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Fragment Screening of GPCRs Using Biophysical Methods: Identification of Ligands of the Adenosine A_{2A} Receptor with Novel Biological Activity

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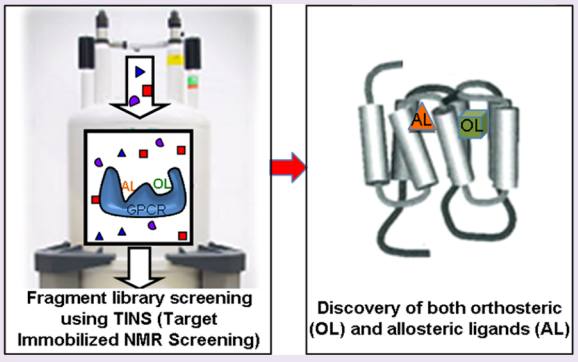
ABSTRACT: Fragment-based drug discovery (FBDD) has proven a powerful method to develop novel drugs with excellent oral bioavailability against challenging pharmaceutical targets such as protein−protein interaction targets. Very recently the underlying biophysical techniques have begun to be successfully applied to membrane proteins. Here we show that novel, ligand efficient small molecules with a variety of biological activities can be found by screening a small fragment library using thermostabilized (StaR) G protein-coupled receptors (GPCRs) and target immobilized NMR screening (TINS). Detergent-solubilized StaR adenosine A_{2A} receptor was immobilized with retention of functionality, and a screen of 531 fragments was performed. Hits from the screen were thoroughly characterized for biochemical activity using the wild-type receptor. Both orthosteric and allosteric modulatory activity has been demonstrated in biochemical validation assays. Allosteric activity was confirmed in cell-based functional assays. The validated fragment hits make excellent starting points for a subsequent hit-to-lead elaboration program.



G protein-coupled receptors (GPCRs) are one of the most popular classes of investigational drug targets due to their central role in a variety of biological and pathological processes, such as neuromodulation, metabolic disorders, inflammation, cancer, and viral infection.[1,2] Thus, it is not surprising that about 30% of all prescribed drugs on the market act on this class of receptors.[3] In the past 10 years, GPCR drug discovery has relied on cell-based assays combined with high-throughput screening (HTS) of large compound libraries[4] for lead discovery as well as optimization. This paradigm has delivered compounds that modulate the behavior of GPCRs as desired, many of which have made it to patients. Nonetheless, only one small molecule drug targeting a novel GPCR has been approved in each of the past 10 years. Moreover, the majority of drugged GPCRs are members of families that are activated by small molecules such as the adrenergic, muscarinic, and histamine receptors.[5] In contrast, peptide and protein hormone receptors have represented a much greater challenge. In addition, achieving subtype selectivity has been difficult and, where successful, has required quite substantial medicinal chemistry efforts. Furthermore, hits from HTS screens frequently must be deconstructed to remove liabilities that result in non-ideal absorption, distribution, metabolism, and excretion (ADME) properties.[6]

Fragment-based drug discovery (FBDD) uses low-molecular-weight (<300 Da), moderately lipophilic (clog P < 3), and highly soluble[7] molecules or "drug fragments" as starting points for developing novel drugs. FBDD is particularly advantageous for its ability to more completely assess "compound space" for molecules that interact with the target of interest.[8] Since the typical interaction of such small molecules with proteins is weaker than 1 μM K_D, the types of assays employed in HTS screening are not easily capable of discriminating between positives and negatives. In order to detect such weak binding, sensitive biophysical techniques such as NMR, SPR, or X-ray crystallography are most commonly used. FBDD has been successful in developing inhibitors of soluble targets such as kinases,[9] proteases,[10,11] and most recently protein−protein interactions.[12] In principle, it could be advantageous to apply FBDD to GPCRs to identify molecules with novel modes of action.[13] However, the application of biophysical techniques to membrane proteins is extremely challenging due to their instability when extracted from the cell membrane and the difficulty of large scale recombinant expression. Even when

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successful, significant levels of non-specific partitioning of compounds into the media used to solubilize membrane proteins remains a significant source of false positives with biophysical studies.

We have developed an NMR-based, fragment screening approach that has been used to find new chemical starting points for further development of compounds targeting membrane proteins, including GPCRs.[14,15] The approach, called target immobilized NMR screening (TINS),[16] uses a target and a reference protein immobilized on a compatible solid support. Binding of fragments to the immobilized protein is monitored by changes in the 1D ^{1}H NMR spectrum of a ligand. A powerful method, called StaRs (stabilized receptors), has been developed to overcome the instability and purification problems of GPCRs. StaR GPCRs, which contain a small number of point mutations for both enhanced thermostability and conformational homogeneity,[17−20] can be produced in significant quantities and purified in correctly folded form in detergent micelles for structural and biophysical studies.[21−23]

We have previously demonstrated the feasibility of the combination of TINS and StaRs to screen fragment libraries for target specific binding.[15] Here we use TINS to find fragment ligands for the human adenosine A_{2A} receptor (A_{2A}R) with novel modes of action. Screening of a moderate sized fragment library using an antagonist stabilized StaR yielded multiple hits that were pharmacologically validated with the wild-type (WT) receptor. Among these hits, we find multiple allosteric modulators of the A_{2A}R with both positive and negative effects on binding and function of the orthosteric ligand.

