

Complementarity between in Silico and Biophysical Screening Approaches in Fragment-Based Lead Discovery against the A_{2A} Adenosine Receptor

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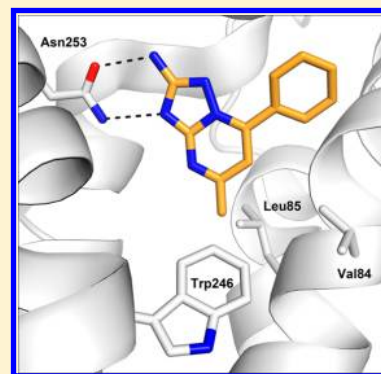
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Supporting Information

ABSTRACT: Fragment-based lead discovery (FBLD) is becoming an increasingly important method in drug development. We have explored the potential to complement NMR-based biophysical screening of chemical libraries with molecular docking in FBLD against the A_{2A} adenosine receptor (A_{2A}AR), a drug target for inflammation and Parkinson's disease. Prior to an NMR-based screen of a fragment library against the A_{2A}AR, molecular docking against a crystal structure was used to rank the same set of molecules by their predicted affinities. Molecular docking was able to predict four out of the five orthosteric ligands discovered by NMR among the top 5% of the ranked library, suggesting that structure-based methods could be used to prioritize among primary hits from biophysical screens. In addition, three fragments that were top-ranked by molecular docking, but had not been picked up by the NMR-based method, were demonstrated to be A_{2A}AR ligands. While biophysical approaches for fragment screening are typically limited to a few thousand compounds, the docking screen was extended to include 328,000 commercially available fragments. Twenty-two top-ranked compounds were tested in radioligand binding assays, and 14 of these were A_{2A}AR ligands with *K_i* values ranging from 2 to 240 μM. Optimization of fragments was guided by molecular dynamics simulations and free energy calculations. The results illuminate strengths and weaknesses of molecular docking and demonstrate that this method can serve as a valuable complementary tool to biophysical screening in FBLD.



■ INTRODUCTION

The adenosine receptors belong to the family of G protein-coupled receptors (GPCRs) and are expressed both in the central nervous system (CNS) and the periphery. The four adenosine receptor subtypes (A₁, A_{2A}, A_{2B}, A₃) are responsible for a wide range of physiological processes by acting on different signaling pathways. The A_{2A} and A_{2B} adenosine receptors increase cAMP levels by coupling to G_s, whereas the A₁ and A₃ subtypes signal via G_i and decrease cAMP levels.¹ A_{2A} adenosine receptor (A_{2A}AR) activation occurs in response to stress or cell damage and protects tissues by controlling inflammation. For this reason, A_{2A}AR agonists are candidates for development of anti-inflammatory drugs.² In the CNS, the A_{2A}AR is expressed in the basal ganglia and counterbalances the action of the D₂ dopamine receptor. The two receptors form heterodimers in the cell membrane, and A_{2A}AR activation leads to a decrease in D₂ dopamine receptor signaling.³ Since Parkinson's disease is characterized by a loss of dopamine receptor activity, A_{2A}AR antagonists have been studied intensively as drug candidates for Parkinson's disease, and several compounds are currently in clinical trials.¹

The rapidly increasing number of crystal structures for G protein-coupled receptors (GPCRs) has made structure-based approaches an attractive strategy for drug design against these pharmaceutically important targets.^{4,5} Twelve high-resolution structures, with bound antagonists or agonists, have been determined for the A_{2A}AR since the first one was solved in 2008.^{6–9} Structure-based computational ligand discovery based on these structures has been surprisingly successful. Two recent molecular docking screens against these structures have resulted in the discovery of several novel A_{2A}AR antagonists.^{10,11} The “hit rates” in these screens, i.e. the percentages of active compounds among those selected for experimental evaluation, were 35 and 41%, which is remarkably good for virtual screening. Similar results were achieved in docking screens against the β₂ adrenergic,^{12,13} D₃ dopamine,¹⁴ CXCR4,¹⁵ and H₁ histamine receptors,¹⁶ which further demonstrated the potential for structure-based drug design against these targets.

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During the past decade, fragment-based lead discovery (FBLD) has steadily increased in popularity as an alternative to high-throughput screening (HTS).^{17,18} The idea behind FBLD is to identify ligands that are about half the size of a drug. The increased coverage of chemical space in fragment libraries makes it possible to identify ligands from screens of only a few thousand compounds. Ligands from fragment screens are often of low affinity and lack selectivity but can serve as a starting point for the development of new lead compounds. There are now a large number of successful examples of where fragment ligands identified by crystallography, surface plasmon resonance (SPR), or nuclear magnetic resonance (NMR) techniques have been used to generate high-affinity leads.¹⁷ In fact, several compounds that originate from fragment screens are now in clinical trials, and the first drug developed from FBLD was approved in 2011.¹⁹ Fragment screens by NMR and SPR have also been carried out against GPCRs,^{20,21} but this is still challenging because of difficulties in expressing these proteins in large quantities and their inherent instability outside the cell membrane. The use of in silico fragment-based screening has also been explored successfully, both for GPCRs^{16,22} and soluble proteins,^{23,24} but structure-based approaches have been questioned in this context.²⁵ Most molecular docking programs were developed for and benchmarked on drug-like compounds and thus may have difficulties to rank fragment ligands, which typically are of low affinity and only occupy subpockets of a binding site.

In this work, we explored fragment-based ligand discovery by carrying out two prospective docking screens against a crystal structure of the A_{2A}AR. The questions that we wanted to address were if molecular docking could be used to discover fragment ligands and if in silico methods in combination with biophysical screening methods could improve the efficiency of FBLD. In a first step, we computationally docked a set of 500 fragments to an A_{2A}AR crystal structure and ranked these compounds by affinity prior to an NMR-based screen of the same library. As the entire library was screened both in silico and experimentally, our blind prediction of the results made it possible to evaluate the utility of molecular docking in FBLD. The comparison of the computational and experimental screens for the small fragment library showed that a majority of the orthosteric ligands were among the top-ranked compounds in the docking screen. In a second step, we extended the docking screen to include several hundred thousand commercially available molecules and tested top-ranked compounds experimentally. The results from this second screen allowed us to directly compare the fragment ligands and hit-rates from biophysical and structure-based computational screening approaches. Fourteen fragment ligands were discovered from the docking screen of this larger set of commercially available molecules. The structure–activity relationships (SAR) were explored for three novel A_{2A}AR ligands, guided by molecular docking and molecular dynamics (MD) simulations.

RESULTS

Ranking of a Fragment Library by Molecular Docking.

To test the performance of molecular docking in FBLD against the A_{2A}AR, parallel experimental and computational screens of an in-house library of 500 chemically diverse fragments were carried out. Prior to screening the fragment library using an NMR-based approach, the same set of molecules was computationally docked to the orthosteric site of an antagonist-bound crystal structure of the A_{2A}AR⁷ using the

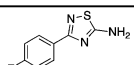
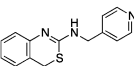
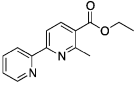
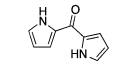
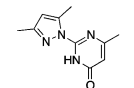
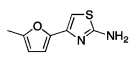
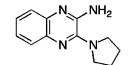
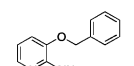
program DOCK3.6.^{26–28} Each molecule was sampled in, on average, 18700 orientations and 16 conformations. For each conformation that did not clash with the receptor, a physics-based scoring function was used to evaluate the complementarity of the fragment to the orthosteric site. The docking score was calculated from the sum of the receptor–ligand electrostatic and van der Waals interaction energy, corrected for desolvation of the ligand. The best scoring conformation for each fragment was used to rank these molecules by their predicted affinity to the A_{2A}AR. In total, 487 of the 500 fragments were successfully scored in the orthosteric binding site. The average computation time per docked molecule was 10 s, and, in total, only 80 min were required to screen the library.

Comparison of NMR-Based and Molecular Docking Screens of a Fragment Library. Given the low affinity and specificity of typical fragment ligands, the molecular docking technique can be expected to have difficulties to rank such compounds by affinity. As every library molecule was tested for binding to the A_{2A}AR in the NMR-based screen, this gave a unique opportunity to assess the ability of molecular docking to discriminate between fragment ligands and experimentally verified nonbinders.

