To bind or not to bind, that is an important question! : Development of covalent probes for adenosine receptors

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Chapter 6

An Affinity-based Probe for the human Adenosine A_{2A} Receptor

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Abstract

Using activity-based protein profiling (ABPP) functional proteins can be interrogated in their native environment. Despite their pharmaceutical relevance G protein-coupled receptors (GPCRs), have been difficult to address through ABPP. In the current study we took the prototypical human adenosine A_{2A} receptor ($hA_{2A}R$) as the starting point for the construction of a chemical toolbox allowing two-step affinity-based labeling of GPCRs.

Firstly, we equipped an irreversibly binding $hA_{2A}R$ ligand with a terminal alkyne to serve as probe. We showed that our probe irreversibly and concentration-dependently labeled purified $hA_{2A}R$. Click-ligation with a sulfonated cyanine-3 fluorophore allowed us to visualize the receptor on SDS-PAGE. We further demonstrated that labeling of the purified $hA_{2A}R$ by our probe could be inhibited by selective antagonists. Lastly, we showed successful labeling of the receptor in cell membranes overexpressing $hA_{2A}R$ making our probe a promising affinity-based probe that sets the stage for the further development of probes for GPCRs.

1. Introduction

The adenosine receptors, belonging to the family of G protein-coupled receptors (GPCRs), have been coined adenosine A_1, A_{2A}, A_{2B}, and A_3. These receptors are widely distributed through the human body and are considered promising targets for a wide range of diseases [1]. Regadenoson, a selective human adenosine A_{2A} receptor ($hA_{2A}R$) agonist used to increase vasodilation during cardiac imaging, has been approved by the FDA, exemplifying the potential therapeutic applications for the $hA_{2A}R$. Likewise, $hA_{2A}R$ antagonists are currently being pursued as potential treatment of Parkinson's disease and as adjuvants in cancer immunotherapy [2, 3].

The $hA_{2A}R$ was one of the first GPCRs for which a crystal structure was elucidated [4]. However, the challenges in structural biology of GPCRs, including the low expression level in native tissue and inherent poor protein stability, still exist [5]. To overcome these obstacles, covalent probes have been developed as useful pharmacological tools. Such probes, also named affinity labels, represent compounds that feature a reactive cross-linking moiety, which can irreversibly and specifically bind to a receptor. For example, an irreversible antagonist was used to stabilize the adenosine A_1 receptor for co-crystallization, resulting in the visualization of key amino acids important for ligand-receptor binding [6].

The design of covalent probes for GPCRs generally follows a similar strategy, which is to incorporate a warhead in a high-affinity, reversibly binding ligand. Based on the type of warhead used, two categories of irreversible ligands can be discerned: photoaffinity- and chemoreactive ligands [7, 8]. Whereas in the former type a photoreactive warhead is employed, the latter is equipped with an electrophilic chemical moiety capable of binding nucleophilic residues in the target protein. A commonly used warhead is aryl sulfonyl fluoride, which is capable of covalently binding to many nucleophilic amino acid residues, such as serine, threonine, lysine, and cysteine [9]. This warhead has been incorporated in several reported covalent ligands for the adenosine receptors, including FSCPX [10], FSPTP [11], fluorosulfonyl functionalized pyrimidine derivatives [12] and LUF7445 [13]. Likewise, fluorescent tags have been incorporated into adenosine receptor ligands to visualize the receptor, which yielded e.g., FITC-ADAC [14], MRS5422 [15] and NBD-NECA [16]. However, fluorescent moieties are of significant size and *a priori* derivatization of a ligand with such a group may negatively affect receptor affinity. Here, two-step affinity-based probes (AfBPs) might be a better alternative, as a reporter tag is added after the reactive ligand has bound its target [17].

