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Structure-Based Screening of Uncharted Chemical Space for Atypical Adenosine Receptor Agonists

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Abstract

Small molecule screening libraries cover only a small fraction of the astronomical number of possible drug-like compounds, limiting the success of ligand discovery efforts. Computational screening of virtual libraries representing unexplored chemical space could potentially bridge this gap. Drug development for adenosine receptors (ARs) as targets for inflammation and cardiovascular diseases has been hampered by the paucity of agonist scaffolds. To identify novel AR agonists, a virtual library of synthetically tractable nucleosides with alternative bases was generated and structure-based virtual screening guided selection of compounds for synthesis. Pharmacological assays were carried out at three AR subtypes for 13 ribosides. Nine compounds displayed significant activity at the ARs, and several of these represented atypical agonist scaffolds. The discovered ligands also provided insights into receptor activation and revealed unknown interactions of endogenous and clinical compounds with the ARs. The results demonstrate that virtual compound databases provide access to bioactive matter from regions of chemical space that are sparsely populated in commercial libraries, an approach transferrable to numerous drug targets.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.6b00357. Supplementary Figure S1, Tables S1–S3, Schemes S1–S3, Supplemental Methods, and NMR spectra (PDF)

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Access to ligands that display an ability to modulate protein activity has contributed greatly to our understanding of physiological processes and resulted in numerous pharmaceuticals.¹ A majority of small molecule probes originate from classical medicinal chemistry efforts and high-throughput screening (HTS) campaigns. A large fraction of these probes are related structurally to an endogenous ligand, reflecting that this was the starting point of chemical optimization.² Ligands also stem from HTS hits identified in libraries containing up to millions of compounds, which can now be routinely assayed at industrial and academic screening centers. Considering the astronomical number of possible drug-like molecules ($\sim 10^{23}$ to 10^{60}), both of these approaches are severely limited by the small fraction of chemical space that is experimentally accessible.³ However, computational methods can explore a significantly larger fraction of the chemical universe *via* generation of virtual libraries with billions of synthetically tractable molecules.^{4,5} Screening libraries of this size may soon be within reach for efficient *in silico* approaches, which offer exciting opportunities to discover ligands in uncharted regions of chemical space.^{6,7} To explore this idea, we designed a virtual chemical library tailored for a family of G protein-coupled receptors (GPCRs) and used structure-based screening to prioritize compounds for synthesis and experimental evaluation.

The human adenosine receptors (hARs) are membrane proteins that belong to the GPCR superfamily and are divided into four distinct subtypes (A_1 , A_{2A} , A_{2B} , and A_3).⁸ Activation of hARs leads to signaling *via* G proteins that either stimulate (G_s or G_{olf} for A_{2A} and A_{2B}) or inhibit (G_i for A_1 and A_3) the production of intracellular cAMP. The adenylyl cyclase and other effector pathways may contribute to cytoprotective effects in response to cellular stress conditions, *via* elevated extracellular levels of adenosine.⁹ For this reason, hAR agonists have been pursued as therapeutic agents for the treatment of pain, inflammation, and cardiovascular diseases.^{9,10} The A_{2A} AR agonists adenosine (**1**) and regadenoson (**2**) are FDA-approved for myocardial perfusion imaging. Furthermore, A_1 and A_3 AR agonists have been the focus of testing in clinical trials for treatment of diabetes-associated hypertriglyceridemia (CVT-3619, **3**) and autoimmune inflammatory conditions (IB-MECA, **4**), respectively (Figure 1).¹¹ Extensive medicinal chemistry efforts focused on the hARs have been based on the structure of adenosine, which can be divided into two individual ring systems, e.g., the adenine and ribosyl groups. Adenine derivatives devoid of a ribose-like group are always antagonists of ARs. Numerous agonists are found among ribose-containing AR ligands, pointing toward a critical role of this moiety in AR activation.⁹ However, with only rare exceptions,¹² all hAR agonists bearing ribose-like groups also have an adenine-like base. Ribosyl ligands with nonadenine bases thus represent unexplored chemical space for

these targets and could potentially lead to the discovery of selective agonists and provide novel insights into receptor activation.

To identify novel AR agonist scaffolds, we designed a virtual library containing synthetically tractable ribosides and used structure-based *in silico* screening to guide the selection of compounds for synthesis. Experimental evaluation of the successfully synthesized compounds led to the discovery of novel riboside agonists with nonadenine bases and identified unknown interactions of endogenous and clinical ligands with the ARs. Analysis of atomic-resolution models for complexes provided new insights into molecular recognition by hARs. The prospects of extending our virtual library and the broader application of this approach in drug discovery will be discussed.