The details of the methodology and experiments used to screen the fragment library against a thermostabilized mutant²⁹ of the A_{2A}AR by NMR have been presented in separate publications.^{21,30} The results of the NMR-based screen of the fragment library are presented briefly in this section to enable a comparison to the docking predictions. Five hundred fragments, which were selected to represent a diverse set of molecules in terms of shape and chemistry,³¹ were screened using the target immobilized NMR screening (TINS) technique.³⁰ The TINS screen resulted in 94 primary hits, which were further evaluated in radioligand displacement assays. Five molecules showed more than 30% displacement of the radioligand at 500 μ M and had well-behaved dose response curves, which corresponds to a hit-rate of 5% for the secondary screen. The five hits are shown in Table 1, and the K_i values for these compounds ranged from 14 to 600 μ M. Another key metric for judging the potential of a fragment ligand is its ligand efficiency (LE). The LE value was calculated as $(RT \ln K_i)/N$, where N is the number of heavy atoms.^{32,33} The best LE value for the fragments discovered in the NMR-based screen was 0.53 kcal mol^{−1} atom^{−1}, which is in the range considered to be promising for further optimization (typically >0.35 kcal mol^{−1} atom^{−1}). Additional experiments demonstrated that 11 of the initial hits were either positive or negative allosteric modulators of the A_{2A}AR.²¹ The remaining hits were found not to displace radioligands from the orthosteric site or allosterically modulate agonist or antagonist binding. These compounds were considered to be less potent fragment ligands, bind to regions that do not influence ligand binding to the A_{2A}AR orthosteric site, or be false positives.

To assess how well the docking was able to predict the results of the fragment screen, we generated receiver operating characteristic (ROC) curves for the identified ligands based on the predicted ranking for the compounds in the library. As the docking calculations only sampled fragment orientations in the orthosteric site, we did not expect to identify any allosteric ligands. For this reason, the ligands were divided into an orthosteric and allosteric group, which made it possible to directly compare the computational and NMR-based screening results. The ROC curves for the ortho- and allosteric ligands

Table 1. Fragment Ligands Discovered in the NMR-Based Screen (1–5)²¹ and False Negatives from the NMR Screen That Were Identified by the Docking Screen (6–8)

ID (Rank) ^a	Ligand structure	K _i (LE ^c) μM (kcal mol ⁻¹ atom ⁻¹)	T _c ^d
1 (26)		14 ± 2.3 (0.53)	0.33
2 (19)		183 ± 20 (0.29)	0.33
3 (7)		324 ± 29 (0.27)	0.39
4 (NS ^b)		472 ± 34 (0.39)	0.42
5 (14)		586 ± 45 (0.31)	0.31
6 (34)		17.6 ± 1.5 (0.56)	0.38
7 (45)		20.6 ± 2.4 (0.42)	0.30
8 (13)		128 ± 11.3 (0.37)	0.44

^aRank by docking screen of fragment library (500 compounds in total). ^bNot scored in molecular docking screen. ^cLigand efficiency. ^dTanimoto similarity (ECFP4 fingerprints) to A_{2A} adenosine receptor ligands in the ChEMBL14 database.

are shown in Figure 1. To quantify the ability of DOCK3.6 to identify fragment ligands, we evaluated enrichment factors at different false positive rates (ROC_EF_{x%}, where *x* denotes the false positive rate).³⁴ For example, the ROC_EF_{5%} value was calculated as the fraction of true positives (ligands) identified

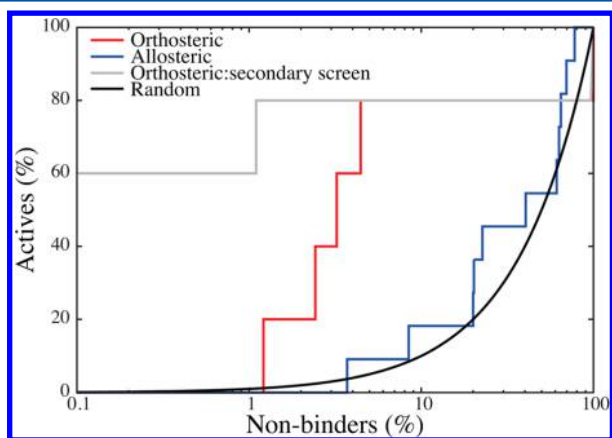


Figure 1. ROC curves for docking enrichment of orthosteric (red line) and allosteric (blue line) ligands discovered in the NMR-based screen. The enrichment of orthosteric ligands among the molecules from the NMR-based screen that were selected for follow-up radioligand assays is also shown (gray line). The black line represents the curve expected from random enrichment.

when 5% of the false positives (nonbinders) had been found in the ranked list divided by the fraction of false positives at that point, i.e. 0.05 in this case.³⁵ No enrichment of orthosteric ligands was found at 0.5%–1% false positive rate. One ligand was identified at a 2% false-positive rate, corresponding to an enrichment factor of 10. Four out of five orthosteric ligands were identified in the top 5% of the ranked database, which corresponded to an ROC_EF_{5%} equal to 16, i.e. a 16-fold improvement over random selection (Figure 1, red curve). The fifth orthosteric ligand, compound 4, was not scored at all, which was due to the sampling scheme used in DOCK3.6. If the sampling parameters were (retrospectively) adjusted to determine a score for compound 4, it was ranked as ~160 and would hence not have affected early enrichment. The enrichment of orthosteric ligands from the subset of 94 primary hits in the NMR-based screen was also calculated. The enrichment in this case was remarkably good, ranking four of the ligands in the very top of the database (Figure 1, gray curve, ROC_EF_{0.5%} = 120). Finally, as may have been expected, the enrichment of allosteric modulators was poor, as reflected by that the ROC curve was closer to random enrichment (Figure 1, blue curve). Predicted binding poses for two of the orthosteric ligands from the NMR-based screen, compounds 1 and 5, are shown in Figure 2. Both compounds interact with Asn253^{6,55} of the A_{2A}AR, a residue that been shown to be critical for recognition of both agonists and antagonists.³⁶

After assessing the ability of molecular docking to predict the results of the NMR-based screen, the top-ranked fragments from the docking calculations were analyzed in more detail. It was encouraging that 80% of the orthosteric ligands were among the top 50 compounds of the ranked library. However, this also suggested that there were 46 top-ranked inactive compounds for which the docking algorithm had predicted similar or better binding energies. To try to understand the origin of these false positives from docking, the pose for each of the top-ranked 50 compounds was inspected visually. If general problems with the scoring function could be identified, this information could be valuable for further development of the molecular docking algorithm. Two observations were made from this analysis. First of all, there were several cases where polar groups of the fragments or the receptor did not form hydrogen bonds but were still desolvated to a large extent, which should be energetically unfavorable. Whereas the ligand desolvation is taken into account by the scoring function in DOCK3.6, the receptor desolvation energy contribution is ignored.³⁷ This may be a reasonable approximation for lead- or drug-like compounds that fill the entire binding site, resulting in a constant desolvation energy contribution. Fragments, on the other hand, often occupy subpockets of the binding site, and in these cases the desolvation term could vary significantly and thus be crucial for accurate ranking.³⁸ The second observation was that there were several top-ranked fragments for which no obvious problems could be identified. Instead, we would likely have predicted several of them to be ligands in a prospective screen against the A_{2A}AR. To test if any of these were false negatives from the NMR-based screen, we selected five compounds from the top 10% of the ranked library for experimental rescreening in radioligand displacement assays. These five molecules had all been screened by the NMR-based method but were not among the 94 compounds that were tested in follow-up assays. Three out of five compounds showed >50% displacement of radioligand binding at 500 μM. The dose–response curves demonstrated that they were

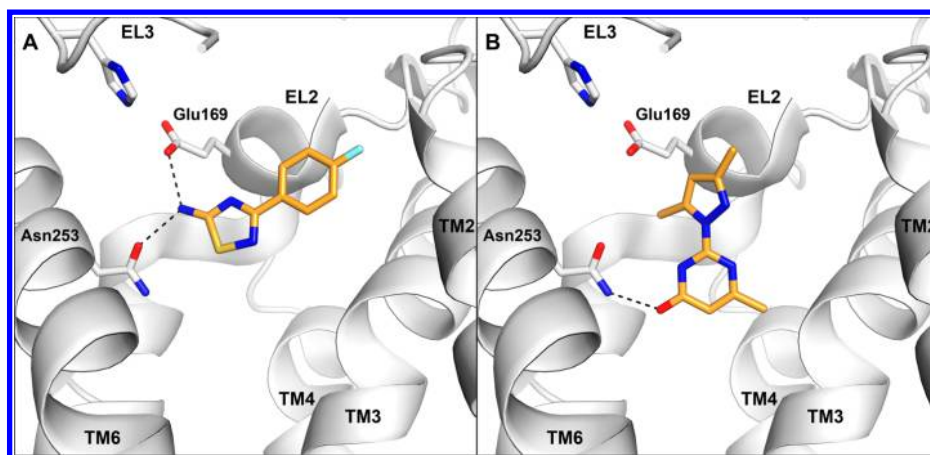


Figure 2. Predicted binding modes for two ligands (1 and 5) that were discovered in the NMR-based screen. The binding site is displayed as a white cartoon with selected side chains shown in sticks. The ligand is depicted with orange carbon atoms. Black dotted lines indicate hydrogen bonds. This figure was generated with PyMOL (version 1.4.1).

orthosteric ligands with K_i values equal to 17.6, 20.6, and 128 μM (compounds 6–8, Table 1). Compound 6 was ranked as number 34 in the docking screen and had the highest LE of all $A_{2A}\text{AR}$ fragment ligands discovered by docking or NMR ($\text{LE} = 0.56 \text{ kcal mol}^{-1} \text{ atom}^{-1}$), and the predicted binding mode for this compound is shown in Figure 3. Given the potency of these three fragments, it is possible that they have relatively slow off-rates rendering them less sensitive to ligand observed NMR techniques such as TINS.