Interestingly, from the field of activity-based protein profiling (ABPP), combined with click chemistry, many techniques have emerged that could potentially be applied to GPCRs using our covalent ligand. Normally in ABPP, an irreversible ligand is equipped with a ligation handle and after binding to the protein of interest is paired with a clickable fluorophore. In this way, via a Huisgen 1,3-dipolar cycloaddition, a stable triazole-linked product is formed, effectively attaching a fluorescent label to the protein [18-20]. Currently, this technique serves as a tool to profile the activities of drug targets (currently mainly enzymes) in native biological systems. One-step labeling, where the reporter group is pre-attached to the probe, has been applied on GPCRs before [21-23]. Moreover, similar two-step labeling strategies have been applied for other targets[24, 25]. However, due to their low abundance GPCRs are difficult to address with this otherwise promising technique. Within the entire GPCR family with over 800 members, until recently, only the $mGlu_5$ receptor had been the subject of this approach, albeit with limited success [26]. Very recently, the type 2 cannabinoid receptor (CB_2R) has been probed with a two-step photoaffinity probe, leading to great insights into receptor localization and target engagement [27].

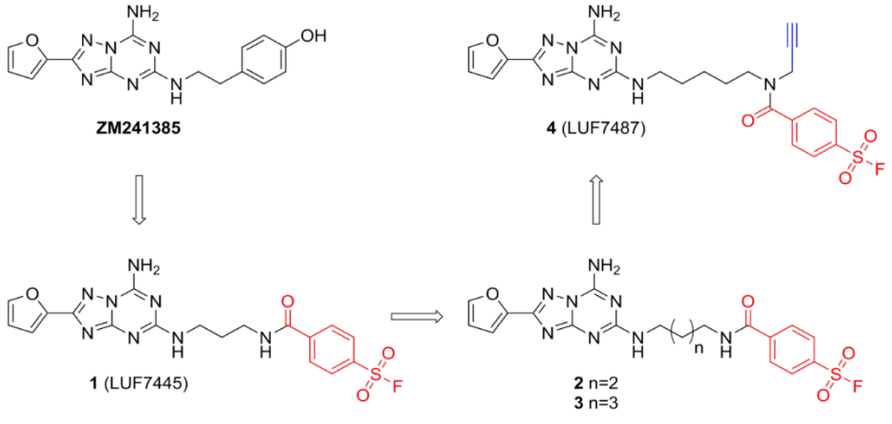


Figure 1. Chemical structures of the $hA_{2A}R$ antagonists examined in this study. The lead compound ZM241385, a selective $hA_{2A}R$ antagonist, inspired the design of covalent antagonist **1**.[13] In the current study the effect of the linker length between scaffold and warhead on affinity was further examined, yielding compound **2** and, preferably, compound **3**. The affinity-based probe **4** was then synthesized from compound **3**, bearing an alkyne ligation-handle and a fluorosulfonyl electrophilic warhead. The electrophilic warhead is in red and the click-ligation handle is in blue.

In this study, we describe our efforts to obtain a clickable affinity-based probe, with an electrophilic warhead, as a logical extension of our previous research on the successful design of a covalent antagonist of $hA_{2A}R$, compound **1** (LUF7445) [13]. We used the antagonist ZM241385 as the starting point in our design efforts, and synthesized a series of fluorosulfonyl derivatives with diverse linker lengths (compounds **1-3**, Figure 1). The most

potent ligand, with low nanomolar affinity, was retained for further structural modification and was equipped with an alkyne-click handle, resulting in probe **4**, as shown in Figure 1. We then validated that the ligand's binding to the receptor was wash-resistant. Additionally, we demonstrated the ligand's covalent labeling capacity of purified receptors via a bioorthogonal copper catalyzed azide-alkyne ligation reaction with a fluorescent moiety, sulfonated cyanine 3 ((E)-2-((E)-3-(1-(6-((3-azidopropyl)amino)-6-oxohexyl)-3,3-dimethyl-5-sulfo-3*H*-indol-1-ium-2-yl)allylidene)-3,3-dimethyl-1-(3-sulfopropyl)indoline-5-sulfonate). Finally, this probe was able to profile the presence of $hA_{2A}R$ in a relatively complex biological sample. Hence, this is one of the first AfBPs for a GPCR, and may set the stage for similar probes to facilitate target discovery and bioanalysis of GPCRs associated with human disease.