RESULTS AND DISCUSSION

Functional Immobilization and Stability. In order to assess the feasibility of ligand screening studies, we first determined whether immobilized, micelle-solubilized A_{2A}-StaR2 retained functionality and if so, whether the preparation was sufficiently stable over time (Figure 1). We used an

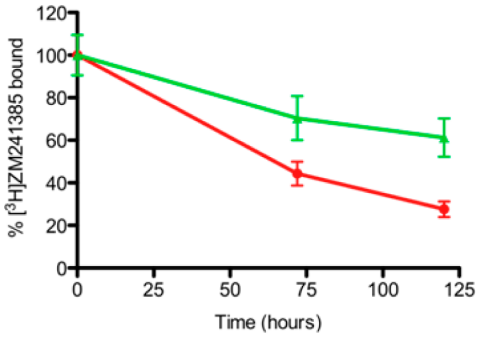

Figure 1. Functional immobilization and stability of micelle-solubilized A_{2A}-StaR2 (green) compared to A_{2A}R-WT in HEK cell membranes (red). A_{2A}-StaR2 was covalently bound to Sepharose resin (see text for details). Functionality was assessed by binding of [^{3}H]ZM241385 upon storage of the protein at 4 °C for the indicated time. Specific [^{3}H]ZM241385 binding at day 0 was set as 100% for both A_{2A}-StaR2 and A_{2A}R-WT.

immobilization method that was successfully applied to the StaR β_1 adrenergic receptor (β_1AR),[15] in which primary amines of the protein form a Schiff's base with an aldehyde group on the solid support. Using this procedure, the DDM-solubilized A_{2A}-StaR2 was efficiently immobilized on a sepharose resin at pH 7.4 with a yield of 70%, and subsequently, unreacted aldehydes were blocked using deuterated Tris buffer. To determine whether the immobilized A_{2A}-StaR2 remained competent for ligand binding, the binding of [^{3}H]ZM241385, an inverse agonist that binds the orthosteric site of StaR and wild-type receptors with a K_D of approximately 1 nM and 1:1 stoichiometry, was assessed as previously described.[24] Initial measurement of [^{3}H]ZM241385 binding after immobilization indicated that nearly 95% of the immobilized A_{2A}-StaR2 was competent. Next, we compared the stability of immobilized A_{2A}-StaR2 with A_{2A}R-WT in HEK cell membranes. After 5 days stored at 4 °C, more than 60% of the immobilized A_{2A}-StaR2 still remained competent for radioligand binding (Figure 1). In contrast, over the same time period, the A_{2A}R-WT lost 75% activity, despite the fact that it was in a native environment.

Fragment Screening. Having determined the functionality and stability of immobilized A_{2A}-StaR2, we set out to screen a collection of fragments for binding to the receptor. A set of 531 fragments was selected for their chemical and shape diversity.[25] The TINS technique uses a reference to cancel out non-specific binding, thereby reducing the amount of false positives. Previously, we had determined that the *E. coli* outer membrane protein OmpA, a β-barrel structure that can be solubilized in various detergents, was an appropriate reference for micelle-solubilized membrane proteins,[14,15] and therefore, we have used it for the current study as well. The fragments were injected into the dual cell sample holder[26] in mixtures of between 3 and 8 compounds. A spatially selective, one-dimensional ^{1}H Hadamard experiment[27] was used to acquire the NMR spectrum of the compounds in solution. Since the NMR relaxation of a spin is approximately 1,000 times more efficient in the solid state than in solution, binding of a ligand to the immobilized protein causes the resonances of that molecule to further broaden into the kHz range. As a result, ligand binding is detected as a simple reduction in the amplitude of the NMR signals of that molecule in the presence of the target in comparison with those in the presence of the reference (Figure 2A).

In order to differentiate hits from nonhits, we analyzed a profile of the entire screen (Figure 2B). In this plot, the T/R ratio (the weighted average of the amplitude of well resolved resonances in the presence of target divided by that in the presence of reference) for each fragment has been bucketed, and the number of fragments in each bucket is presented as a histogram. Similarly to the β_1 adrenergic receptor,[15] the profile shows a peak at a T/R ratio of 1 indicating that the bulk of the fragments do not bind or bind non-specifically to both target and reference. Further, very few compounds have a T/R greater than 1, recapitulating that OmpA has minimal specific small molecule binding capacity and is therefore a good reference. In contrast, there are large numbers of fragments with a T/R ratio significantly less than 1, indicating that they preferentially bind to the A_{2A}-StaR2. In order to make a selection of positives, a discontinuity in the histogram is chosen and that value is used as a cutoff. Such a discontinuity can be found at a T/R ratio of about 0.7. Since the goal was to characterize how the interaction of small molecules with the micelle-solubilized GPCR is reflected in the TINS assay, we opted for a very conservative hit selection (*i.e.*, a larger than normal number of compounds were designated as hits), which resulted in 94 fragments defined as A_{2A}R hits. Application of the same criteria to OmpA binding identified six compounds as hits.

Pharmacological Characterization of TINS Hits. *1. Probing for Orthosteric Site Ligands.* A biophysical assay