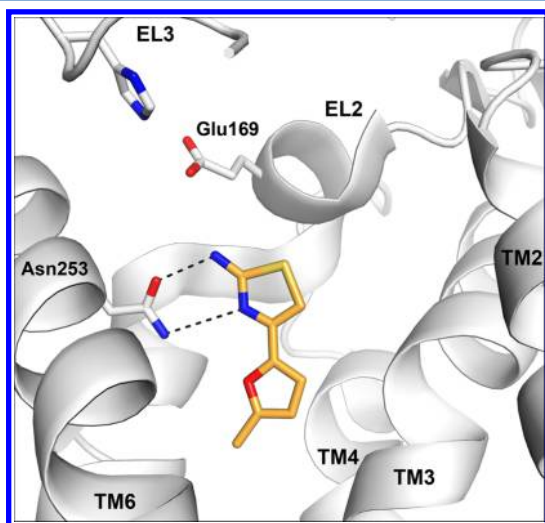


Figure 3. Predicted binding mode for a fragment ligand (compound 6) from the small fragment library that was discovered by docking. The binding site is displayed as a white cartoon with selected side chains shown in sticks. The ligand is depicted with orange carbon atoms. Black dotted lines indicate hydrogen bonds. This figure was generated with PyMOL (version 1.4.1).

Molecular Docking Screen of Commercially Available Fragments against an $A_{2A}\text{AR}$ Crystal Structure. Although the positive enrichment of $A_{2A}\text{AR}$ ligands in the fragment library was encouraging, a more interesting test of docking performance would be to extend the number of molecules screened to all commercially available or even all synthetically feasible fragments, e.g. the 166 billion compounds in the GDB-17.³⁹ One major strength of the in silico approach comes from

the ability to screen large libraries and reduce the fraction that is tested experimentally to a small number of chemicals. This would enable a more realistic comparison of a typical experimental fragment-based screen to a computational structure-based approach. Screening of a larger chemical library could be also advantageous because of better coverage of chemical space, which gives the opportunity to discover ligand chemotypes that were not available to the NMR-based screen. On the other hand, ranking several hundred thousand disparate fragments by affinity is a tremendous challenge, and, as only a few hundred top-ranked compounds are considered for experimental testing, one may actually expect that molecular docking would perform worse for screening of large chemical libraries.

To test the utility of large-scale docking screens in FBLD, we docked 328,000 commercially available fragments from the ZINC library⁴⁰ to an $A_{2A}\text{AR}$ crystal structure using DOCK3.6. The same receptor structure, sampling scheme, and physics-based scoring function as in the first screen were used. The docking of the library was completed in seven hours on 222 cores at a local computer cluster, and each molecule was sampled in, on average, 12800 orientations and 40 conformations. The top-ranked 500 compounds, corresponding to 0.15% of the screened library, were considered for experimental testing. There was essentially no overlap of compounds between this set of molecules and the library screened by the NMR-based method. Only one molecule from the small library screened by NMR would have ranked among the top 500 in the docking screen. Twenty-two top ranked molecules from the docking screen of the large fragment library (0.007%) were selected for experimental testing based on their complementarity to the orthosteric site and availability from commercial vendors.

The twenty-two predicted fragment ligands were first tested in radioligand assays at 500 μM , and 14 molecules showed significant radioligand displacement. Subsequent dose–response curves were well behaved (Figure 4), and the K_i values ranged from 2 to 240 μM (Table 2, compounds 9–22). With the same affinity cutoff as in the NMR-based screen ($K_i < 600 \mu\text{M}$), the hit-rate from the docking screen was 64%. Although it is difficult to compare screens against different GPCRs, our results were similar to the fragment-based docking campaign against the histamine H_1 receptor, which achieved an

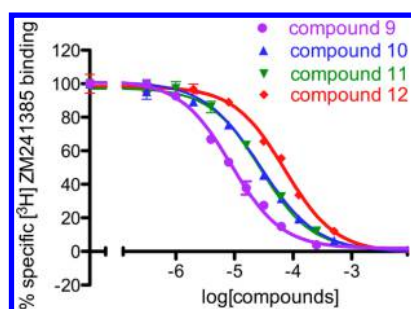


Figure 4. Representative concentration-effect curves for displacement of binding of the radiolabeled A_{2A} R inverse agonist [3H]ZM241385 by compounds 9, 10, 11, and 12.

unprecedented hit-rate of 73% and identified several potent ligands.¹⁶ Compared to the two previous docking screens of lead-like libraries against the A_{2A} AR,^{10,11} we achieved higher hit-rates, but the affinities of the discovered ligands were typically lower, as expected for fragment screening. To enable a comparison between the hits from our fragment screen and those discovered from docking of lead-like libraries against the same crystal structure, LE values (at 310 K) were calculated for each study. The LE values for the fragments from our in silico screen ranged from 0.29 to 0.56 kcal mol⁻¹ atom⁻¹ with an average equal to 0.40, which was similar to the results of Carlsson et al. (0.29–0.44 kcal mol⁻¹ atom⁻¹, average = 0.37)¹⁰ and Katrich et al. (0.32–0.52 kcal mol⁻¹ atom⁻¹, average = 0.41).¹¹

The NMR-based and docking screens are summarized in Figure 5. Five orthosteric ligands were identified in the NMR-based screen, corresponding to a hit-rate of 5% from the secondary screen, and 14 fragment ligands or 64% of those tested experimentally were discovered by docking. Compared to the NMR-based screen, the hits from the A_{2A} AR docking screen had lower K_i values. Four of the hits from the docking screen were more potent than the compound with the highest affinity from the NMR-based screen. A similar trend was observed for the LE values. Ten compounds from the docking screen had LE values >0.35 kcal mol⁻¹ atom⁻¹. In comparison, two compounds from the NMR-based screen reached this level. To investigate the novelty of the discovered fragments, we calculated the pairwise Tanimoto similarity with extended chemical fingerprints for four atoms (ECFP4) of each hit to the thousands of known A_{2A} AR ligands in the ChEMBL14⁴¹ database. The Tanimoto coefficient (T_c) quantifies the two-dimensional chemical similarity between two molecules by a value between 0 and 1. A T_c value close to zero suggests no chemical similarity between a pair of molecules, whereas a value equal to one represents two identical molecules. For each discovered ligand, the highest T_c value among the previously characterized A_{2A} AR ligands is presented in Tables 1 and 2. Some of the hits from the NMR-based and docking screens were relatively similar to known adenosine receptor ligands, e.g., compounds 4 and 9 ($T_c > 0.4$), but in both cases relatively novel chemotypes for the A_{2A} AR were also discovered (e.g., compounds 2, 5, 11, and 14), as reflected by their lower Tanimoto coefficients ($T_c = 0.28$ –0.33). Predicted binding modes for compounds 10, 11, 12, and 13 are shown in Figures 6 and 7. As shown in Figure 6, these compounds were predicted to bind deep in the orthosteric binding site. The key interactions for the fragments are hydrogen bonds to Asn253^{6,55} and Glu169^{5,30} together with π -stacking against the side chain

Table 2. Structures and Experimental Data for the Fragment Ligands Discovered in the Docking Screen against the A_{2A} Adenosine Receptor

ID (Rank) ^a	Ligand structure	K _i (LE ^b) μ M or % displacement at 10 μ M (kcal mol ⁻¹ atom ⁻¹)		T _c ^c
		A _{2A}	A ₁	
9 (27)		2.2 ± 0.2 (0.47)	0.9 ± 0.1	0.41
10 (348)		6.4 ± 0.6 (0.46)	4.0 ± 0.5	0.40
11 (288)		6.3 ± 0.5 (0.39)	11.1 ± 1.0	0.28
12 (224)		6.8 ± 0.4 (0.43)	1.2 ± 0.3	0.37
13 (468)		16 ± 3.0 (0.43)	8.0 ± 0.6	0.36
14 (499)		19.3 ± 4.2 (0.45)	13 ± 2	0.28
15 (233)		22.1 ± 3.1 (0.37)	5.5 ± 0.5	0.33
16 (386)		29.7 ± 6.1 (0.36)	22 ± 2	0.41
17 (235)		33.5 ± 6.0 (0.35)	10 ± 2	0.36
18 (10)		39.3 ± 5.3 (0.35)	7.2 ± 0.5	0.32
19 (492)		46.4 ± 4.7 (0.34)	40 ± 5	0.37
20 (82)		95 ± 5.4 (0.36)	80 ± 4	0.34
21 (203)		100 ± 7 (0.32)	104 ± 8	0.36
22 (206)		240 ± 8 (0.29)	173 ± 6	0.38