2. Results and discussions

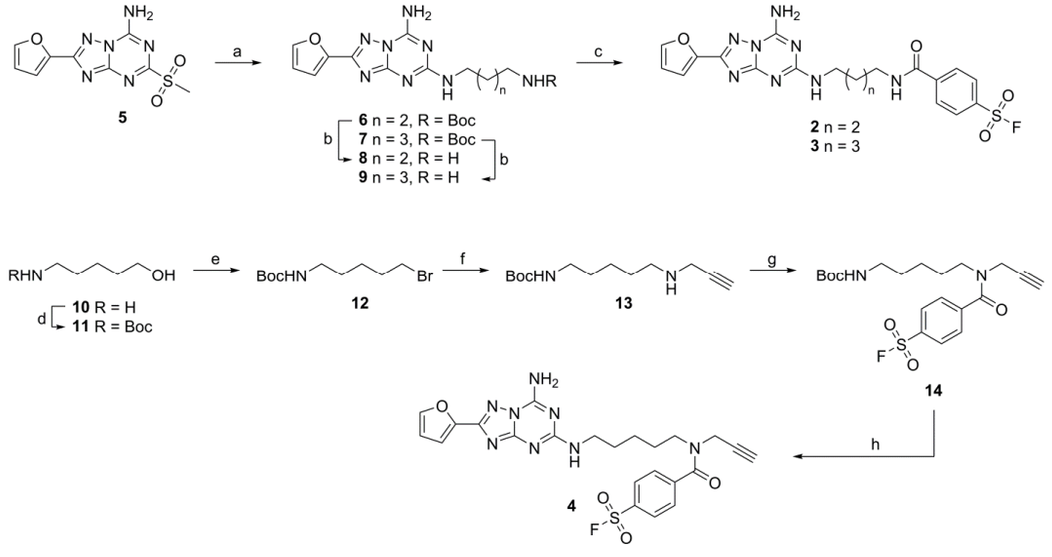
2.1. Chemistry.

In the past, our research group has been evaluating structural modifications of triazolotriazine derivatives based on the selective adenosine A_{2A} antagonist 4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazin-5-ylamino)ethyl)phenol (ZM241385), to obtain a covalent ligand for the $hA_{2A}R$. The rational design of this covalent ligand originated from a reported $hA_{2A}R$ crystal structure (PDB: 4EIY) in complex with ZM241385 [4]. In it, the ligand binding pocket demonstrated a deep, planar and narrow cavity embracing the aromatic core and furan ring of ZM241385. Therefore, an extension of the hydroxyphenethylamine moiety into the extracellular domain of the receptor offered us the playground for integration of the electrophilic reactive groups. Our earlier covalent antagonist, compound **1** (Figure 1), in which the 4-hydroxyphenylethylamine-side chain in ZM241385 was replaced with a similar side chain harboring an electrophilic fluorosulfonyl moiety, was recognized by $hA_{2A}R$ with an apparent pK_i of 8.99 [13]. In order to optimize the irreversible binding potential of our compound, with our current aim of developing an AfBP in mind, an exploration of linker length was performed, varying the linker between the fluorosulfonyl warhead moiety and the aromatic recognition element from three to five carbon atoms. To this end, compounds **2** and **3** were synthesized as detailed in Scheme 1. The synthesis starts from 2-(furan-2-yl)-5-(methylsulfonyl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-7-amine **5**, synthesized as previously reported [13], and involves a linear sequence comprising aromatic substitution with either commercially available mono-Boc-protected butyldiamine or pentyldiamine and subsequent Boc-deprotection towards intermediates **8** and **9**. Introduction of the fluorosulfonylbenzoyl

warhead proceeded with low yields due to difficult purification, providing ligands **2** and **3** in 4% and 2% yield respectively.