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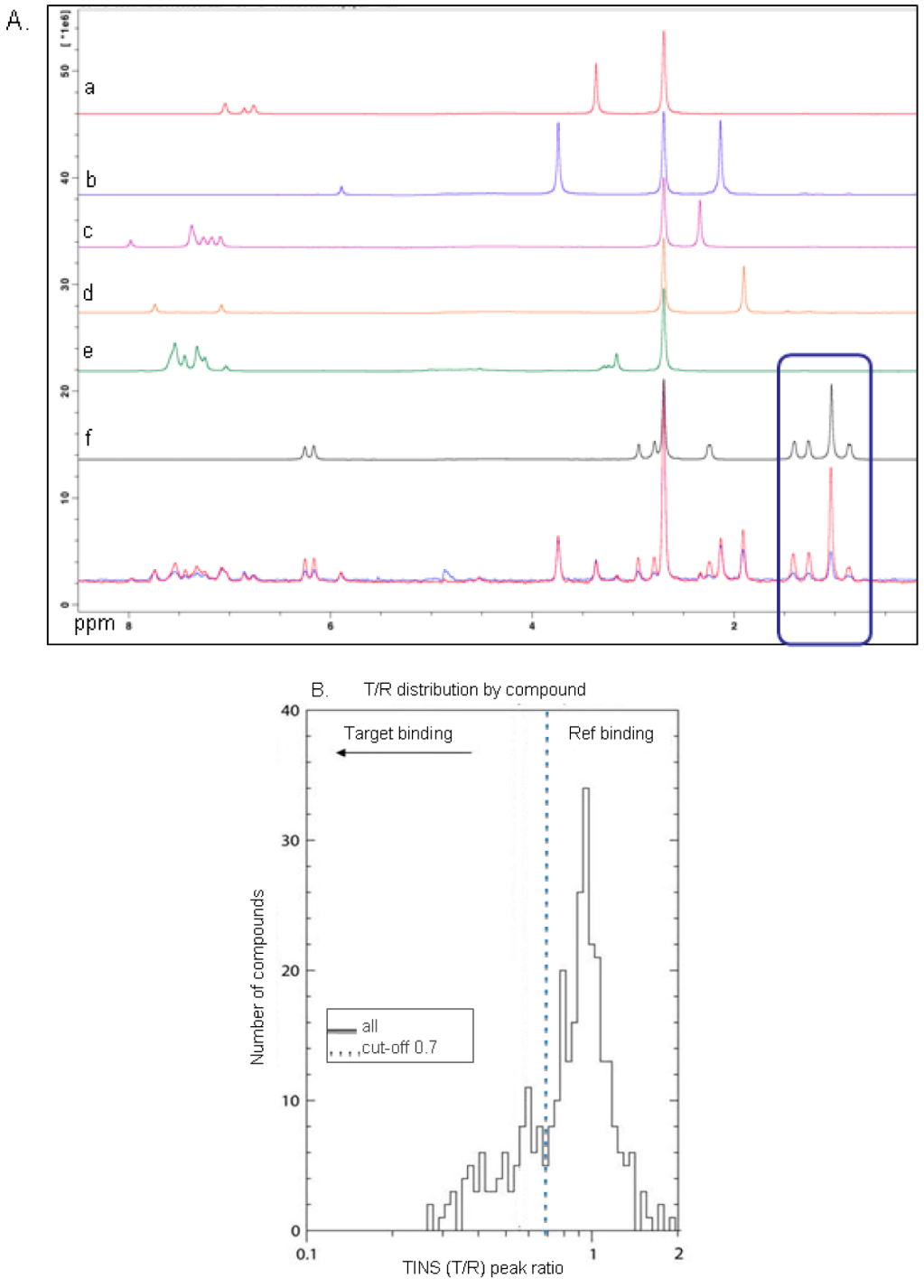

Figure 2. (A) An example of a TINS experiment applied to a mixture of 6 fragments. The ^{1}H NMR spectrum of each component in the mixture is presented, where extensive line broadening has been applied to simulate the line width of resonances in the heterogeneous TINS assay (a–f). The spectrum of the mixture in the presence of the reference protein (red) and A_{2A}-StaR2 (blue) are overlaid. The reduction of peaks in the presence of A_{2A}R indicates that primarily f binds. (B) Profile of the complete screen. The ratio of peaks in the presence of target and reference (T/R) for each compound has been binned, and the number of compounds in each bin is shown. The cutoff for hit selection is shown.

such as TINS simply discovers fragments that specifically bind to the target. We wished to validate this binding and determine whether or not the fragments exhibited a biochemical activity. For these studies we used HEK293 cell membranes transiently expressing the wild-type A_{2A} receptor. Initially we assessed the ability of each of the 94 fragment hits to displace the high affinity, orthosteric inverse agonist [^{3}H]ZM241385 in an equilibrium binding assay. This assay primarily detects ligands binding to the orthosteric site (*i.e.*, the same site as [^{3}H]ZM241385). Five of the fragment hits inhibited binding of ZM241385 by ≥30% at 500 μM, suggesting a biologically relevant interaction between the fragments and the wild-type A_{2A}R. Subsequent dose–response curves of these five hits were well behaved, with IC_{50} values ranging from 70 μM to 1.9 mM (Table 1 and Figure 3). Analysis of the dose–response curves revealed that the Hill coefficient for each of the five compounds

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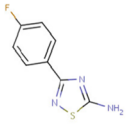
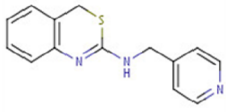
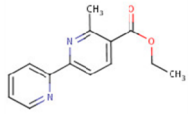
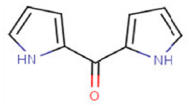
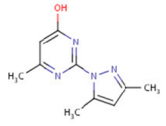
Table 1. Characterization of Fragment Hits by Equilibrium Radioligand Displacement Assays on Wild-Type A_{2A} and A_1 Receptors

ID	Fragment structure	Displacement of [^{3}H]ZM241385 on $A_{2A}R$[a]			Displacement of [^{3}H]NECA on $A_{2A}R$[a]			% displacement of [^{3}H]DPCPX on A_1R[b]
		IC_{50} (mM) (% displacement)	Hill Slope[c]	Ligand Efficiency[d] (LEAN)	IC_{50} (mM) (% displacement)	Hill Slope[c]	Ligand Efficiency[d] (LEAN)	
ZB643		0.07 ± 0.02 (91%)	-0.87 ± 0.12	0.43	0.08 ± 0.03 (90%)	-1.02 ± 0.12	0.42	92%
ZB1703		0.79 ± 0.32 (65%)	-1.23 ± 0.19	0.23	1.07 ± 0.04 (57%)	-1.11 ± 0.15	0.22	52%
ZB1166		1.32 ± 0.26 (40%)	-1.07 ± 0.24	0.22	0.25 ± 0.02 (68%)	-0.92 ± 0.09	0.27	48%
ZB2086		1.73 ± 0.47 (34%)	-1.07 ± 0.26	0.30	0.76 ± 0.05 (37%)	-1.04 ± 0.07	0.35	21%
ZB1396		1.88 ± 0.26 (30%)	-1.13 ± 0.21	0.23	7.71 ± 0.04 (26%)	-1.24 ± 0.24	0.19	22%