^aRank by docking screen of fragment library with 328,000 commercially available compounds. ^bLigand efficiency. ^cTanimoto coefficient (ECFP4 fingerprints) to A_{2A} adenosine receptor ligands in the ChEMBL14 database.

of Phe168^{5,29} (superscripts denote Ballesteros-Weinstein numbers⁴²), which is consistent with the ligand interactions observed in several of the twelve available A_{2A} AR crystal structures (Figure 7).^{6–9}

Structure–Activity Relationships and Molecular Dynamics Simulations for Analogs of Compounds 10, 11,

Library size	500 compounds	328,000 compounds
	NMR/TINS	Docking/DOCK3.6
Primary screen	94	22
	RLA	RLA
Secondary screen (hit-rate)	5 (5%)	14 (64%)

Figure 5. Summary of the discovery of orthosteric ligands from the NMR-based and docking screens. Primary screens were carried out using NMR-based (NMR/TINS) and docking (Docking/DOCK3.6) screens. Secondary screens were performed using radioligand binding assays (RLA).

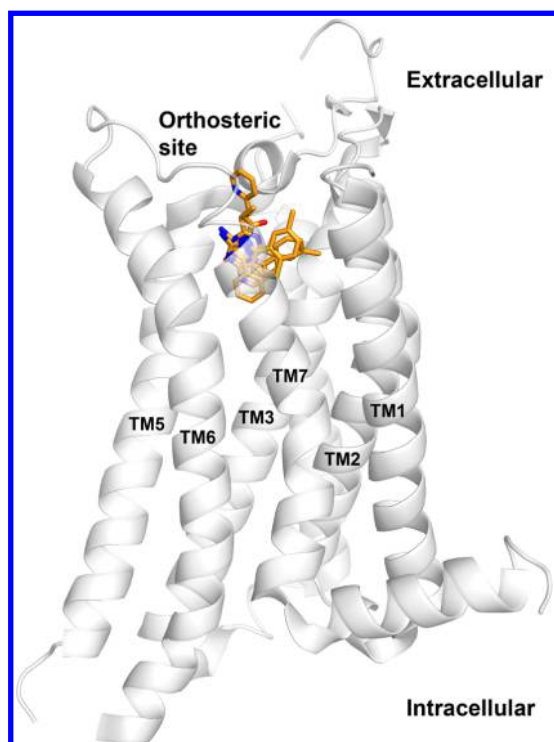


Figure 6. Predicted binding modes for compounds **10**, **11**, **12**, and **13** in the orthosteric site of the $A_{2A}AR$ crystal structure. The $A_{2A}AR$ is displayed as a white cartoon, and the four ligands are depicted with orange carbon atoms. This figure was generated with PyMOL (version 1.4.1).

and **12**. To explore the SAR for the ligands and investigate how the fragments could be further optimized, we docked commercially available analogs of the three potent compounds **10**, **11**, and **12** to the $A_{2A}AR$ orthosteric site. A series of 23 compounds were selected based on their predicted interactions in the orthosteric site and were tested in radioligand binding assays. Molecular docking in combination with MD simulations was used to guide analog selection and to investigate the predicted binding modes of the screening hits.

Compound **10** was one of the most potent fragment ligands from the screen with a K_i value of $6.4 \mu M$. The compound also had a promising LE value equal to $0.46 \text{ kcal mol}^{-1} \text{ atom}^{-1}$. The binding mode of compound **10** remained stable over one ns of MD simulation with no significant conformational changes in the orthosteric site. The exocyclic amine and triazole group formed strong interactions with Asn253^{6,55} in the simulation, whereas the phenyl group was deeply buried in a hydrophobic pocket created by Val84^{3,32}, Leu85^{3,33}, and Trp246^{6,48}. MD simulations of compound **10b** suggested that substituents that extended further toward the extracellular loops could be

accommodated by the binding pocket. Six analogs of compound **10** were tested to further investigate the binding mode of the ligand (Table 3). Possibilities for optimization of this fragment were first explored by replacing the ethyl with a methyl and propyl substituent (**10a** and **10b**). Reducing the alkyl chain to a methyl led to a 2-fold reduction of affinity, whereas the propyl substituent did not affect the potency. The retention of the potency by the compound with the longer substituent agreed with the predicted interactions with hydrophobic side chains in the opening of the orthosteric site, e.g. Ile274^{7,44} and Met270^{7,35}. This suggests that further optimization could be achieved by combining **10b** with fragments that extend further toward the extracellular loops. Substitutions on the phenyl ring in the para-position with a methyl-, chlorine-, or methoxy-group led to reductions of the binding affinity in all cases (**10c–e**). The decrease in affinity for the largest substituent was consistent with the prediction that this group was located in an enclosed pocket, in agreement with the predicted binding mode (Figure 7A). Replacing the phenyl group of compound **10b** with a pyridine ring led to a 10-fold decrease in binding affinity to $50 \mu M$ (**10f**). To investigate the reasons for this large reduction of affinity, we calculated the difference in free energy of binding for these compounds using MD free energy calculations. MD simulations were used to alchemically transform compound **10b** to **10f** in the $A_{2A}AR$ orthosteric site and in aqueous solution by employing the free energy perturbation (FEP) technique. From these simulations, the difference in binding free energy between the two ligands can be calculated from a thermodynamic cycle.⁴³ The calculated free energy difference between compounds **10b** and **10f** was $+1.2 \pm 0.1 \text{ kcal/mol}$, in close agreement with the experimental 10-fold loss of affinity caused by the pyridine group. Inspection of the MD trajectories suggested that the reason for the calculated reduction of binding affinity was a loss of favorable solvent interactions for the pyridine group in the bound state (Figure 8).

Compound **11** was one of the more novel fragment ligands identified in the docking screen ($T_c = 0.28$) and relatively potent with a K_i value of $6.3 \mu M$. Based on molecular docking and MD simulations of selected fragments, eleven compounds were selected for experimental testing in radioligand binding assays, which are summarized in Table 4. This congeneric series of fragments explored variations on the pyridine group of compound **11**, which was predicted to interact with residues in the opening of the orthosteric site. Compound **11a**, for which the nitrogen of the pyridine ring was moved to the ortho position, was slightly more potent than the initial hit. On the contrary, replacing the pyridine with a phenyl led to a 3-fold reduction of the K_i value to $18 \mu M$ (**11b**). For compounds **11c–i**, the effects of polar and nonpolar substituents on the phenyl ring of compound **11b** were explored. In the ortho-position, polar (**11c**), nonpolar (**11d**), and halogen substituents (**11e–f**) improved binding affinity with K_i values ranging from 5.5 to $8.8 \mu M$. In the meta-position, a methyl substituent (**11g**) did not affect affinity relative to compound **11b**, whereas an increase in polarity at this position was found to improve the affinity (**11a**). In the para-position, a polar substituent (**11h**) led to a 2-fold improvement of affinity, whereas a methyl group was not tolerated and reduced the K_i value to $60 \mu M$ (**11i**). The observed effects on affinity for ortho- and meta- substitutions on the phenyl ring could be explained by the interactions with residues in the opening of the orthosteric site, as this position is close to both polar and nonpolar groups in the opening of the