RESULTS AND DISCUSSION

Design and Molecular Docking Screens of a Virtual Riboside Library

To assess the potential of designing ribosides bearing novel bases, we inspected crystal structures of the human A_{2A}AR, the only subtype from this receptor family with available atomic-resolution X-ray data.^{13,14} In the adenosine-bound A_{2A}AR crystal structure,¹⁵ the adenine group was involved in hydrogen bonds with the family conserved residue Asn253^{6,55} (generic GPCR residue numbers indicated in superscript¹⁶). The ribosyl moiety of adenosine extended into a deeply buried pocket, where the 3'- and 2'-hydroxyl groups established hydrogen bonds to Ser277^{7,42×41} and His278^{7,43×42}, respectively (Figure 2b). The adenine formed well-defined polar and π -stacking interactions with Asn253^{6,55} and Phe168^{45,52}, respectively, which suggested that many nucleobase-like moieties could replace this planar group while maintaining the ability of the resulting riboside to bind to the receptor. Analysis of the A₁ and A₃AR homology models also revealed differences in the adenine-recognizing region, most notably the Val169^{45,53} residue present in the latter subtype (compared to Glu172/169^{45,53} of A₁ and A_{2A}AR), which suggested that alternative nucleobases could lead to the discovery of selective scaffolds.

Motivated by the lack of riboside scaffolds with alternative bases, we investigated the availability of such compounds in commercial databases. To this end, substructure searches for adenine- and/or ribosyl-containing molecules were performed in the ZINC database of commercially available molecules.¹⁷ There were only 339 ribosides with nonadenine bases in the ZINC lead-like library (Figure 3), which was >13-fold less than the number of compounds containing only adenine. This probably reflects focus in drug discovery on an abundance of proteins that recognize purine-containing compounds, on one hand, and on the synthetic challenges involved in carbohydrate chemistry, on the other.¹⁸ To circumvent the shortage of purchasable ribosides, we identified nucleobase-sized heterocycles in commercial libraries that could be coupled *via* a nitrogen atom to the ribosyl group through a β -glycosidic bond (Figure 4). The resulting ribosides were also required to have a base containing both hydrogen bond donor and acceptor groups to favor interactions with the conserved binding site residue Asn253^{6,55}. There were 5452 compounds that complied with these constraints, which resulted in a focused library of 6847 unique ribosides after considering multiple attachment points on each building block. The final virtual library was thus 17-fold larger than the set of ribosides that was available in stock from commercial

vendors and 99% had nonadenine bases (Figure 3). Although the library only covered a small fraction of all theoretically possible ribosides, a point to which we will return, it provided an excellent starting point to identify agonists with novel base scaffolds.

As synthesizing the thousands of ribosides in the virtual library would be prohibitively challenging, we used molecular docking screening to identify candidates for experimental evaluation. The virtual library was screened against a crystal structure of the A_{2A}AR in an active-like conformation (PDB code 2YDO).¹⁵ This receptor structure was selected because all the compounds in the virtual library contained a ribose group, which was also part of the cocrystallized ligand adenosine. These calculations were also complemented by screening against homology models of the A₁ and A₃ARs. Docking screens against the three receptor subtypes were carried out with the program DOCK3.6.^{20,21} All structures were prepared in the presence and absence of a crystallographic water molecule (wat2016) that formed hydrogen bonds with both the adenine and ribose moiety of adenosine in the A_{2A}AR crystal structure (Figure 2b). Molecular docking against the A_{2A}AR crystal structure strongly enriched known agonists over property-matched decoys.²² Addition of the water molecule both resulted in an improved docking energy for adenosine and led to a 2-fold increase of the enrichment factor in the top 1% of the database. The two additional crystallographic waters that formed hydrogen bonds with adenosine (wat2017 and wat2018) were not included in the docking screens because they either did not interact with the adenine base (wat2017) or were solvent exposed (wat2018). The sampling of the ribosides in the rigid binding site was restricted to reproduce the orientation observed for the sugar moiety in the crystal structure. The base was sampled in a large number of conformations, and each pose was evaluated by a physics-based energy function.^{20,21} The top 10% of the ranked library for the A_{2A}AR was inspected visually and guided compound selection. Compound rankings were also calculated for the A₁ and A₃ARs based on median ranks over 100 receptor conformations for each subtype. Encouragingly, several adenosine-like molecules were found among the top-ranked compounds. There were also a large number of ribosides with bases that were dissimilar to adenine but had favorable binding energies and formed hydrogen bonds to Asn^{6.55}, a key residue for ligand recognition.^{15,23} Ribosides with good complementarity to the binding site were selected based on manual inspection of the docking results for the A_{2A}AR crystal structure, taking into account energy terms neglected by the docking scoring function, as described previously,²² and commercial availability. The selected compounds all scored in the top 10% of the virtual library for this subtype and were typically also top-ranked for the A₁ and A₃ARs. In a second step, a subset of 18 compounds, which explored several different chemotypes, was finally selected for further consideration. On the basis of visual inspection, the bases of the selected compounds could be grouped into purine- (**5–11**, and **19**), pyrimidine- (**12–14**), andazole-like (**15–17**, and **22**) chemotypes, whereas the remaining three compounds (**18**, **20**, and **21**) explored other scaffolds (Supporting Information Table S1). Six of these were available directly from commercial sources, providing an opportunity to compare activities for compounds originating from commercial and virtual chemical space.