[a]Displacement of specific [^{3}H]ZM241385 or [^{3}H]NECA binding in HEK293 cell membranes transiently expressing human adenosine A_{2A}Rs expressed as IC_{50} ± SEM (n = 3) or % displacement of specific binding at 0.5 mM fragment concentration (n = 2). [b]Percent displacement of specific [^{3}H]DPCPX binding in CHO cell membranes stably expressing human adenosine A_1Rs at 0.5 mM fragment concentration. [c]Hill slope ± SEM (n = 3). [d]LEAN ligand efficiency is −log IC_{50} divided by the heavy atom count.[32]

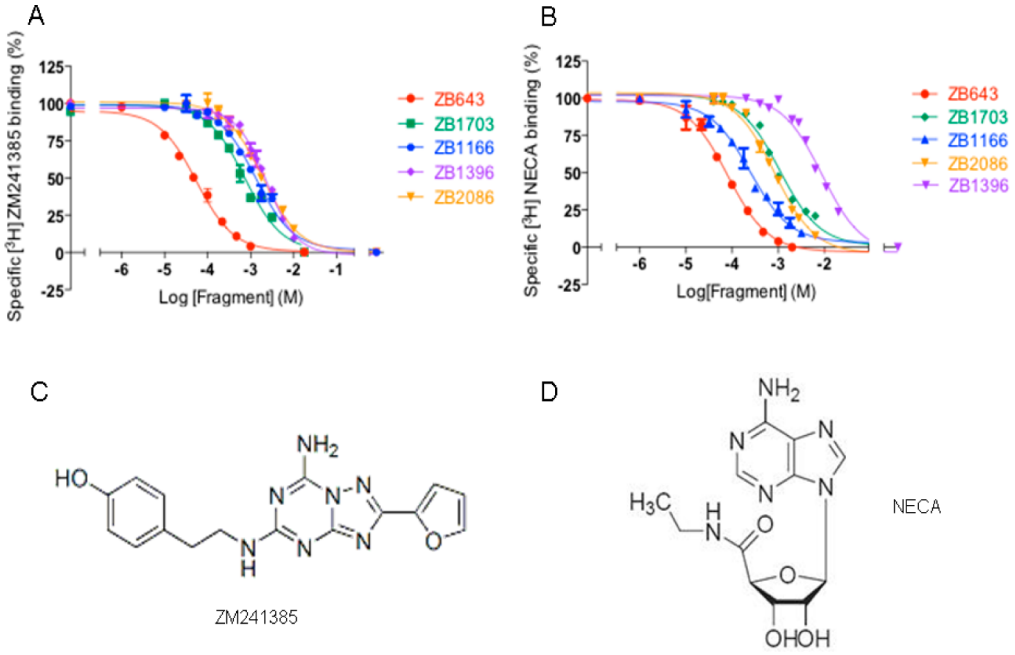


Figure 3. Representative dose−response curves for displacement of binding of the radiolabeled A_{2A}R inverse agonist ZM241385 (A) and agonist NECA (B) by A_{2A} hits identified in the TINS screen. Each curve was determined in triplicate in three independent experiments. The structures of the radiolabeled ligands are shown in panels C and D.

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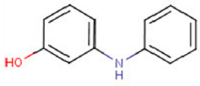
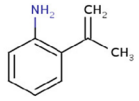
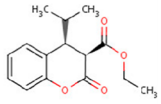
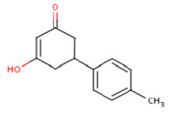
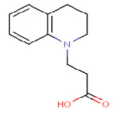
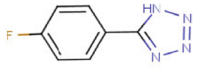
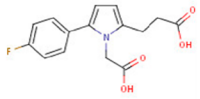
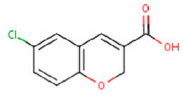
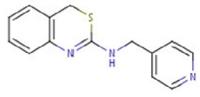
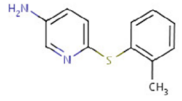
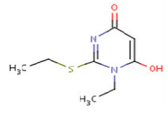
Table 2. Characterization of Fragment Hits by Kinetic Radioligand Dissociation Assays on the Wild-Type A_{2A} and A_1 Receptors

ID	Fragment structure	k_{off} (min^{-1}) or % allosteric modulation at 2 mM[a]			
		$A_{2A}R$[b]		A_1R[c]	
		[^{3}H] ZM241385	[^{3}H] NECA	[^{3}H] DPCPX	[^{3}H] CCPA
Control		0.011 ± 0.002 (0%)	0.015 ± 0.002 (0%)	0%	0%
Negative Allosteric Modulators for both [^{3}H]ZM241385 and [^{3}H]NECA binding					
ZB418		0.039 ± 0.001 (-36%)	0.022 ± 0.003 (-27%)	-11%	-15%
ZB2044		0.019 ± 0.001 (-21%)	0.032 ± 0.003 (-32%)	-30%	-22%
ZB1153		0.017 ± 0.004 (-18%)	0.021 ± 0.002 (-26%)	-23%	-8%
Positive Allosteric Modulators for [^{3}H]ZM241385 and/or [^{3}H]NECA binding					
ZB335		0.0071 ± 0.0002 (20%)	0.0051 ± 0.0009 (41%)	16%	-6%
ZB1854		0.0063 ± 0.0005 (31%)	0.0095 ± 0.0005 (18%)	1%	-29%
ZB391		0.0066 ± 0.0006 (25%)	0.0085 ± 0.0005 (24%)	2%	-41%
ZB1250		0.0062 ± 0.0004 (30%)	0.0089± 0.0007 (22%)	3%	-14%
ZB268		0.0061 ± 0.0005 (31%)	(8%)	17%	-43%
ZB1703		14%	0.0079 ± 0.0006 (30%)	-8%	48%
ZB114		12%	0.0081 ± 0.0006 (28%)	11%	22%
ZB337		17%	0.0082 ± 0.0005 (26%)	3%	-4%