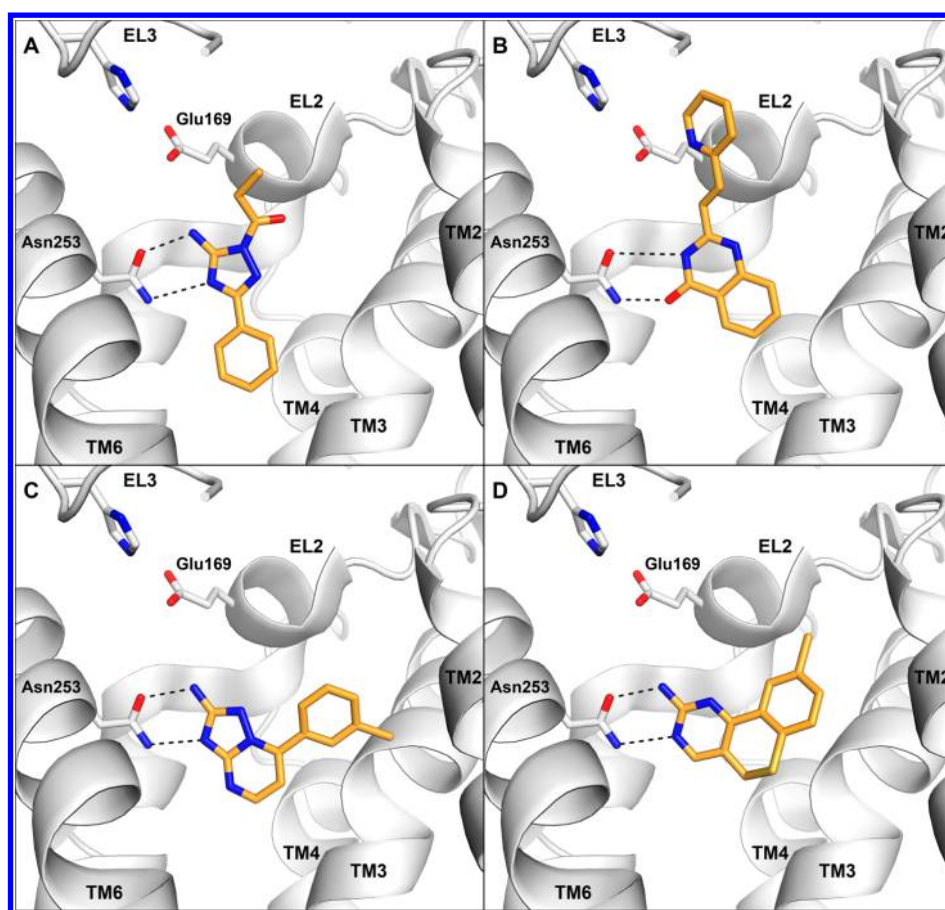


Figure 7. Predicted binding modes for four ligands discovered in the molecular docking screen: (A) 10, (B) 11, (C) 12, (D) 13. The binding site is displayed as a white cartoon with selected side chains shown in sticks. The ligand is depicted with orange carbon atoms. Black dotted lines indicate hydrogen bonds. This figure was generated with PyMOL (version 1.4.1).

Table 3. Structures and Experimental Data for Analogs of Compound 10

			K_i (LE ^a) μ M or % displacement at 10 μ M (kcal mol ⁻¹ atom ⁻¹)	
ID	X	R	A _{2A}	A ₁
10a	H	CH ₃	11.0 \pm 0.5 (0.47)	11.4 \pm 1.0
10b	H	(CH ₂) ₂ CH ₃	5.1 \pm 0.2 (0.44)	1.0 \pm 0.2
10c	Cl	(CH ₂)CH ₃	17.0 \pm 3.2 (0.40)	44%
10d	CH ₃	(CH ₂)CH ₃	10.6 \pm 0.6 (0.41)	6.4 \pm 0.3
10e	OCH ₃	(CH ₂) ₂ CH ₃	42 \pm 4 (0.33)	8.1 \pm 0.3
10f	-	-	50 \pm 5 (0.36)	47%

^aLigand efficiency.

orthosteric site. However, the changes in affinity for polar and nonpolar substituents in the para-position could not be explained by our model because this position was typically solvent exposed in the predicted binding mode and in the MD simulations (Figure 7B). This may be related to interactions with flexible parts of extracellular loop two, e.g. two rotamers of Glu169^{5,30} that have been observed in crystal structures.^{6–9} Two additional compounds were designed by combining

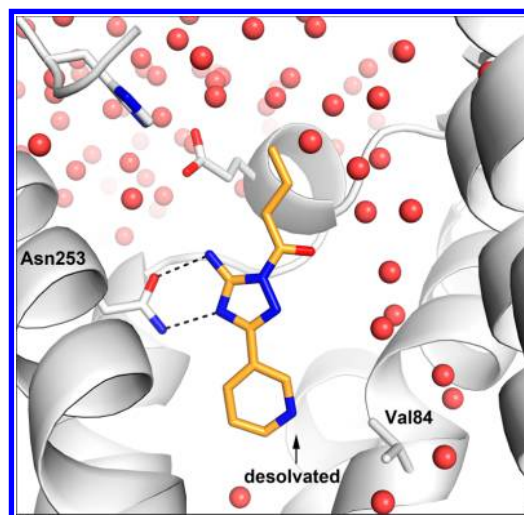
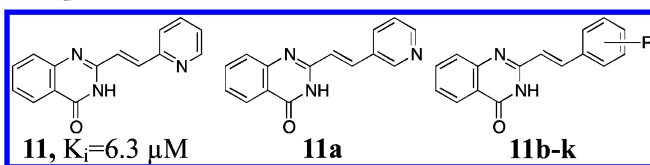


Figure 8. Representative molecular dynamics snapshot of the A_{2A}AR in complex with compound 10f. The binding site is shown in white ribbons and selected side chains shown in sticks. The ligand is depicted with orange carbon atoms. Water molecules are shown as red spheres. Black dotted lines indicate hydrogen bonds. This figure was generated with PyMOL (version 1.4.1).

hydroxyl substituents in ortho- and para-positions with a methoxy group in the meta-position (compounds 11j and 11k, respectively). The most potent compound, 11k, had an affinity

Table 4. Structures and Experimental Data for Analogs of Compound 11



ID	ligand structure R	K_i (LE^a) μM or % displacement at 10 μM ($\text{kcal mol}^{-1} \text{atom}^{-1}$)	
		A_{2A}	A_1
11a	-	4.1 ± 0.3 (0.40)	20 ± 2
11b	H	18.0 ± 1.3 (0.35)	29%
11c	2-OH	8.8 ± 0.6 (0.36)	26%
11d	2-CH ₃	7.8 ± 0.5 (0.36)	19%
11e	2-Cl	7.0 ± 0.4 (0.37)	20 ± 3
11f	2-F	5.5 ± 0.3 (0.37)	3.5 ± 0.4
11g	3-CH ₃	22 ± 1 (0.33)	24%
11h	4-OH	7.4 ± 0.8 (0.36)	34%
11i	4-CH ₃	60 ± 5 (0.30)	18%
11j	2-OH, 3-OCH ₃	9.6 ± 0.5 (0.32)	25%
11k	3-OCH ₃ , 4-OH	2.4 ± 0.2 (0.36)	9.6 ± 0.4

^aLigand efficiency.

of $2.4 \mu\text{M}$, which is 8-fold better than compound **11b** and a 2-fold improvement compared to the initial hit from the docking screen. The MD simulation of compound **11k** suggested that the methoxy substituent complements the shape of the orthosteric site very well, which is shown in Figure 9.

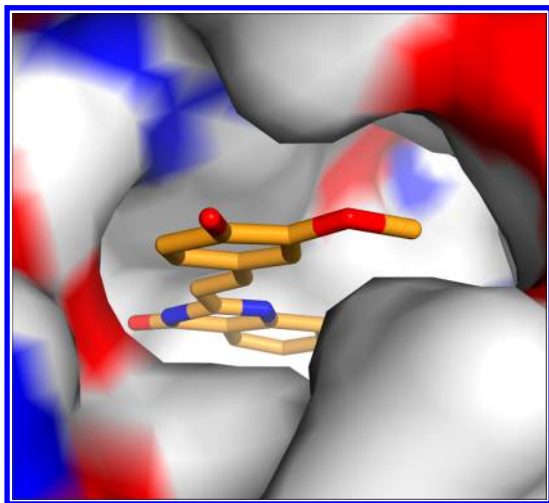


Figure 9. Representative snapshot from molecular dynamics simulation of the A_{2A} AR in complex with compound **11k**. The extracellular part of the A_{2A} AR is shown in surface representation, and the ligand is depicted with orange carbon atoms. This figure was generated with PyMOL (version 1.4.1).