Synthesis and Pharmacological Evaluation of Predicted Riboside Ligands

The building blocks needed for synthesis of 12 compounds (**7**, **8**, **10**, **11**, **14**, **15**, **17**, and **18–22**) were sourced from commercial vendors. Vorbrüggen glycosylation reactions were successful in some cases,²⁴ including a variation consisting of a one-step microwave-assisted reaction.²⁵ Where several possibilities existed, the regioselectivity of the glycosylation reactions was confirmed using 1D NOESY or HMBC (Heteronuclear Multiple Bond Correlation) NMR experiments (Supporting Information). Single products with the desired chemical identity were successfully obtained for seven ribosides (Table 1). In attempts to synthesize compounds **10**, **18**, and **19**, the undesired products **23–25** were first obtained due to linkage to alternative nitrogens of the building block (Supporting Information Table S2). Interestingly, whereas the synthesis of compound **23** required microwave conditions, the targeted compound **10** was successfully obtained using conventional reaction conditions. Despite several synthesis attempts using both conventional and microwave conditions, no products corresponding to compounds **18–22** were obtained. Details for synthesizing all compounds can be found in the Supporting Information (Supplemental Methods and Schemes S1–S3).

Radioligand binding assays were performed at the human A₁, A_{2A}, and A₃ARs (expressed in CHO or HEK cells) for the 16 ribosides that were either purchased or obtained from synthesis. Full dose–response curves were determined for nine compounds displaying significant displacement of the radioligand at 10 μM. Seven compounds were ligands of the A₁AR, and the most potent of these (**5**) had a K_i value of 84 nM (Table 1). Of the seven A₃AR ligands, five had submicromolar affinities for this receptor (**5**, **7**, **9**, **11**, and **14**). Two ribosides displayed significant binding to the A_{2A}AR (**5** and **9**). Several ligands were found to be subtype-selective for either the A₁ (e.g., **16**) or the A₃AR (e.g., **7**) or both of these subtypes over the A_{2A}AR (e.g., **11**). Finally, none of the three undesired products (**23–25**) showed significant displacement of the radioligand at any of the three AR subtypes examined (Supporting Information Table S2).

As the largest number of ligands was identified for the A₁ and A₃AR subtypes, assessment of functional activity was mainly focused on G_i-mediated effects by measuring inhibition of forskolin-stimulated intracellular cAMP production in response to the most potent compounds. The adenosine-like ligands **5**, **7**, and **9** showed efficacy corresponding to 86–106% of the maximal effect observed for nonselective agonist 5'-N-ethylcarboxamidoadenosine (NECA) at 10 μM and were thus full agonists for A₁ and/or A₃ARs, consistent with binding affinities. However, no influence on cAMP levels was detected for compound **6** at the A₁AR despite its very close similarity to adenosine. Similarly, compound **11** was an agonist of the A₁AR but did not show significant activity for the A₃ subtype at 10 μM. Two ribosides with atypical bases, compounds **12** and **14**, were evaluated in concentration–response experiments (Figure 5). Compound **12** was a partial agonist (~70% maximal efficacy) of the A₁AR with an EC₅₀ value of 1.5 μM, whereas this relatively weak A₃ ligand only showed activation of this subtype (~30% maximal efficacy) at the highest tested concentration. Compound **14** was a full agonist of both the A₁ and A₃AR subtypes with EC₅₀ values of 0.6 and 0.4 μM, respectively. Compounds **5**, **6**, **9**, and **14** were also tested in functional assays for the A_{2B}AR. Compounds **5** and **9** were full

agonists with EC₅₀ values of 1.5 and 3.1 μM, respectively, whereas compounds **6** and **14** were inactive at 10 μM, which was in agreement with the binding affinities measured for the A_{2A}AR.