[a] k_{off} ± SEM (n = 3), % allosteric modulation (n = 2). [b] The values of the kinetic dissociation rate constants were obtained by analysis of the exponential dissociation curve of [^{3}H]ZM241385 or [^{3}H]NECA bound to human adenosine A_{2A}Rs. [c] Percent allosteric modulation of [^{3}H]DPCPX and [^{3}H]CCPA binding at human adenosine A_1Rs in the absence (control; 0%) or presence of 2 mM of the fragment.

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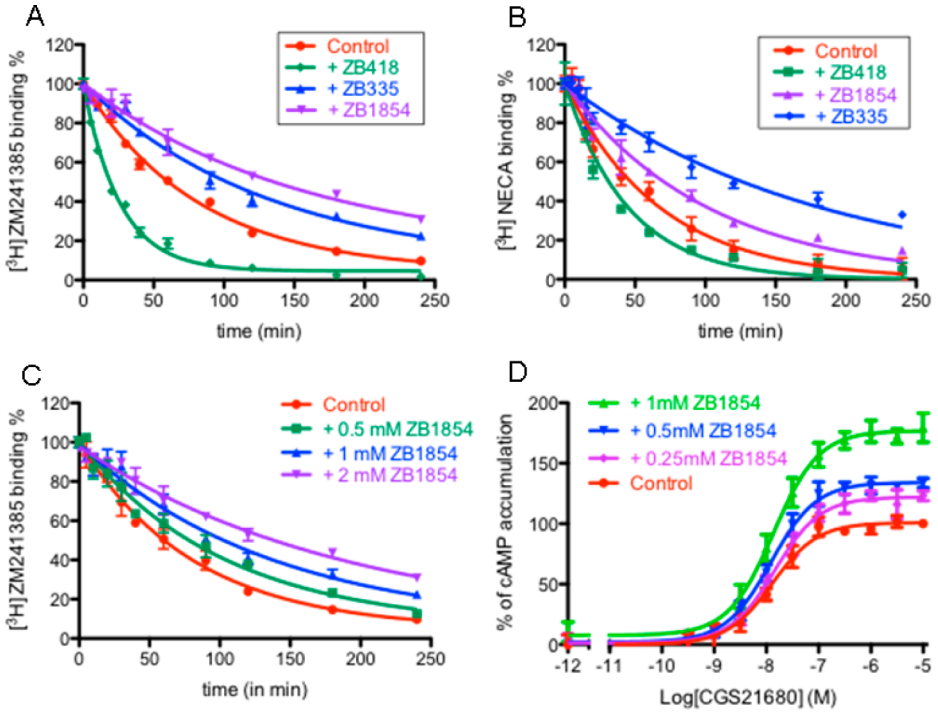

Figure 4. Allosteric effects of fragment hits on the $A_{2A}R$. (A) [^{3}H]ZM241385 and (B) [^{3}H]NECA kinetic dissociation assay. Fragments were assayed for their effect on the dissociation rate of two $A_{2A}R$ orthosteric ligands: [^{3}H]ZM241385 and [^{3}H]NECA. Dissociation was initiated by the addition of 10 μM ZM241385 or NECA in the absence (control) or presence of 2 mM of the indicated fragment. (C) Dissociation kinetics of [^{3}H]ZM241385 binding to $A_{2A}R$. Dissociation was initiated by the addition of 10 μM ZM241385 mixed with buffer (control) or the indicated concentration of the fragment. (D) The effect of increasing concentration of the fragment ZB1854 on the cAMP production induced by the $A_{2A}R$ specific agonist CGS21680 in HEK293 cells transiently expressing the human A_{2A} receptor. The % of cAMP accumulation is normalized to the effect of CGS21680 at 10 μM.

is not significantly different from unity, which in conjunction with the TINS data indicates that these fragments reversibly bind $A_{2A}R$ with a 1:1 stoichiometry. As expected for compounds binding to the orthosteric site, the five hits also significantly inhibited the binding of [^{3}H]NECA, an adenosine receptor agonist, with IC_{50} values ranging from 80 μM to 7.7 mM (Table 1). Achieving receptor subtype selectivity is desirable but can be a challenge for fragments targeting the orthosteric site. Perhaps not unsurprisingly, the five compounds also displaced [^{3}H]DPCPX, a well-characterized orthosteric antagonist for the closely related adenosine A_1 receptor subtype. Moreover, the potency of each of the five compounds in the A_1R assay was similar to that of the $A_{2A}R$. It is not unexpected at this stage in hit finding for fragments to show binding to two closely related receptors. During a chemistry lead optimization program, such fragment hits could be optimized for selectivity using a variety of structure-based design approaches. Many known adenosine receptor ligands have been developed based on the adenosine[28] and xanthine[29] chemotypes. Newer adenosine receptor antagonists have modifications based on tricyclic derivatives of triazolopyrimidine,[30] including Preladenant,[31] which has been evaluated in clinical trial (phase III) for the treatment of Parkinson's disease, and exhibit high selectivity for $A_{2A}R$. As shown in Table 1, the five fragment $A_{2A}R$ orthosteric ligands represent a diversity and novelty of chemical scaffolds. A hallmark of FBDD is that it generates ligands in which most of the atoms contribute to target binding, a characteristic termed ligand efficiency. Indeed, two of the fragments in Table 1 have high ligand efficiency (LEAN[32] (pIC50/heavy atom count) $\geq$ 0.3) and are potentially suitable as starting points for lead generation, even with their low potency.