The affinity of compound **12** was $6.8 \mu\text{M}$, and this fragment had a LE value of $0.43 \text{ kcal mol}^{-1} \text{atom}^{-1}$. In the predicted binding mode of this ligand, it is almost completely buried in the orthosteric site with the 1,2,4-triazolo[1,5-a]pyrimidine-2-amine group hydrogen bonding with Asn253 and forming stacking interactions with the side chain of Phe168 (Figure 7C). The phenyl group of compound **12** occupied part of the subpocket where the ribose ring of A_{2A} AR agonists is located in crystal structures. MD simulations of this fragment suggested

that the compound likely explores multiple conformations in the site. In these simulations hydrogen bonds to either Asn253 or Glu169 were conserved, but the phenyl ring explored several subpockets. Six additional compounds in this series were tested experimentally in radioligand binding assays, and the results are summarized in Table 5. The compound without any

Table 5. Structures and Experimental Data for Analogs of Compound 12



ID	ligand structure		K_i (LE^a) μM or % displacement at 10 μM ($\text{kcal mol}^{-1} \text{atom}^{-1}$)	
	X	R	A_{2A}	A_1
12a	H	H	101 ± 6 (0.35)	2%
12b	H	4-Cl	99 ± 5 (0.33)	17%
12c	CH ₃	H	2.1 ± 0.2 (0.47)	3.5 ± 0.3
12d	CH ₃	4-OCH ₃	8.7 ± 0.1 (0.40)	2.8 ± 0.2
12e	CH ₃	4-CH ₃	3.4 ± 0.4 (0.41)	2.4 ± 0.3
12f	CH ₃	4-Cl	9.1 ± 0.2 (0.38)	2.5 ± 0.2

^aLigand efficiency.

substituents on the phenyl ring was 15-fold less potent than the initial hit, $K_i = 101 \mu\text{M}$ (**12a**), demonstrating that the Cl in the meta-position of compound **12** was essential for binding. In the next step, a Cl in the para-position was tested, but this compound was also inactive. Subsequently, a methyl group was added to the pyrimidine group, which resulted in a 50-fold improvement of affinity to $2 \mu\text{M}$ and an increase of the LE to $0.47 \text{ kcal mol}^{-1} \text{atom}^{-1}$ (**12c**). Substituents in the para-position on the phenyl group on this compound (**12d–f**) did not further improve the affinity of the series. The predicted binding mode for compound **12c** was in good agreement with the observed changes in affinity for the series. The methyl substituent that improved the affinity of compound **12c** to $2.1 \mu\text{M}$ was positioned in a hydrophobic pocket formed by Val84^{3,32}, Leu85^{3,33}, and Trp246^{6,48}. Almost complete burial of the compound in the orthosteric site was likely responsible for its high efficiency ($\text{LE} = 0.47 \text{ kcal mol}^{-1} \text{atom}^{-1}$, Figure 10).

Selectivity and Efficacy for the Discovered Fragment Ligands. One advantage of fragment-based screening is that high hit-rates are often achieved, but the ligands that are discovered are typically not selective for their target. To test the selectivity profile for the fragment ligands that emerged from the in silico screen, all compounds were also tested against the A_1 AR subtype in radioligand binding assays. Two of the more potent fragments ligands were also tested in cyclic-AMP (cAMP) assays. For the fourteen fragments discovered in the docking screen (Table 2), no significant selectivity for the A_{2A} AR was found. On the contrary, several ligands actually had better affinities for the A_1 AR, with an average 2-fold selectivity for this subtype. The maximal A_1 selectivity among the more potent compounds was a 4-fold difference in affinity for compound **15**. A similar trend was observed for the analogs of compounds **10**, **11**, and **12** (Tables 3, 4, and 5). For the two smaller fragments, **10** and **12**, no significant selectivity for A_{2A} over the A_1 subtype was found for any of the analogs. Several of

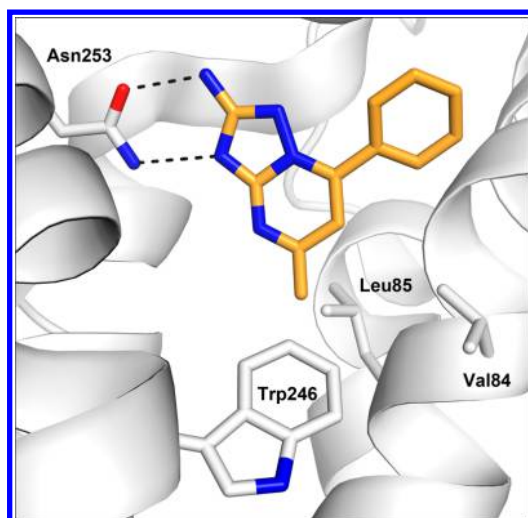


Figure 10. Predicted binding mode for compound 12c. The binding site is displayed as a white cartoon with selected side chains shown in sticks. The ligand is depicted with orange carbon atoms. Black dotted lines indicate hydrogen bonds. This figure was generated with PyMOL (version 1.4.1).

the analogs of compound 11 approached the size of lead-like compounds, and it was also among these compounds that some selectivity for the A_{2A} AR was observed. The most potent compound in the series (11k) had an affinity of 2.4 μ M at the A_{2A} AR, but it was a 4-fold weaker ligand for the A_1 subtype.

Two of the more potent fragments from the docking screen, compounds 10 and 11, were also tested for their behavior in cAMP assays. Addition of 10 μ M of each compound resulted in a rightward shift of the agonist (CGS21680) concentration-effect curve as expected for antagonists (Figure 11). This result

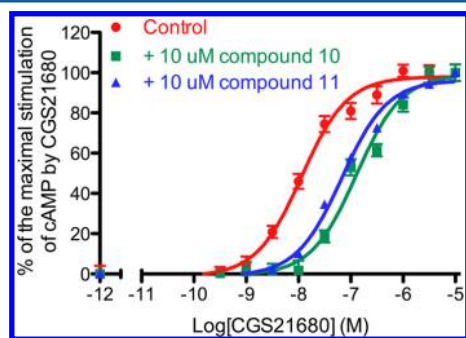


Figure 11. Functional assay based on measuring the concentration-dependent production of cAMP by A_{2A} AR agonist CGS21680 for compounds 10 and 11.

is in agreement with previous docking screens of lead-like libraries against A_{2A} AR crystal structures in the inactive state that, to our knowledge, only have resulted in the discovery of antagonists.^{10,11,44}

DISCUSSION

The revolution in GPCR structural biology during the last years has made it possible to take advantage of structure-based methods in ligand discovery against these pharmaceutically important targets. During the same period, FBLD has become widely used in drug discovery, but there are only a few examples of the use of experimental^{20,21,45,46} and in silico^{16,47} fragment screens against GPCRs. To assess the potential

benefits of complementing biophysical screens against GPCRs with structure-based discovery of fragment ligands, we carried out two prospective molecular docking screens against the A_{2A} AR, a drug target for development of drugs against inflammation and Parkinson's disease.^{2,3} The parallel screen of a small chemical library against the A_{2A} AR allowed us to evaluate the ability of molecular docking to distinguish fragment ligands of the receptor. A second comparison was made possible by the large-scale screen of commercially available fragments and subsequent experimental testing of top-ranked compounds.

The first encouraging result was the ability of molecular docking to identify all but one of the hits from the NMR-based screen among the 30 top-ranked compounds. The results are similar to the parallel docking and HTS screen of 200,000 drug-like compounds against Cruzain by Ferreira et al., in which several inhibitors were among the top-ranked compounds.⁴⁸ It should be noted that significantly higher enrichment of ligands has been reported by Katritch et al. in a benchmark of molecular docking against the same A_{2A} AR crystal structure.¹¹ As only lead- to drug-like compounds were used in these retrospective screens, this result could suggest that it is more challenging for docking to identify fragment ligands. However, it could also reflect differences in the scoring functions used and how the nonbinders (decoys) were selected. The main finding from the two in silico screens that were undertaken in this work was the ability of the molecular docking to complement the biophysical screen for fragment ligands of the A_{2A} AR. One of the main advantages of fragment-based screening compared to HTS is that the high hit-rates provide numerous starting points for development of lead compounds.^{17,18} However, prioritizing between a large number of primary hits from biophysical screens, a total of 94 from the NMR-based method analyzed here, is often difficult and time-consuming.²¹ The successful prediction of the NMR-based screening results strongly suggests that it is advantageous to carry out docking screens in parallel and use the results to select hits for follow-up testing to reduce the number of false positives. This approach would also provide a starting point for structure-based optimization of fragments. Interestingly, a recent comparison between fragment-based screens with different biophysical approaches showed that different sets of ligands are identified using the two methods.⁴⁹ To some extent, this likely reflects challenges in detecting low-affinity fragments and suggests that a combination of several orthogonal methods could reduce the number of false negatives and positives from biophysical screens. Our results demonstrate that molecular docking also could play such a role. Several fragments that were top-ranked by docking, but had not been identified by the NMR-based screen, were micromolar ligands with high ligand efficiencies.