As synthesis involved a significant effort, we were interested in exploring if binding of the discovered ligands to the ARs could have been predicted based on the properties of the bases used as reagents. To investigate this, adenine (**26**) and the bases used to synthesize ribosides **7**, **11**, and **14** (compounds **27–29**) were evaluated in radioligand binding assays at 30 μM for the A₁, A_{2A}, and A₃ARs (Table 2). Some displacement of radioligand was observed for compound **27** (base of riboside **7**) at the A₃AR (48%), but not for the other two subtypes. Compound **28** (base of riboside **11**) showed the largest displacement of radioligand at the A₁AR subtype (78%) and, to some extent, also at the A_{2A} (40%) and A₃ (36%) subtypes. No significant displacement was observed for adenine (**26**) or compound **29** (base of riboside **14**) at any of the three subtypes examined.

Structure–Activity Relationships for Discovered Ligands

Several of the discovered riboside ligands represented novel scaffolds, and structure–activity relationships (SAR) provided new insights into nucleobase recognition by the ARs. Compounds **5–7** and **9** probed the molecular recognition of ribosides with adenine-like bases, and the predicted binding modes for these compounds matched that of adenosine in the crystal structure of the A_{2A}AR (Figure 6). The binding affinities for the close analogs **5–7** revealed an intriguing SAR around positions N1 and N3 of the adenine scaffold. Compound **5** (1-deazaadenosine) was a potent and nonselective ligand, but 3-deaza- and 1,3-didezaadenosine (**6** and **7**, respectively) completely lost affinity for the A_{2A}AR (Figure 6a,b). Interestingly, the N1 and N3 atoms of adenosine did not have any polar interactions with the receptor in the A_{2A}AR crystal structure. However, a network of ordered waters resolved in the binding site appeared to play a crucial role in agonist recognition. In particular, wat2016 was found to stabilize the complex by coordinating both the N3 and O2' atoms of the adenine and ribose moieties, respectively, in the A_{2A}AR crystal structure (Figure 2b). The lack of hydrogen bonding groups in position 3 disrupts this hydrogen bond network, which provides an explanation to why compounds **6** and **7** were inactive at the A_{2A}AR. The importance of the N3 nitrogen was further supported by the functional data for the A₁AR. Although significant A₁AR affinity was maintained for compound **6**, the compound lost the ability to activate the receptor, suggesting an important role of this water molecule in the activation mechanism. Interestingly, the A₃ subtype was less affected by modifications at the N1 and N3 atoms, and compound **7** was a strongly selective agonist for this subtype. One possible explanation is the presence of the bulkier residue Leu90^{3,32} (Val^{3,32} for the other three AR subtypes) in the ribose binding site of the A₃AR, which may displace wat2016. In summary, the series of adenosine-like agonists provided insights into recognition of the endogenous agonist and demonstrated that changes in selectivity and efficacy could be achieved *via* subtle changes to this scaffold.

The novelty of the discovered ligands was quantified by calculating the Tanimoto similarity to known hAR ligands from the ChEMBL database.²⁶ The Tanimoto similarity coefficient (*T_c*) is equal to 1 for identical pairs of compounds, whereas values close to zero are obtained

for unrelated molecules. The calculated T_c values were relatively high for the tested compounds, ranging from 0.39 (**10**) to 0.69 (**5**). However, in most cases, the high similarity appeared to reflect that the compounds contained the ribose group, which was further supported by all of the closest hAR ligands having adenine-like bases (Supporting Information Table S3). The Tanimoto similarity of the bases of the discovered ligands to adenine was instead calculated to assess novelty. The low T_c values obtained in this case demonstrated that the docking screen identified diverse and novel riboside agonists with purine- (compound **11**, $T_c = 0.21$), pyrimidine- (compound **14**, $T_c = 0.31$), andazole-like (compound **16**, $T_c = 0.21$) bases (Table 1). The most potent of these, compound **11**, contained 1-methylxanthine as a nucleobase and was an agonist of the A₁AR with a K_i value of 130 nM (Figure 6d). Several novel AR ligands had pyrimidine-related bases (e.g., 4-amino-1,3,5-triazin-2(1*H*)-one **12**, 5-methylcytosine **13**, and 6-amino-5-chloropyrimidin-4(3*H*)-one **14**). Similar to adenosine, these compounds were predicted to hydrogen bond to Asn^{6.55} (Figure 6e–g). Notably, several of the discovered ligands with nonadenine bases had polar atoms positioned to interact with the wat2016 molecule, in agreement with the observations made for adenosine (Figure 2b and Figure 6). The most potent pyrimidine-containing agonist was compound **14**, which had K_i values of 359 and 557 nM at the A₁AR and A₃AR, respectively. To further probe the SAR for this scaffold, an analog without the chlorine on the pyrimidine group was evaluated, but this compound (**37**) was inactive (Supporting Information Table S2), suggesting that this substituent is important for high affinity binding. The nucleoside analogue and drug candidate 5-azacytidine (**12**) had K_i values of 2.0 and 3.7 μ M at the A₁ and A₃ARs, respectively, whereas the endogenous 5-methylcytidine²⁷ (**13**) had an affinity of 8.0 μ M for the A₃ subtype. Finally, theazole-like base of compound **16** could potentially represent a promising starting point for the development of subtype-selective agonists of the A₁AR.