The synthetic route towards probe **4** (LUF7487, Figure 1) is depicted in Scheme 1. First, the amino group of 5-aminopentanol was protected with a Boc group and the hydroxyl was converted to a bromide using an Appel reaction, providing intermediate **12**. Nucleophilic substitution of the bromide with propargylamine afforded amine **13**, which was acylated with 4-fluorosulfonylbenzoylchloride to give Boc-protected bi-functional spacer **14** uneventfully. Finally, in a two-step process, the spacer was deprotected and coupled to scaffold **5**, to provide probe **4** in 45% yield.

Scheme 1. Synthesis of compounds **2**, **3** and **4.**



Reagents and conditions: a) *tert*-butyl (4-aminobutyl)carbamate or *tert*-butyl (5-aminopentyl)carbamate, DiPEA, MeCN, 70-85˚C, 46-74%; b) TFA, quant; c) 4-fluorosulfonylbenzoylchloride, DiPEA, MeCN, 70˚C, 2-4%; d) Boc_2O, DCM, quant; e) PPh_3, CBr_4, 90%; f) propargylamine, DiPEA, 46%; g) 4-fluorosulfonylbenzoylchloride, DiPEA, MeCN, quant; h) i. **5**, TFA, DCM, ii. DiPEA, MeCN, 70˚C, 45%

2.2. Biology

To assess the affinity for the $hA_{2A}R$, compounds **2** and **3** were tested in [^{3}H]ZM241385 displacement experiments (n = 3), which demonstrated a concentration-dependent inhibition of radioligand binding to $hA_{2A}R$ overexpressed in HEK293 cells. To better understand the time-dependent binding characteristics of these compounds, we then carried out displacement assays performed with two different incubation times. Representative graphs for these

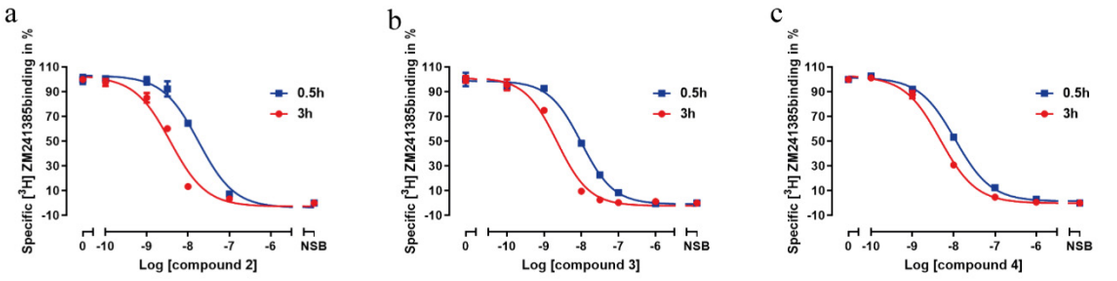


Figure 2. Displacement of specific [3H]ZM241385 binding from HEK293 cell membranes stably expressing the $hA_{2A}R$ receptor at 25°C by compound **2** (a), **3** (b) and **4** (c) with an incubation time of 0.5h (blue curve) and 3h (red curve), respectively. Representative graphs are from one experiment performed in duplicate. ndependent experiments performed in duplicate with error bars representing SEM values