The parallel screen also demonstrated major weaknesses of molecular docking screens. As in a majority of all structure-based virtual screens, the receptor volume searched by the docking algorithm was reduced to one pocket on the protein surface. The consequence of this approximation was that allosteric modulators could not be identified. On the contrary, the NMR-based screen led to one of the first discoveries of compounds with positive and negative allosteric effects on ligand binding to the A_{2A} AR.^{2,3} The obvious solution would be to extend the searched volume to include the entire receptor surface, but this would be too computationally expensive for screening of large chemical libraries. Multiple pockets could be

considered, but it is not yet clear where the allosteric ligands bind to the A_{2A}AR, and it would also be a major challenge for docking scoring functions to rank molecules in sites of different sizes and chemical properties. Modulation of receptors via allosteric interactions may provide novel opportunities for development of completely new classes of drugs against the A_{2A}AR.⁵⁰

The second docking screen unleashed the full potential of the structure-based approach. Several hundred thousand fragments were docked to the A_{2A}AR crystal structure to identify compounds that complement this receptor conformation. As only a few dozen compounds are evaluated experimentally in this case, more extensive testing can be carried out for each compound, which reduces the risk for false negatives. As in earlier *in silico* screens against A_{2A}AR and other GPCR crystal structures, the hit-rates were unusually high.^{10–16} It may be the combination of a highly ligandable orthosteric site⁴⁸ and bias in chemical libraries toward GPCR chemotypes that make structure-based screens against these receptors so successful.^{10,12} If this is true, the successes for deep and enclosed binding sites, which are characteristic for the adenosine and aminergic receptors, may not be transferrable to GPCRs with more shallow and solvent exposed pockets. The observation that biophysical screens of fragments generally yield higher hit-rates than those that rely on lead- or drug-like libraries was also demonstrated to be true for *in silico* screens. Several ligands from the docking screen were also more potent than expected from the typical fragment screens, both in comparison to screens against soluble proteins and the NMR-based screen against the same target.^{20,21,45}

Successful FBLD relies on the possibility to optimize the initial hits, and, in this step, access to atomic resolution structures provides valuable information. However, in the case of predicting affinities for a series of analogs, subtle structural changes of the receptor may be responsible for differences in potency, which may require a more accurate representation of protein–ligand interactions than molecular docking can provide.⁵¹ Initial attempts to use more rigorous methods in lead optimization of GPCR ligands have been encouraging, and such approaches will likely also play an important role in FBLD against these receptors.^{52,53} An increasing amount of computational resources has made it possible to use atomistic simulations to compute the relative affinities for large series of protein–ligand complexes.^{54,55} The small size and relatively good availability of force field parameters for fragment-like molecules make them ideal cases for MD simulations and free energy calculations. Here, three new scaffolds from the *in silico* screen were further explored via analogs and MD simulations of several complexes. The MD simulations provided a deeper understanding of the SAR for analogs of the docking hits, and free energy calculations were used to explain changes in binding affinity due to changes in ligand structure. Two of the scaffolds were optimized to an affinity of 2 μ M. In particular these compounds, as well as the 15 other discovered fragments that were left unexplored, provide new starting points for development of new lead compounds against this medically important target.

Drug design against GPCRs also involves identifying compounds with a specific efficacy and selectivity profile. The fragments that were tested for efficacy were all antagonists, and this was consistent with that the screen was carried out against a crystal structure in the inactive state. Fragment ligands are typically not selective for their targets, and this is also a general

challenge for the A_{2A}AR because of the strong sequence conservation in the orthosteric site among the four subtypes.¹ None of the ligands discovered in the NMR-based or docking screens showed significant selectivity for A_{2A}AR over the closely related A₁ subtype, which likely reflected that the fragment hits explored the conserved core of the binding site. If anything, the styryl moiety in compound series **11** had been found as a chemical substructure contributing to A_{2A} receptor selectivity before, and indeed in this series there was some selectivity for this receptor subtype too.⁵⁶ The refined binding modes for the most potent compounds now provide starting points for optimization of selectivity. In this case, future crystal structures and models of other adenosine receptor subtypes will be crucial for optimizing the selectivity of fragment ligands discovered in biophysical and docking screens.^{22,57,58}

CONCLUSIONS

The picture that emerges from this study is that fragment-based ligand discovery via biophysical and computational structure-based screens are highly complementary approaches. Both the NMR- and molecular docking based screens identified novel A_{2A}AR fragment ligands with no overlap between the two sets of hits. Molecular docking was shown to be a highly efficient method for the identification of fragments ligands, as reflected by the 64% hit-rate achieved in this screen. On the other hand, the unbiased experimental screen of a relatively small fragment library unexpectedly discovered allosteric modulators of the A_{2A}AR, which will provide new opportunities for drug development against this receptor. The opportunities to combine biophysical and *in silico* screening methods with structure determination for GPCRs will likely position FBLD as one of the principal approaches for drug development against this pharmaceutically important class of targets.

METHODS

Molecular Docking Calculations. All docking calculations were carried out with the program DOCK3.6^{26–28} using a 2.6 Å resolution crystal structure of the human A_{2A}AR in complex with an antagonist (PDB accession: 3EML⁷). The receptor was prepared by removing all nonprotein atoms and the intracellular T4-lysozyme insertion. The protonation states of ionizable residues of all Asp, Glu, Arg, and Lys residues were set to the most probable in aqueous solution at pH 7. The protonation states of the histidines were set by manual inspection (His278^{7,43}:protonated in delta position, His250^{6,52}:protonated in epsilon position, His264^{6,66}:double protonated).

The flexible ligand sampling algorithm in DOCK3.6 superimposes atoms of the docked molecule onto binding site matching spheres, which indicate putative ligand atom positions. Sixty matching spheres were used, and these were either based on the atoms of the crystallographic ligand or positioned manually.^{27,28} The spheres were also labeled for chemical matching based on the local receptor environment.⁵⁹ The degree of ligand sampling was determined by the bin size, bin size overlap, and distance tolerance. These three parameters were set to 0.4 Å, 0.2 Å, and 1.5 Å, respectively, for both the binding site matching spheres and the docked molecules. An initial filter discarded conformations that had more than one overlapping heavy atom with the receptor. An overlap between two atoms was defined as a distance shorter than 2.3 and 2.6 Å for polar and nonpolar atoms, respectively, and was calculated

from a precalculated grid. For ligand conformations passing this steric filter, a physics-based scoring function was used to evaluate the fit to the binding site. For the best scoring conformation of each docked molecule, 100 steps of rigid-body minimization were carried out.^{27,28} The score for each conformation was calculated as the sum of the receptor–ligand electrostatic and van der Waals interaction energy, corrected for ligand desolvation. These three terms were evaluated from precalculated grids. The three-dimensional map of the electrostatic potential in the binding site was prepared using the program Delphi.⁶⁰ In this calculation, partial charges from the united atom AMBER force field⁶¹ were used for all receptor atoms except the side chain amide of Asn253^{6,55}, for which the dipole moment was increased to favor hydrogen bonding to this residue.¹⁰ The program CHEMGRID was used to generate a van der Waals grid, which is based on a united atom version of the AMBER force field.⁶² The desolvation penalty for a ligand conformation was estimated from a precalculated transfer free energy of the molecule between solvents of dielectrics 78 and 2. The desolvation energy was obtained by weighting the transfer free energy with a scaling factor that reflects the degree of burial of the ligand in the receptor binding site.^{37,63}

Prior to the DOCK3.6 calculation, each docked library was prepared for docking by pregenerating up to 600 conformations using the program OMEGA.⁶⁴ Partial atomic charges and transfer free energies were calculated using AMSOL,^{65,66} and van der Waals parameters were derived from an all-atom AMBER potential.⁶⁷ Two libraries were screened. The first was an in-house fragment library of 500 compounds.³¹ The second was the ZINC fragment-like library⁴⁰ of 328,000 commercially available molecules (molecular weight <250, LogP < 3.5, and rotatable bonds <5).

Similarity Calculations. Similarity calculations for the fragments were carried out using Screenmd program from Chemaxon.⁶⁸ We calculated the maximum Tanimoto coefficient with ECFP4 fingerprints between each discovered fragment ligand to the 6509 compounds with recorded activity against the human A_{2A}R in the ChEMBL14⁴¹ database.

Molecular Dynamics Simulations and Free Energy Calculations. The molecular dynamics simulations (MD) and binding free energy calculations for the A_{2A}R were performed using a recently published high-resolution crystal structure (PDB accession code: 4EIY,⁹ 1.9 Å). In the first step, a hydrated POPC membrane was first equilibrated around the A_{2A}R structure without any ligand using periodic boundary conditions in GROMACS⁶⁹ using the OPLSAA force field,⁷⁰ TIP3P waters,⁷¹ and Berger lipids.⁷² In this simulation, all protein atoms were tightly restrained to their initial coordinates, and the hydrated membrane was equilibrated for 40 ns at 300 K. All other MD simulations and free energy calculations of the protein–ligand complexes were carried out starting from the membrane equilibrated A_{2A}R system using spherical boundary conditions in the program Q.⁷³ All ligand force field parameters were obtained using Schrodinger's program hetgrp_ffgen.⁷⁴ The simulations were carried out at a constant temperature of 310 K in a sphere of 18 Å radius centered on the ligand. All protein, water, and ligand atoms within 18 Å of the center of the sphere were explicitly included in the simulations. All atoms outside the sphere were tightly restrained to their initial coordinates and excluded from nonbonded interactions. Asp, Glu, His, Lys, and Arg residues within the spherical system were protonated as in the docking calculation. All other

ionizable residues close to the sphere edge or further away than 18 Å were set to their neutral state. The SHAKE⁷⁵ algorithm was applied to all solvent bonds and angles, and the water molecules at the sphere surface were subjected to radial and polarization restraints.^{73,76} A nonbonded cutoff of 10 Å was used for all atoms except the ligand, for which no cutoff was applied. Long-range electrostatic interactions were treated with the local reaction field (LRF) multipole expansion method.⁷⁷ The time step was set to 1 fs, and nonbonded pair lists were updated every 25 steps.