Theoretical Size of the Virtual Library and Origin of Commercially Available Ribosides

By identifying commercially available heterocyclic compounds that could be linked to a ribosyl group, we were able to create a library of synthetically tractable molecules that was an order of magnitude larger than the set of commercially available ribosides. The library could be further expanded by considering the vast number of nucleobase-like molecules that are currently not available commercially as building blocks. To estimate the maximal size of the tailored virtual library, we turned to the chemical universe database GDB-13, which enumerates chemically stable molecules up to a 13 heavy atom count (HAC) based on the elements C, N, O, S, and Cl.²⁸ As the bases of all ribosides synthesized in this work were part of GDB-13, the theoretical size of the tailored library could be computed using our virtual protocol (Supporting Information Supplemental Methods). More than 18.0 million GDB compounds fulfilled our criteria for synthetic feasibility and physicochemical properties, leading to a tailored virtual library with nearly 19.6 million compounds after consideration of multiple attachment points.

Of the 19.6 million synthesizable ribosides, only 323 were available in the ZINC database. To investigate the origin of the commercially available compounds, we analyzed how many ribosides from the ZINC and GDB-derived libraries were available in the Human Metabolome Database (HMDB),²⁹ which contains endogenous and xenobiotic (e.g., drugs)

compounds that can be found in the human body, or the Dictionary of Natural Products (DNP).³⁰ HMDB and DNP contained a set of 113 ribosides, of which 50 (44%) were present in the ZINC database. Interestingly, if the 323 compounds from ZINC had been selected at random from the GDB-derived database, the probability of obtaining any 50 ribosides from HMDB or DNP would equal 4.8×10^{-209} . These results demonstrate that there is a strong bias toward human metabolites, drugs, and natural products among commercially available ribosides. Indeed, the six ribosides purchased directly from vendors were either natural products (**9**, **13**),³⁰ drugs used against cancer or viral infections (**12**, **16**),^{31,32} or ligands explored for those indications (**5** and **6**).^{33–35} All of these displayed significant binding to at least one of the ARs. Of the seven synthesized ribosides that were selected from the virtual chemical space (Table 1), five (**8**, **10**, **11**, **14**, and **15**) were novel compounds that were not present in the HMDB, DNP, ChEMBL, or ZINC databases.^{17,26,29,30} Whereas all the submicromolar AR ligands identified from commercial sources had adenine-like bases, the most potent of the synthesized compounds explored novel scaffolds, e.g., the agonists **11** and **14**.

Structure-Based Screening of Uncharted Chemical Space Enables Discovery of Atypical AR Agonists

Three key findings emerge from this study. First, virtual compound databases can provide access to molecules from regions of chemical space that are sparsely populated in commercial libraries. In order to explore the role of the adenine base of adenosine in AR activation, we searched for ribosides with alternative bases in commercial compound databases. Surprisingly, despite ribosides playing key roles in numerous physiological processes, only a small number of such compounds were available. By designing a virtual library that could be synthesized from commercial building blocks, we were able to access thousands of ribosides with novel bases. Second, structure-based screening successfully guided compound selection from the virtual library. Nine of the 13 predicted ligands displayed significant binding, and several of these compounds represented novel agonist scaffolds. Finally, the discovered ligands provided new insights into receptor activation and suggested that endogenous ribosides other than adenosine may be involved in signaling mediated by this receptor family.

Considering the limited fraction of drug-like space that can currently be screened for ligands, the composition of the chemical library will be crucial for success. Efforts to synthesize biologically active molecules have populated chemical libraries with compounds that are similar to biogenic substances.³⁶ During the past decade, high-throughput techniques have contributed to a rapid expansion of screening libraries and have increased the fraction of aromatic and achiral molecules in commercial chemical space.³⁷ The biogenic bias and low molecular complexity of screening collections have been instrumental for the success of HTS, as molecules from these two sources are likely to display activity at a given target.³⁸ In this spirit, medicinal chemistry efforts for the ARs have mainly been based on planar scaffolds rather than stereochemically rich ribose-like moieties. This strategy has resulted in the discovery of thousands of AR antagonists, of which several have reached clinical trials.^{9,10} In contrast, only two classes of AR agonists have been identified.⁹ The determination of crystal structures of GPCRs has provided unique opportunities for the