experiments are given in Figure 2a and 2b, in which the concentration-dependent inhibition of specific [^{3}H]ZM241385 binding shifted to the left with an incubation time extension from 0.5 h (standard) to 3 h. As detailed in Table 1, the affinities of both compound **2** and **3** significantly increased by approximately 5-fold to sub-nanomolar values with longer incubation times. In other words, both designed covalent ligands became more potent in displacing the radioligand [^{3}H]ZM241385 from the receptor over time. Similarly, to **1** [13], this pronounced affinity increase may be attributed to an irreversible binding nature of the compounds, leading to a higher receptor occupancy with a longer incubation time. It should be kept in mind that due to the covalent nature of the interaction; affinity values can only be apparent as no dynamic equilibrium can be reached. Compound **3** inhibited the specific [^{3}H]ZM241385 binding to the $hA_{2A}R$ with a pK_i of 9.21, compared to the affinity of compound **2** (pK_i = 9.05 ± 0.07) and **1** (pK_i = 8.99 ± 0.01). Thus, the extension of the linker to five carbon atoms slightly increased the apparent affinity. This could be caused by more steric freedom allowing the fluorosulfonyl group to orient towards the adjacent nucleophilic residue in the receptor binding site compared to ligands with a shorter linker. A similar example is an electrophilic probe for the cannabinoid CB_1 receptor, 7'-NCS-1',1'-DMH-Δ^8-THC, in which lengthening the C-3 alkyl side chain to seven carbons, resulted in a significantly improved affinity [28]. Above all, high affinity is a key requirement for the development of irreversible ligands, as it increases the presence of the chemoreactive moiety in proximity to a nucleophilic residue in the binding site, thereby improving receptor occupancy and causing a

Table 1. (Apparent) affinities of synthesized ligands for the human A_{2A} adenosine receptor[a]

Compound[b]	pK_i[c] (0.5h)	pK_i[d] (3h)	pK_i shift[e]
1[f]	8.27 ± 0.04	8.99 ± 0.01***	0.72
2	8.20 ± 0.13	9.05 ± 0.07***	0.85
3	8.56 ± 0.03	9.21 ± 0.01***	0.65
4	8.41 ± 0.02	8.82 ± 0.02***	0.41

[a] Data are expressed as means ± SEM of three separate experiments each performed in duplicate. ***P < 0.001 compared with the pK_i values in displacement experiments with a 0.5h incubation time; Student's t-test.

[b] For all the designed covalent antagonists, pK_i values can only be apparent, as true equilibrium cannot be reached;

[c] Affinity, expressed as pK_i value, determined from displacement of specific [^{3}H]ZM241385 binding from the $hA_{2A}R$ at 25°C during a 0.5h incubation;

[d] Affinity, expressed as pK_i value, determined from displacement of specific [^{3}H]ZM241385 binding from the $hA_{2A}R$ at 25°C during a 3h incubation;

[e] Affinity shift was calculated as [pK_i (3h)-pK_i (0.5h)];

[f] Data previously reported provided for comparison [13].

decrease in non-specific binding to other unrelated targets. As we anticipated a greater demand for steric freedom for the incorporation of the alkyne group and the subsequent ligation between the alkyne moiety and a bulky fluorescent dye, we retained the preferable five carbon atom linker length for the design of our probe.

Inspired by the most promising compound **3**, we incorporated the alkyne click-handle to afford a novel covalent probe, compound **4** (LUF7487, Figure 1). As detailed in Table 1, affinity-based probe **4** demonstrated a high affinity, displacing [^{3}H]ZM241385 with an apparent pK_i value of 8.82. Under these conditions **4** was at least 10-fold selective over human A_1 and A_3 receptors (SI Table S1). In a time-dependent study, probe **4** generated a significant increase in specific [^{3}H]ZM241385 displacement over time (Table 1). In analogy to the covalent ligand **3**, the designed probe was markedly influenced by prolonged incubation times (Figure 2c), suggesting an increasing level of covalent binding over time. However, compared to **3**, the slight decrease in affinity may be attributed to the incorporation of the click handle, possibly leading to a steric hindrance in the ligand-receptor complex, and/or the formation of a covalent bond between the warhead and other nucleophilic residues.