2. Probing for Allosteric Site Ligands. We next looked at whether any of the 94 fragment hits bind to allosteric site(s) on the $A_{2A}R$. In order to do so, we employed an assay that measures the effect of a fragment on the dissociation rate (k_{off}) of an orthosteric ligand but is not sensitive for competitive binding at the orthosteric site. Each of the 94 fragment hits were screened for their effect on the off rate of the inverse agonist [^{3}H]ZM241385 and the agonist [^{3}H]NECA dissociating from wild-type A_{2A} receptor using a single time point at which 50% of the radioligand has dissociated under control conditions. Three fragments significantly (at least 30%) increased k_{off} of the orthosteric ligand and hence are negative allosteric modulators (NAMs), while four significantly decreased k_{off} and hence are positive allosteric modulators (PAMs) of both ZM241385 and NECA (Table 2). In addition, another four fragments allosterically enhanced either NECA binding or ZM241385 binding (Table 2). Subsequently, full dissociation curves of [^{3}H]ZM241385 and [^{3}H]NECA were measured in the absence (control) or presence of 2 mM of each of the active fragment hits. As shown in Table 2 and Figure 4A and B, the intrinsic dissociation rate of the radioligand was 0.011 $\pm$ 0.002 min^{-1} for [^{3}H]ZM241385 and 0.015 $\pm$ 0.002 min^{-1} for [^{3}H]NECA. Most of the fragment hits showed at least 2-fold modulation of radioligand dissociation. For example, in the presence of 2 mM ZB335, the k_{off} of [^{3}H]NECA was decreased 2.9-fold to 0.0051 $\pm$ 0.0009 min^{-1}. In combination with the lack of competitive binding, this data strongly suggests that ZB335 is acting at an allosteric site.

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Similarly, addition of 2 mM ZB418 resulted in an increase of the k_{off} of [^{3}H]ZM241385 by 3.5-fold to 0.039 ± 0.001 min^{-1}, suggesting this fragment is a NAM. Interestingly, ZB418 showed a hill slope of about −2 in the equilibrium displacement assay for both [^{3}H]ZM241385 and [^{3}H]NECA, which can also be indicative of an allosteric mechanism.[33] Importantly, the allosteric modulation by the fragment hits was concentration-dependent (Figure 4C and Table 3). In contrast to the fragments that were competitive with orthosteric ligands, the most potent allosteric modulators, such as ZB335 and ZB418, exhibited considerably reduced effects on the A_1R, suggesting that they have some A_{2A} subtype specificity.

Table 3. Effect of ZB1854 on the Dissociation of [^{3}H]ZM241385 from A_{2A}R

condition	k_{off} (min^{-1})[a]	shift[b]
control ([^{3}H] ZM241385)	0.0121 ± 0.0010	
+ 0.5 mM ZB1854	0.0102 ± 0.0009	1.2
+ 1 mM ZB1854	0.0072 ± 0.0011	1.7
+ 2 mM ZB1854	0.0058 ± 0.0015	2.1

[a] k_{off} ± SEM (n = 3). [b] The shift is defined as the ratio of the k_{off} values in the absence (control) and presence of ZB1854, respectively.

Finally, we determined whether the fragments hits were active in a cell-based assay. We therefore investigated the effect of fragments on A_{2A}R activation by the agonist CGS21680 in a cyclic AMP accumulation assay (Table 4 and Figure 4D).

Table 4. Receptor Activation by CGS21680 in the Presence of ZB1854

	cAMP accumulation assay[a]	
ID	EC_{50} (nM)	E_{max} (%)[b]
CGS21680	14.1 ± 0.3	100 ± 2
CGS21680 + 0.25 mM ZB1854	14.1 ± 0.3	120 ± 3
CGS21680 + 0.5 mM ZB1854	13.8 ± 0.4	134 ± 9
CGS21680 + 1 mM ZB1854	14.0 ± 0.3	177 ± 13

[a] cAMP accumulation in HEK cells that transiently express the human adenosine A_{2A}R. [b] Maximal effect of CGS21680 in the absence or presence of modulator, where CGS21680 in the absence of modulator was set at 100%. Values are means ± SEM (n = 3).

Addition of ZB1854 at three concentrations did not cause a shift in potency of CGS21680 (EC_{50} = 14.1 ± 0.3 nM). However, increasing concentrations of ZB1854 did result in a dose-dependent increase of the maximal effect (E_{max}) of CGS21680. The change in E_{max} is consistent with the previous data, suggesting that ZB1854 acts *via* an allosteric mechanism.[33,34] For other compounds, such as ZB418 (NAM) and ZB335 (PAM), that were tested at these high concentrations, we observed some cell toxicity, and therefore it was not possible to confirm their allosteric effects in the cellular assay.