design of ligands with specific signaling properties.¹⁴ Seven class A GPCRs have been crystallized both with agonists and antagonists, revealing interactions responsible for receptor activation.^{15,19,23} In the active-like A_{2A}AR structure, adenosine is deeply buried in the binding site and is involved in an intricate hydrogen bonding network, which suggests that compounds with a high molecular complexity are required for receptor activation.²² On the basis of the crystal structure, we hypothesized that ribosides should have a high probability of achieving activation. Remarkably, close to 20 million ribosides (HAC 22) could theoretically be synthesized, but only 0.002% of these were commercially available. To explore uncharted parts of chemical space, we designed a focused virtual library of ribosides that could be synthesized by combining the ribosyl group with a purchasable building block. Seven of the 12 compounds that were prioritized for synthesis were successfully obtained, and three of these were confirmed as active in AR binding. A strategy that could potentially improve the efficiency of our approach is to evaluate binding of the base before synthesizing the riboside, which was explored for four AR ligands. For the compounds representing the bases of **7** and **11**, significant binding was observed, and their subtype selectivity partially agreed with that of the corresponding riboside. However, for the bases of compound **14** and adenosine, no significant binding was found, suggesting that a fragment-based approach would only be partially predictive of riboside activity. Overall, prioritizing ribosides from virtual chemical space that were not available commercially or previously explored in the literature proved worth the effort as several novel agonists originated from synthetic efforts and had submicromolar binding affinities.

New agonist scaffolds can provide novel insights into the structural basis of receptor activation. To investigate the role of the base of riboside ligands, we first evaluated a series of compounds with varying similarity to adenosine. Remarkably, subtle modifications to the adenine group led to large changes in ligand affinity, selectivity, and efficacy. Despite the fact that a crystal structure of the A_{2A}AR guided compound selection, this receptor was found to bind the lowest number of tested compounds, while the A₁ and A₃ subtypes were more tolerant. Unexpectedly, an efficacy switch was observed for the A₁AR when a single nitrogen (N3) in the adenine ring of adenosine was replaced by a carbon atom. This revealed that an ordered water molecule is likely to play a key role in binding and activation at the A₁ and A_{2A}ARs, a finding that will be important to consider in future design of AR agonists. For example, the design of ligands with substituents that displace this water molecule could lead to the discovery of agonists with improved affinity and subtype selectivity. These results are also in agreement with previous studies demonstrating that consideration of explicit water molecules can improve predictions of ligand binding energies and enhance virtual screening performance.^{39,40} We have shown that isoguanosine (**9**) binds to all of the ARs and acts as an agonist. This compound, also known as crotonoside, has antibiotic and anticancer activity.^{41,42} Two of the discovered A₃AR ligands with nonadenine bases were the antineoplastic drug 5-azacytidine (**12**) and the metabolite 5-methylcytidine (**13**). The mechanism of action of 5-azacytidine is poorly understood,³¹ and our results suggest that interactions with the A₃AR, a target for cancer,⁴³ could be partially responsible for its therapeutic effect. The physiological concentrations of 5-methylcytidine have been found to be elevated during cancer⁴⁴ and may reach concentrations in plasma that could activate the

A₃AR. The potential role of the A₃AR in mice tumor reduction upon treatment with 5-methylcytidine⁴⁵ may thus merit further investigation.

The most important result of this study is the use of virtual chemical libraries to discover novel riboside agonists **11** and **14**, which both have nonadenine bases and are potent A₁ and A₃AR ligands. Interestingly, the base of **11** is a xanthine, a naturally occurring scaffold extensively explored as AR antagonists.⁹ Ribosides bearing bases with a 1,3-dialkylxanthine scaffold have previously been found to be agonists of the rat A₃AR.⁴⁶ Compound **11** identified in this work achieved submicromolar affinities for both hA₁ and hA₃ARs, but only activated the A₁ subtype. Pyrimidine ribosides have not been described as AR ligands, and the new scaffolds represented by compounds **12** and **14** could contribute to the development of lead candidates for the A₁ and A₃ subtypes. In addition, compound **12** is a partial A₁AR agonist, which is an advantageous pharmacological profile for cardioprotection with a reduced risk of bradycardia.⁴⁷ Together, our results demonstrate that structure-based screening of virtual libraries can be a powerful approach to the discovery of novel lead candidates. In this work, we greatly expanded the range of candidate molecules for virtual screening by considering separate domains of the nucleoside to be joined chemically. By further extending virtual libraries to the billions of compounds that remain unexplored for biological activity, our approach is directly transferrable to numerous other drug targets.