To better understand the receptor-ligand binding nature, the novel affinity-based probe was then evaluated for its covalent nature by determining its capacity to irreversibly block [^{3}H]ZM241385 to $hA_{2A}R$ binding sites. Membranes overexpressing $hA_{2A}R$ were pretreated with probe **4** or ZM241385 at the indicated concentration (IC_{50} or 0.3-fold IC_{50}) for 3 hours,

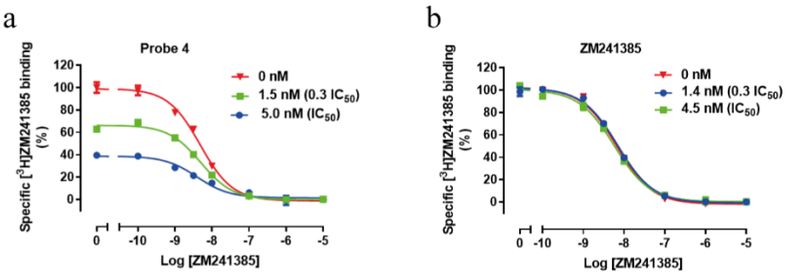


Figure 3. Probe **4** irreversibly binds to $hA_{2A}R$ HEK293 cell membranes stably expressing $hA_{2A}R$ were pre-incubated with probe **4** (a) or ZM241385 (b) at the indicated comparable concentrations. Pretreated membranes were washed three times extensively before further displacement studies of specific [^{3}H]ZM241385 binding from the $hA_{2A}R$ at 25°C by non-labelled ZM241385 were performed. Representative graphs are from three independent experiments performed in duplicate with error bars representing SEM values

followed by a three-cycle washing step to remove the non-covalently bound material. The membranes pretreated with probe **4** (Figure 3a) at increasing concentrations revealed a concomitant decline in specific [^{3}H]ZM241385 binding, which was reduced from 65 ± 2% to 43 ± 2%. However, membranes pretreated with the reversible antagonist ZM241385 (Figure 3b) at increasing concentrations showed no decrease in specific [^{3}H]ZM241385 binding, proving that the washing procedure was extensive enough to remove all non-covalently binding compound. Meanwhile, the affinity of unlabeled ZM241385 was not influenced significantly by the preincubation and washing procedure, indicating that the extensive washing did not damage the membrane integrity or alter the membrane binding sites (SI Table S2). Therefore, it could be concluded that the concentration-dependent decrease in specific [^{3}H]ZM241385 binding observed with probe **4** resulted from an irreversible occupancy of the hA_{2A} receptor binding pocket. Similar results have been obtained on other GPCRs, e.g., for the adenosine A_1 receptor irreversible antagonist FSCPX [29, 30] and the covalent histamine H_4 receptor partial agonist VUF14480 [31], although these compounds lack the alkyne moiety to perform a click chemistry approach.

2.3. Fluorescent labeling of the $hA_{2A}R$.

Having shown that the designed probe **4** meets the requirement of covalent binding, we then set out to evaluate its ability to function as an affinity-based probe. Purified $hA_{2A}R$ was first incubated with the alkyne-containing probe **4** to ensure formation of a covalent probe-$hA_{2A}R$ adduct. Then all samples were subjected to a copper (I)-catalyzed sulfonated cyanine 3-azide