Discussion. Traditionally, small molecule modulators of GPCRs have been discovered through pharmacological studies. More recently functional, cell-based assays have been coupled to high-throughput screening to discover novel molecules with selected activities such as positive allosteric modulation.[35,36] However, for a number of reasons such molecules have failed to gain marketing approval. The combination of structure-based drug discovery and fragment-based drug discovery has demonstrated the ability to generate approved drugs with excellent pharmacological properties[37] against soluble targets. It is becoming apparent that FBDD/SBDD can now be applied to GPCRs with great potential advantages.[5]

Use of a direct binding assay, such as TINS, selects for fragments that specifically bind to the target, regardless of the site. In principle it is possible to discover ligands with many different biological activities. Indeed here we have found orthosteric ligands (although we have not investigated their pharmacology; Table 1), positive allosteric modulators, and negative allosteric modulators (Table 2), thereby substantiating the claim. One of the orthosteric ligands, ZB643, constitutes a scaffold in a series of thiadiazole derivatives previously found to interact with other adenosine receptor subtypes.[38] Whether ZB643 can be a lead for further exploration needs careful consideration as other thiadiazoles have been reported to be protein modifiers due to their reactivity.[39] Moreover, this study is one of the first reports of allosteric modulators for the adenosine A_{2A} receptor, with indeed previously unknown chemistry.[40] Giorgi *et al.* have provided evidence of allosteric enhancement in a class of substituted 8-azaadenines before.[41] As ZB1854 potentiates the action of an adenosine A_{2A} receptor agonist, its therapeutic potential may be in wound healing and in combating inflammation.[42] Given their apparent low potency, it is not clear whether the allosteric modulators would have been discovered by other methods. However, fragments typically bind in the micromolar to millimolar range from where they are developed to the potency ultimately required for *in vivo* efficacy.

The present study used a biophysical technique to discover novel fragment ligands. However, the various biochemical activities of these ligands were characterized by traditional radioligand binding methods and a functional assay. It is likely that these methods missed multiple, valid fragment ligands simply because their potency was too low to register in the selected assay. In order to take true advantage of the biophysical screening technique, it will be necessary to implement biophysical techniques to characterize the binding mode and discriminate orthosteric from allosteric ligands. This can be accomplished using a competition binding assay in TINS as we have demonstrated for the β_1 adrenergic receptor.[15] Furthermore, the biophysical mapping technique developed by Zhukov *et al.*[24] offers the possibility to refine the binding site to significantly better resolution than competition binding studies.

Evolving weakly binding hits to lead-like compounds remains a significant challenge for FBDD. It is well established that with the availability of high-resolution 3D structures of target-fragment complexes, the success and efficiency of fragment elaboration is quite high.[9] One particular advantage of StaR GPCRs is that they are amenable to X-ray crystallographic analysis, and indeed structure-based drug design is beginning to be applied to this target class.[5,43] In addition to X-ray, StaRs may enable NMR analysis of protein-small molecule complexes. The NMR resonance assignment and structure elucidation of sensory rhodopsin has recently been accomplished.[44] Although the latter was a significant achievement, it is plausible that the enhanced thermostability and reduced conformational exchange of StaRs may enable resonance assignment, at least for putative allosteric sites at or near the extracellular loop region. If so, the ligand binding site can be mapped at low resolution using chemical shift perturbation techniques.[45] The tremendous increase in crystal structures of GPCRs in various states has had a positive impact on the ability to model small molecule-GPCR interactions.[46] The combination of biophysical

mapping and/or NMR-based structural constraints with modeling should also provide considerable aid to the fragment elaboration process.

METHODS

Materials. 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}H]ZM241385, specific activity 30 Ci mmol^{-1}) and tritiated 5′-*N*-ethylcarboxamido adenosine ([^{3}H]NECA, specific activity 33 Ci mmol^{-1}) were purchased from ARC Inc. (St. Louis, USA). Unlabeled ZM241385, NECA, and 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethyl-carboxamido adenosine (CGS21680) were purchased from Sigma-Aldrich (Steinheim, Germany). All compounds in the fragment library are commercially available. A 1D ^{1}H spectrum of each fragment has been collected[25] and is consistent with the manufacturer's claim of >95% purity. Bovine serum albumin (BSA, fraction V) was obtained from Sigma (St. Louis, MO, USA) and BCA protein assay reagent was from Pierce Chemical Company (Rockford, IL, USA). Human embryonic kidney 293 cells transiently expressing the wild-type A_{2A}R (HEK293 A_{2A}R) were generated as described below. All other chemicals were of analytical grade and obtained from standard commercial sources.

Protein Purification. For biophysical studies, the inverse agonist stabilized A_{2A}-StaR2,[47] including a C-terminal His-10 tag, was expressed in *Trichoplusia ni* (Tni) cells and purified as previously described.[18] OmpA, including an N-terminal His-6 tag, was expressed in BL21(DE3) cells under the control of the T7 promoter and purified as previously described.[48] Successful refolding of OmpA from inclusion bodies was monitored by SDS-PAGE analysis.[48] Both A_{2A}R and OmpA were buffer-exchanged to PBS (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.4) containing 0.47% *n*-dodecylphosphocholine (DPC) for OmpA or 0.1% *n*-decyl-*β*-D-maltoside (DM) for A_{2A}R.

Protein Immobilization. DM-solubilized A_{2A}R and DPC-solubilized OmpA were immobilized on Actigel ALD resin (Sterogene, Carlsbad, CA, USA) *via* Schiff's base chemistry using the manufacturer's protocol. After overnight incubation at 4 °C, residual unreacted aldehydes on the resin were blocked by addition of 50 mM d11-Tris buffer. Quantification of immobilized protein was monitored by absorption of the supernatant at 280 nm before and after immobilization, and by SDS-page gel with a known standard curve and band volume analysis. This data indicated that a final concentration of 50 μM of immobilized A_{2A}R and 65 μM OmpA was achieved (nmol protein/mL settled bed volume), equating to an 80% and 75% yield, respectively. Subsequently, the buffer of both protein samples was exchanged to PBS containing 0.01% *n*-dodecyl-*β*-D-maltopyranoside (DDM) for ligand screening experiments.