METHODS

Generation of a Virtual Riboside Library

A virtual library of synthetically tractable ribosides was generated by replacing the adenine moiety of adenosine with commercially available compounds. The ZINC library was filtered using the software FILTER⁴⁸ to identify building blocks containing an aromatic nitrogen donor (SMARTS pattern: *a[nh;r]a*) that had all the following properties: (i) MW < 200, (ii) neutral and nonzwitterionic, (iii) 1–2 ring systems, (iv) hydrogen bond acceptors > 1 and hydrogen bond donors > 2. All tautomeric forms considered in the ZINC library were filtered to take into account multiple attachment points for each building block. The library of ribosides was generated by attaching a ribose moiety to each protonated aromatic nitrogen of the compounds satisfying the filtering criteria using the software Reactor.⁴⁹ The resulting virtual chemical library was prepared for molecular docking using the ZINC database protocol.¹⁷

Homology Modeling of the A₁ and A₃ARs

MODELLER 9.11⁵⁰ was used to generate homology models of the human A₁ and A₃ARs in active-like conformations based on a manually edited multiple-sequence alignment of the human ARs (Supporting Information Figure S1 and Supplemental Methods). The main template was the structure of the A_{2A}AR bound to the agonist UK-432097 (PDB code 3QAK).⁵¹ A total of 250 models were generated for each subtype, and the 100 top-ranked models according to the DOPE scoring function were used in the molecular docking screens.

Molecular Docking Screens

The crystal structure of the human A_{2A}AR in complex with adenosine (PDB code 2YDO)¹⁵ and homology models of the A₁AR and A₃AR were prepared for docking with DOCK3.6.^{20,21} The protonation states of ionizable residues were set to the most probable at pH 7. Tautomeric states of binding site histidines were set by manual inspection based on the local environment.²² His251/250^{6,52} (A₁ and A_{2A}ARs) and His278/278/272^{7,43×42} (A₁, A_{2A}, and A₃ARs) were protonated at Ne and Nδ, respectively. His264/264^{EL3} (A₁ and A_{2A}AR) was treated as doubly protonated. All receptors were prepared for docking in the presence and absence of a crystallographic water molecule. Sampling parameters were selected to favor the conformation of the ribose moiety observed for agonists in A_{2A}AR crystal structures. For each ligand conformation, the binding energy was estimated as the sum of the electrostatic and van der Waals interaction energies,²¹ corrected for ligand desolvation.²⁰ The side chain dipole moment for Asn^{6,55} was increased to favor hydrogen bonding with this residue, as previously described.^{22,52–54}

Synthesis of Nucleosides

Six ribosides were sourced directly from commercial vendors: compounds **6**, **12**, **13** and **16** (Sigma-Aldrich); compound **5** (Tocris); and **9** (CarbonSynth). Starting material for the synthesis of the remaining compounds was purchased from vendors Enamine, Molport, VitasM, and Scientific Exchange. Targeted ribosides were typically obtained through Vorbrüggen glycosylation reactions. The purity of all ribosides tested was determined as 95% by LC/MS spectra, and chemical identity was confirmed by ¹H NMR. Details about synthesis and structural assessments for relevant compounds can be found in the Supporting Information.

Radioligand Binding and Functional Experiments

Radioligand binding assays were performed using membranes prepared from CHO (hA₁AR or hA₃AR) or HEK293 (hA_{2A}AR) cells stably expressing a single hAR subtype (Supporting Information Supplemental Methods). CHO cells were used for functional assays at all hARs. The binding affinity for hA₁, A_{2A}, and A₃ARs was expressed as *K_i* values (*n* = 3, 4). Binding parameters were calculated using Prism 6 software (GraphPAD, San Diego, CA, USA). IC₅₀ values obtained from competition curves were converted to *K_i* values using the Cheng–Prusoff equation. All data are expressed as mean ± standard error resulting from three independent experiments. For determination of cAMP production, an ALPHAScreen cAMP kit (PerkinElmer) was used according to the manufacturer's instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

HTS	high-throughput screening
GPCR	G protein-coupled receptor
AR	adenosine receptor
HMBC	heteronuclear multiple bond correlation
cAMP	3',5'-cyclic adenosine monophosphate
NECA	5'-N-ethylcarboxamidoadenosine
SAR	structure–activity relationship
HAC	heavy atom count
DNP	dictionary of natural products
HMDB	human metabolome database

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regadenoson (2)

A_1AR : >10,000 nM

$A_{2A}AR$: 290 nM

A_3AR : >10,000 nM

CVT-3619 (3)

A_1AR : 55 nM

$A_{2A}AR$: >10,000 nM

A_3AR : >1000 nM

IB-MECA (CF101, 4)

A_1AR : 51 nM

$A_{2A}AR$: 2900 nM

A_3AR : 1.8 nM

Figure 1.