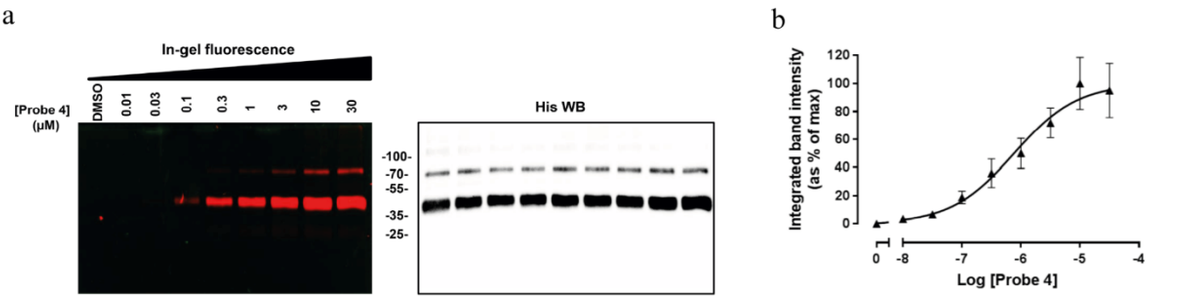


Figure 4. Concentration-dependent affinity labeling of purified, His-tagged $hA_{2A}R$ by probe **4**. (a) Purified $hA_{2A}R$ material was incubated with the indicated concentrations of probe **4** or vehicle (1% DMSO), and subjected to click chemistry ligation with Cy3-azide, followed by SDS-PAGE separation and in-gel fluorescence scanning (left). The blotted membranes were probed with anti-histidine antibody, wherein bands corresponding to purified $hA_{2A}R$ molecular weight (~47 kDa) were evident in all samples (right). (b) Quantification of fluorescence intensity from purified $hA_{2A}R$ labeled by probe **4** clicked to Cy3-azide. Representative graphs are from three independent experiments, with errors bars representing SEM values. In-gel fluorescence of the $hA_{2A}R$ band at ~47 kDa was normalized to the corresponding $hA_{2A}R$ immunoreactivity in each sample.

(Cy3-azide) attachment to the terminal alkyne [32, 33]. The subsequent fluorescence scanning of a SDS-PAGE showed that in the presence of fluorescent dye Cy3-azide (Figure 4a), probe **4** was concentration-dependently incorporated into a fixed amount of purified $hA_{2A}R$, while in the absence of probe, little fluorescence intensity was detected. Importantly, western blot analysis using the purified $hA_{2A}R$ receptor and specific anti-histidine antibodies unambiguously validated the labeling band was $hA_{2A}R$ (Figure 4a). Interestingly, a second band was observed in both affinity labeling results and western blots, most likely resulting from posttranslationally modified receptors,[34] as has been shown before on CB_2R [27]. Quantification of the fluorescence intensity of the main labeling bands in the $hA_{2A}R$ is reflected in the concentration-effect curve in Figure 4b. This revealed that clickable probe **4** labeled $hA_{2A}R$ with a pEC_{50} value of 6.10 ± 0.04, resulting in a maximal labeling achieved with 10 μM probe **4** when incubated with 0.1 mg mL^{-1} of purified $hA_{2A}R$. Collectively, these data demonstrate that probe **4** can be used as an affinity-based probe for purified $hA_{2A}R$.

To further characterize our affinity-based probe, we then investigated whether competitive antagonists could inhibit the labelling of purified receptors by probe **4**. We chose to evaluate