For the docking hits and analogs that were studied with MD simulations, each protein–ligand complex was first equilibrated for 810 ps. During the equilibration, harmonic restraints on the protein and ligand atoms were gradually released, which was followed by one ns of unrestrained simulation. To compute the relative free energy of binding from a thermodynamic cycle, the ligands have to be transformed to each other in water and the A_{2A}R binding site. The free energy of transforming ligand **10b** to **10f** was computed using the free energy perturbation (FEP) technique, and the calculation was carried out via several intermediates.⁷⁸ The potentials governing the intermediate states are defined by $U_m = \lambda_m U_A + (1 - \lambda_m) U_B$ where *A* and *B* represent two different ligands, respectively, and λ_m is a mapping parameter which varies from $\lambda_1 = 0$ to $\lambda_n = 1$. The free energy difference between states *A* and *B* can be calculated by summing up the free energy differences of the *n* intermediate states using

$$\Delta G_{A \rightarrow B}^{\text{FEP}} = -kT \sum_{m=1}^{n-1} \ln \langle e^{-(U_{m+1} - U_m)/kT} \rangle_m$$

where $\langle \dots \rangle_m$ represents an ensemble average on the potential U_m , which is calculated from MD simulations.⁴³ The free energy was computed in two steps. First the partial charges of the phenyl ring of compound **10b** were transformed to the pyridine of compound **10f**. In a second step, the van der Waals parameters for the same group were changed to those representing pyridine. These calculations were carried out both for the ligand in aqueous solution and in the A_{2A}R orthosteric site. The calculations in aqueous solution were carried out by solvating the ligand in a water droplet of radius 18 Å. In these simulations a weak harmonic restraint was applied to keep the ligand close to the center of the sphere. Each FEP calculation comprised a 700 ps heating scheme through eight steps of equilibration. The transformation of partial charges and LJ parameters were carried out with 11 λ states (from $\lambda = 0$ to $\lambda = 1$) each, with a production run time of 500 ps for each lambda point and energies were extracted every 50 steps.

Cell Culture and Membrane Preparation. HEK293 cells were maintained in culture in DMEM supplemented with 10% newborn calf serum at 37 °C in a moist, 7% CO₂ atmosphere and passaged twice weekly. Cells were transfected with plasmids containing wild-type A_{2A}R construct using the calcium phosphate precipitation method and harvested after 48 h. Cells were pelleted, resuspended in 20 mL of ice-cold 50 mM Tris-HCl buffer, pH 7.4. An ULTRA-TURRAX was used to homogenize the cell suspension. The cytosolic and membrane fractions were separated using a high-speed centrifugation step of 100,000g for 20 min at 4 °C. This process was repeated twice, and subsequently the pellet was resuspended in a 50 mM Tris-HCl buffer, pH 7.4, the protein

concentration was determined by the BCA method,⁷⁹ and samples were aliquoted and stored at -80°C until further use.

Radioligand Binding Studies. Tritiated 4-(2-[7-amino-2-(2-furyl)-[1,2,4]-triazolo-[2,3-a]-[1,3,5]-triazin-5-yl-amino]ethyl)phenol ($[^3\text{H}]$ ZM241385, specific activity 30 Ci/mmol) and tritiated 1,3-dipropyl-8-cyclopentyl-xanthine ($[^3\text{H}]$ DPCPX, specific activity 120 Ci/mmol) were purchased from ARC Inc. (St. Louis, USA). Unlabeled ZM241385 and -cyclopentyladenosine (CPA) were purchased from Sigma-Aldrich (Steinheim, Germany). The tested compounds were purchased from five different vendors (Asinex, ChemBridge, Vitas-M, Pharmeks, and KeyOrganics). The identity of all tested compounds was verified by ^1H -NMR (Table S1), and HPLC/MS was used to assess the purity for all of the molecular docking screening hits (Table 2 and Table S2, >95% in all cases).

For the $\text{A}_{2\text{A}}$ ARs, membranes containing 20 μg of protein were incubated in a total volume of 100 μL of Tris-HCl buffer (50 mM, pH 7.4), 10 mM MgCl_2 , increasing concentrations of the test compounds (5% DMSO) and $[^3\text{H}]$ ZM241385 at a final concentration of 3 nM. Nonspecific binding was determined using a final concentration of 10 μM ZM241385 and represented less than 10% of the total binding. For the A_1 ARs, membranes containing 5 μg of protein were incubated in a total volume of 100 μL of Tris-HCl buffer (50 mM, pH 7.4), $[^3\text{H}]$ DPCPX (final concentration 1.6 nM), and increasing concentrations of the test compounds (5% DMSO). Nonspecific binding was determined using a final concentration of 100 μM CPA. Total binding was determined in the presence of buffer and was set at 100% in all experiments, whereas nonspecific binding was set at 0%. After incubation for 1 h at 25°C in a shaking water bath, assays were terminated by rapid filtration through 96-well GF/B UniFilter plates (PerkinElmer) followed by washing with 7×0.25 mL of ice-cold Tris-HCl buffer (50 mM, pH 7.4). Plates were dried, 25 μL of P-E Microscint 20 was added per well, and after 3 h, bound radioactivity was measured using a Packard Microbeta counter (PerkinElmer). Data were analyzed using GraphPad Prism v5, normalized as ‘% specific binding’ from which the K_i values were calculated. Experiments were performed three times in duplicate, unless stated otherwise.

Cyclic AMP Accumulation Assay. HEK293 cells were grown and transfected as described above. Experiments were performed 48 h after transfection. The cells were harvested and centrifuged two times at 1,000 rpm for 5 min. For cyclic AMP production and determination, 1500 cells/well were used in 384-well Optiplates (PerkinElmer). The cells were incubated for 30 min at 37°C with either the $\text{A}_{2\text{A}}$ R agonist CGS21680 alone or CGS21680 together with test compounds in different concentrations. The assay buffer used was PBS with the addition of 5 mM HEPES, 0.1% BSA, 50 μM rolipram, 50 μM cilostamide, and 0.8 IU/mL adenosine deaminase. Basal activity was determined in the presence of assay buffer and was set at 0% in all experiments. Maximal receptor activity was determined in the presence of 10 μM CGS21680 and was set at 100% in all experiments. Cells were then lysed, and the amount of cAMP produced was quantified using a LANCE ultra cAMP kit (PerkinElmer) according to the instructions of the manufacturer. Following addition of the detection mixture, plates were left for 1 h at RT prior to reading using an EnVision plate reader (PerkinElmer Life Sciences).

HPLC/MS Protocol. Analytical purity of the compounds 9–15, 16, 17, 19, and 22 was determined by high-pressure liquid chromatography (HPLC) with a Phenomenex Gemini 3u C18

110A column (50×4.6 mm, 3 μm), measuring UV absorbance at 254 nm. Sample preparation and HPLC method is - unless stated otherwise - as follows: 0.3–0.8 mg of compound was dissolved in 1 mL of a 1:1:1 mixture of $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{tBuOH}$ and eluted from the column within 15 min, with a three component system of $\text{H}_2\text{O}/\text{CH}_3\text{CN}/1\%$ TFA in H_2O , decreasing polarity of the solvent mixture in time from 80/10/10 to 0/90/10.

LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C18 50×4.60 mm column (detection at 200–600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI using the same procedure as mentioned above.

■ ASSOCIATED CONTENT

Supporting Information

Tables S1 and S2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ ABBREVIATIONS

FBLD, fragment-based lead discovery; GPCR, G-protein coupled receptor; $\text{A}_{2\text{A}}$ AR, $\text{A}_{2\text{A}}$ adenosine receptor; NMR, nuclear magnetic resonance; SPR, surface plasmon resonance; CNS, central nervous system; TINS, target immobilized NMR screening; MD, molecular dynamics; FEP, free energy perturbation; SAR, structure–activity relationship; T_{c} , Tanimoto coefficient; ECFP4, extended chemical fingerprints up to 4 atoms; TM, transmembrane helix; EL, extracellular loop

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