Examples of AR agonists. K_i values for the human A_1 , A_{2A} , and A_3AR s are reported elsewhere.⁹

Figure 2. Antagonist- and agonist-bound structures of the A_{2A}AR. Crystal structures of the A_{2A}AR bound to the antagonist ZM241385 (PDB code 4EIY)²³ and to the endogenous agonist adenosine (PDB code 2YDO)¹⁹ are shown in panels a and b, respectively.

Figure 3.

Presence of adenine and ribosyl moieties in commercially available and virtual compound libraries. Numbers of compounds resulting from substructure searches for adenine and ribosyl groups in the ZINC database and in the virtual riboside library are represented with Venn diagrams. In the ZINC database, 4,489 compounds contained adenine and 339 had a ribosyl moiety. Of these, 68 contained both an adenine and ribosyl group. The virtual library contained 6,784 compounds. All of these had a ribosyl group, and 63 contained an adenine moiety.

Figure 4.

Generation of a tailored virtual library of synthesizable ribosides. Commercially available building blocks containing a ring system (R) with an aromatic nitrogen (n) donor bound to any two atoms (a) were attached to a ribosyl moiety (SMARTS search pattern: *a[nh;r]a*).

Figure 5. Functional cAMP assays for compounds **12** and **14**. Dose-response curves show levels of inhibition of forskolin-stimulated intracellular cAMP production relative to NECA in cells overexpressing either A_1 or A_3 AR.

Figure 6. Predicted binding modes for discovered AR ligands. Docking poses for compounds **5**, **7**, **9**, **11–14**, and **16** (panels a–h, respectively) in structures of relevant AR subtypes. The ligands and selected receptor side chains are shown in sticks.

Table 1

Experimental Data for Predicted Ligands^d

ID	2D structure	% inhibition at 10 μ M of K_i (nM) ^b			T_c of base to adenine ^c
		A ₁ AR	A _{2A} AR	A ₃ AR	
5		84 $\pm$ 9	645 $\pm$ 151	583 $\pm$ 23	0.48
6		461 $\pm$ 19	24 $\pm$ 8%	51 $\pm$ 11%	0.43
7 ^a		15 $\pm$ 2%	5 $\pm$ 2%	823 $\pm$ 172	0.34
8 ^a		47 $\pm$ 6%	6 $\pm$ 2%	14 $\pm$ 4%	0.41
9		467 $\pm$ 126	495 $\pm$ 10	348 $\pm$ 36	0.39
10 ^a		2 $\pm$ 2%	3 $\pm$ 8%	49%	0.18

ID	2D structure	% inhibition at 10 μ M of K _i (nM) ^b			T _c of base to adenine ^c
		A ₁ AR	A _{2A} AR	A ₃ AR	
11^a		130 $\pm$ 46	25 $\pm$ 11%	917 $\pm$ 42	0.21
12		2,010 $\pm$ 830	34 $\pm$ 2%	3,680 $\pm$ 1,110	0.29
13		10 $\pm$ 3%	13 $\pm$ 9%	8,020 $\pm$ 1,420	0.20
14^a		359 $\pm$ 121	35 $\pm$ 11%	557 $\pm$ 84	0.31
15^a		34 $\pm$ 4%	25 $\pm$ 1%	7 $\pm$ 4%	0.31
16		7,410 $\pm$ 100	23 $\pm$ 6%	16 $\pm$ 5%	0.21
17^a		4 $\pm$ 3%	10 $\pm$ 7%	15 $\pm$ 6%	0.27

^aSynthesized compound. The remaining ribosides were sourced from commercial vendors.

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^b All data are expressed as mean $\pm$ standard error resulting from three independent experiments.

^c The 2D Tanimoto similarity (T_c , ECFP4 fingerprints) of the base of the riboside to adenine.

^d Ribosides selected from docking screens of a virtual chemical library were tested in radioligand binding experiments using membranes of mammalian cells overexpressing one of the three hAR subtypes. Experimental data for undesired products from synthesis (23–25) can be found in Supporting Information Table S2.

Table 2Experimental Data for the Bases of Compounds 7, 11, 14, and Adenosine^b

ID	2D structure	% inhibition at 30 μ M ^a		
		A ₁ AR	A _{2A} AR	A ₃ AR
26		19 $\pm$ 8%	19 $\pm$ 8%	13 $\pm$ 0%
27		21 $\pm$ 4%	25 $\pm$ 4%	48 $\pm$ 1%
28		78 $\pm$ 8%	40 $\pm$ 8%	36 $\pm$ 6%
29		11 $\pm$ 3%	23 $\pm$ 0%	19 $\pm$ 5%

^a All data are expressed as mean $\pm$ standard error resulting from three independent experiments.^b Radioligand binding experiments were performed using membranes of mammalian cells overexpressing one of the three hAR subtypes.