Target Immobilized NMR Screening. Immobilized, DDM solubilized A_{2A}R (50 μM) and OmpA (65 μM) were each packed into a separate cell of a dual-cell sample holder.[26] Mixes of the 531 fragments were made by 200-fold dilution of a 100 mM stock of each compound in d_6-DMSO such that the final DMSO concentration was never greater than 5%. Upon injection of each mix into the dual-cell sample holder, flow was stopped, and spatially selective Hadamard spectroscopy was used to acquire a 1D ^{1}H spectrum of each sample separately.[27] A CPMG T2 filter of 80 ms was used to remove residual broad resonances from the sepharose resin. To maintain the proper fold of each protein, the screen was performed at 15 °C, and 0.01% DDM was included in the buffer (PBS in D_2O) used to wash the fragment mixes from the sample holder.

Pharmacological Characterization of TINS Hits. *Radioligand Binding and Kinetic Dissociation Studies on the Adenosine A_{2A} Receptor.* HEK293 cells were maintained in culture in DMEM supplemented with 10% newborn calf serum at 37 °C in a moist, 7% CO_2 atmosphere and passaged twice weekly. Cells were transfected with plasmids containing wild-type A_{2A}R construct using the calcium phosphate precipitation method[49] and harvested after 48 h. Cells were pelleted and 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 100,000*g* for 20 min at 4 °C. This process was repeated twice, and subsequently the pellet was resuspended in 50 mM Tris-HCl buffer pH 7.4, the protein concentration was determined by the BCA method,[50] and samples were aliquoted and stored at −80 °C until further use.

First, all fragment hits from the TINS screen were assayed in a [^{3}H]ZM241385 displacement assay at 500 μM. Fragments with more than 30% displacement of [^{3}H]ZM241385 were titrated to determine an IC_{50} curve. For displacement binding studies, membranes from cells transiently expressing A_{2A}R-WT (20 μg proteins/well) were incubated in a total volume of 100 μL Tris-HCl buffer (50 mM, pH 7.4) and 5% DMSO with fragments at either a single final concentration of 500 μM or 10 × 0.25 log unit dilutions and a final [^{3}H]ZM241385 (an inverse agonist of the A_{2A}R) concentration of 3 nM or 20 nM of [^{3}H]NECA (an agonist of the A_{2A}R).[42] Non-specific binding was determined using a final concentration of 10 μM ZM241385 and represented less than 10% of the total binding. Total binding was determined in the presence of buffer and was set at 100% in all experiments, whereas non-specific binding was set at 0%. After incubation for 60 min (with [^{3}H]ZM241385) or 150 min (with [^{3}H]NECA) at 25 °C, assays were terminated by rapid filtration through 96-well GF/B UniFilter plates (PerkinElmer) followed by washing with 7 × 0.25 mL 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 bound radioactivity measured using a Packard Microbeta counter (PerkinElmer). Data were analyzed using GraphPad Prism v5, normalized as "% specific binding" from which the IC_{50} values were calculated. Experiments were performed three times in duplicate, unless stated otherwise. Experimental ligand efficiency (LEAN)[32] was defined as $LE = -RT \ln IC_{50}/HAC$, where HAC is the number of heavy atoms in the compounds.

Second, all TINS fragment hits were assayed in a kinetic A_{2A}R-WT orthosteric radioligand dissociation assay using a single time point at which 50% of the radioligand has dissociated (under control conditions). Fragments that resulted in more than 30% increased or decreased radioligand dissociation (*i.e.*, negative or positive allosteric modulation, respectively) were subsequently analyzed at multiple time points to determine the complete dissociation curve. For kinetic dissociation experiments, 3 nM [^{3}H]ZM241385 or 20 nM [^{3}H]NECA was pre-equilibrated with membranes (30 μg proteins/well) for 3 h on ice; dissociation was initiated by addition of 10 μM ZM241385 in the absence (control) or presence of 2 mM test compound in a total volume of 5 μL. The amount of radioligand (~50%) still bound to the receptor was measured after 50 min of dissociation. The amount of specific radioligand binding obtained under control conditions was set at 0% for both positive and negative allosteric modulators (PAMs and NAMs); the total binding (t = 0 min) was set at 100% for PAMs, whereas non-specific binding (in the presence of 10 μM ZM241385) was set at −100% for NAMs. The amount of remaining radioligand bound to the receptor was measured at various time intervals for a total of 4 h. Incubations were terminated, and samples were obtained as described above. The dissociation data were fit globally in GraphPad Prism v5 to determine k_{off}. Experiments were performed three times in duplicate, unless stated otherwise.

Selectivity Assays Using CHO Cell Membranes Stably Expressing Human Adenosine A_1 Receptor. The receptor subtype selectivity of compounds that were positive in the kinetic dissociation assay with A_{2A}R-WT using [^{3}H]NECA was tested in [^{3}H]DPCPX[42] (an antagonist of the A_1R) and [^{3}H]CCPA[42] (an agonist of the A_1R) kinetic dissociation assays using wild-type human adenosine A_1 receptors stably expressed in CHO cell membranes as described previously in the literature.[51]

Characterization of Allosteric Modulation of Orthosteric Ligand Activity by Intracellular cAMP 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 275*g* for 5 min. For cyclic AMP production and determination, 3000 cells/well were used in 384-well Optiplates (PerkinElmer). The cells were incubated for 45 min at 37 °C with either the A_{2A}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).

Fragment Collection. The fragment collection consists of about 1,500 compounds derived from a variety of vendors. The philosophy used to design the collection as well as the physicochemical properties have been described.[25] For quality control purposes as well as for formatting the library for TINS, a 1D ^{1}H NMR spectrum of each compound was acquired and compared to the expected spectrum based on the structure provided by the vendor. Compounds whose purity was less than the 95% indicated by the vendor were rejected. A randomly selected subset of the complete collection was chosen to provide proof of principle while reducing the amount of subsequent characterization work to a practical level.

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