms (LE_{pred} = -score/*N*_{HA}). Values of log *P* (octanol/water partition) and log *S* (solubility) were also predicted using algorithms implemented in the ICM software. In each chemical cluster, from 1 to 10 compounds were selected for experimental assays according to their binding score, LE_{pred} values, chemical properties, and on-shelf availability from

vendors, resulting in 56 compounds purchased for the binding assays.

2. In Vitro and In Vivo Assays. The compounds selected by virtual screening were purchased from available screening collections of four vendors, Chembridge (www.Hit2Lead.com), Chemdiv (www.chemdiv.com), Enamine (www.enamine.com), and Sigma-Aldrich (www.Sigmaaldrich.com). Purity of compounds was equal to or greater than 96% as verified by liquid chromatography (HPLC) experiments performed by the vendors.

Binding Assays in Sf9 Membranes. Membranes highly expressing human A_{2A}AR receptor were produced from the Sf9-baculoviral expression system as previously described.⁵⁵ Frozen aliquots of cells were thawed and then resuspended in homogenization buffer (50 mM Hepes, pH 7.4). The cells were subjected to homogenization using a nitrogen cavitation pump (30 min, 800 psi) following 30 strokes with a Dounce homogenizer. The cell debris and nucleolus were removed by centrifugation at 900g for 10 min, followed by centrifugation at 100000g for 45 min to isolate the raw membrane fraction. The resulting membrane pellet was resuspended in the buffer containing 50 mM Hepes, pH 7.4, 800 mM NaCl, and the protein concentration was assayed using the BCA protein assay kit from Pierce (Rockford, IL) using bovine serum albumin (BSA) as a standard for the protein assay. The centrifugation–resuspension cycle was repeated until supernatant fraction did not contain any trace of soluble protein based on the BCA protein assay (typically six cycles). Prior to the ligand binding assays, the membrane pellets were resuspended in ligand binding buffer (TME: 50 mM Tris–HCl, 10 mM MgCl₂, 0.5 mM EDTA, pH 7.4). The samples were tested for binding with tritiated compound **1** ([³H]ZM241385) (27.4 Ci/mmol) obtained from ARC Inc., St. Louis, MO. Crude plasma membranes (0.2–1 μg of total protein per reaction) were incubated for 30 min at room temperature with serial dilutions of the radioligand (0.05–10 nM). Incubations were rapidly terminated by filtration using a Tomtec Mach III cell harvester (Tomtec) through a 96-well GF/B filter plate (MultiScreen Harvest plate, Millipore Corp.) and rinsed five times with 500 μL of ice-cold buffer (50 mM Tris–HCl, pH 7.4). The harvest plates were dried, and 30 μL of OptiPhase–HiSafe III scintillation liquid (Perkin-Elmer Life Sciences) was added. The bound radioactivity was measured using a Perkin-Elmer Wallac Jet 1450 Microbeta scintillation counter. Nonspecific binding was determined in parallel reactions in the presence of an excess of compound **2** (100 μM, Sigma–Aldrich, USA), and specific binding was defined as the difference between total and nonspecific binding. All incubations were performed in triplicate, and independent experiments were repeated at least twice. Equilibrium dissociation constants (*K_i*) and maximal receptor levels (*B_{max}*) were calculated from the results of saturation experiments using GraphPad Prism version 5 Software. For competition binding studies, the crude membranes were resuspended in ice-cold binding buffer (TME: 50 mM Tris–HCl, 10 mM MgCl₂, 1 mM EDTA, pH 7.4) containing protease inhibitors (Complete protease inhibitor cocktail tablet, Roche Applied Science) and homogenized for 30 strokes with a Dounce homogenizer. Test ligand stock solutions were prepared in DMSO at a final concentration of 10 mM. Some of the test ligands have strong dye property that might cause a problem in the assays at the high concentration of the compound, and therefore we tested ligands using full completion range. Identical aliquots of crude plasma membranes (0.2–5 μg of total protein per reaction) containing 10–14 different concentrations of the competing unlabeled ligands with 20 nM of tritiated compound **1** in 0.1 mL total volume. The reaction components were added in order of crude membrane, test ligand, and radioligand. The final DMSO concentration was 10%. Reference compounds **1**, **2**, and **3** were tested with and without 10% DMSO in the reactions. After 60 min at room temperature with shaking, reactions were rapidly terminated by filtration and counted as

mentioned above. Initial testing was performed with in triplicate and was verified with six replicates. Individual assay results are represented.

Experimental *ligand efficiency*⁴⁵ (LE) was defined as $LE = \Delta G/N_{HA}$, where ΔG was the free energy of ligand binding ($\Delta G = -RT \ln K_i$), and N_{HA} is the number of heavy atoms in the compound.

Binding and Selectivity Assays in Mammalian Membranes. [³H]DPCPX and [¹²⁵I]AB-MECA were purchased from Amersham Biosciences (Netherlands), and tritiated compound **1** was obtained from Tocris Cookson, Ltd. (UK). CHO cells expressing the human adenosine A₁ receptor were provided by Dr. Andrea Townsend-Nicholson, University College London. HEK293 cells stably expressing the human adenosine A_{2A} receptor and CHO cells expressing the human adenosine A₃ receptor were kind gifts from Dr. J. Wang (Biogen/IDEC, Cambridge, MA) and Dr. K. N. Klotz (University of Wurzburg, Germany), respectively.

All compounds were tested in radioligand binding assays to determine their affinities at human adenosine A₁ ([³H]DPCPX), A_{2A} (tritiated compound **1**), and A₃ ([¹²⁵I]AB-MECA) receptors as described previously in literature⁵⁴ with the exception that nonspecific binding to the A_{2A} receptor was determined in the presence of 10 μM concentration of **3a** instead of 100 μM CPA and nonspecific binding to the A₃ receptor was determined by the addition of 100 μM compound **3**. The incubation was terminated by filtration over Whatman GF/C filters under reduced pressure with a Brandel harvester (Gaithersburg, MD). Filters were washed three times with ice cold 50 mM Tris/HCl pH 7.4, placed in vials, and counted.

Functional cAMP Accumulation Assays. HEK293 cells expressing the human adenosine A_{2A} receptor were grown as a monolayer on 10 cm culture plates. For cAMP production and determination, 7500 cells/well were used on 384-well plates. The cells were incubated for 45 min at room temperature with or without test compounds at a 10 nM concentration of the agonist **3a** (approximately an EC₂₀ concentration) and with or without test compounds with a saturating concentration of **3a** (10 μM). The assay medium contained cilostamide (50 μM), rolipram (50 μM), and adenosine deaminase (0.8 IU/mL). Incubation was stopped with detection mix and antibody solution was added, these two steps according to the instructions of the supplier. The assay was performed with the Lance cAMP 384 kit from Perkin-Elmer based on the competition of the sample's cAMP with a europium-labeled cAMP tracer complex for binding sites on cAMP-specific antibodies labeled with Alexa Fluor dye.

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Supporting Information Available: Chemical structures, binding assay results, and 3D binding models for all 56 experimentally tested compounds; ZIP file with 56 models of candidate compounds (PDB). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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