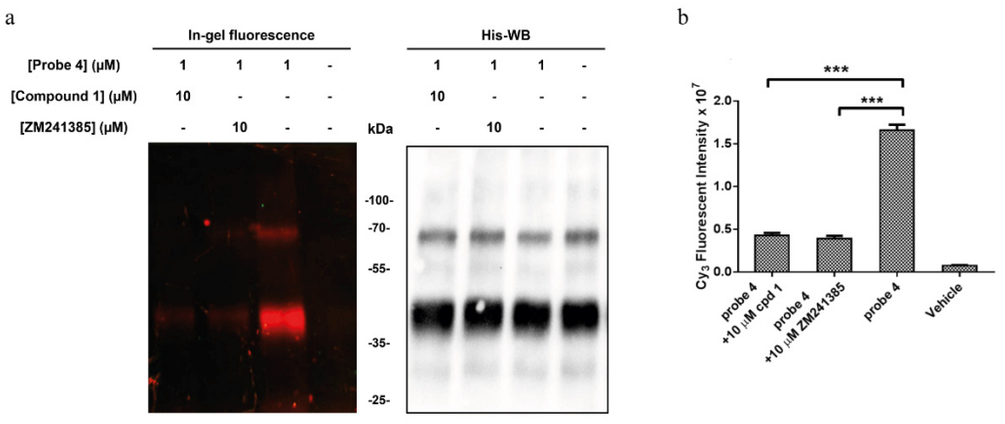


Figure 5. Competitive affinity labeling of the purified $hA_{2A}R$ by probe **4** (a) Affinity labeling of purified $hA_{2A}R$ by probe **4** (1 μM) is inhibited by preincubation with either compound **1** (10 μM) or ZM241385 (10 μM) (left). The blotted membranes were probed with anti-histidine antibody, wherein bands corresponding to purified $hA_{2A}R$ molecular weight (~47 kDa) were evident in all samples (right). (b) Quantification of fluorescence intensity from pretreated purified $hA_{2A}R$ labeled by probe **4** clicked to Cy3-azide. Representative graphs are from three independent experiments, with errors bars representing SEM values. ***$P < 0.001$ compared with the fluorescent intensity of purified $hA_{2A}R$ labeled by probe **4** (1 μM); Student's t-test. In-gel fluorescence of the $hA_{2A}R$ band at ~47 kDa was normalized to the corresponding $hA_{2A}R$ immunoreactivity in each sample

reversible antagonist ZM241385 and irreversible compound **1**, at saturating concentrations (10 μM, i.e. 10 times higher than the concentration of the clickable probe **4**). Purified $hA_{2A}R$, preincubated with the competitors and subsequently treated as mentioned before to incorporate the sulfonated cyanine 3 fluorophore, showed little if any fluorescence intensity of labelling bands under these conditions. This revealed that both a reversible and an irreversible antagonist competed with probe **4** (Figure 5a, left panel) for the same binding site at the $hA_{2A}R$, which was available at identical amounts in all conditions (as evidenced by His-tagging: Figure 5a, right panel). Theoretically, both reversible and irreversible ligands inhibit affinity labelling, provided that they target the same receptor binding site and are present in a sufficient concentration. Of note, in practice, this is not always easily observed, as in the competition between reversible ligand and covalently binding probe there is an inherent bias towards the irreversible pathway, hindering the interaction between the receptor and a reversible ligand. For instance, in the few other studies where an AfBP has been used on GPCRs it was found that a reversible $mGlu_5$ negative allosteric modulator, MPEP, could not inhibit the tandem photoaffinity labeling of purified $mGlu_5$, whereas on CB_2R, inhibition of labeling by various competitors was observed [26, 27]. Apparently, this was less of a problem on the $hA_{2A}R$. Our results demonstrate that the developed AfBP system can serve as an effective chemical tool for profiling the purified $hA_{2A}R$ in vitro, prompting us to further

evaluate the potency and selectivity of probe **4** in profiling the activity of the adenosine A_{2A} receptor in more complex biological samples.

We further explored the ability of probe **4** to label $hA_{2A}R$ in cell membranes prepared from HEK293 cells, which were transiently transfected with N-terminally FLAG-tagged and C-terminally His-tagged human adenosine A_{2A} receptors (FLAG-$hA_{2A}R$-His). Therefore, FLAG-$hA_{2A}R$-His cell membranes were incubated with probe **4** at room temperature for 1 h, followed by click ligation to Cy3-azide treatment. As detailed in Figure 6, a band corresponding to the molecular weight of the FLAG-$hA_{2A}R$-His was observed on fluorescent SDS-PAGE scanning, which was then validated by Western blot using specific anti-FLAG antibodies. In these initial proof-of-concept experiments we highlighted the versatility of probe **4**, which can be efficiently used to label the adenosine A_{2A} receptor in cell membrane samples. Background signals caused by nonspecific labeling of abundant proteins in complex proteomes may sometimes confound the analysis of on-target labeling of low expression proteins such as GPCRs.

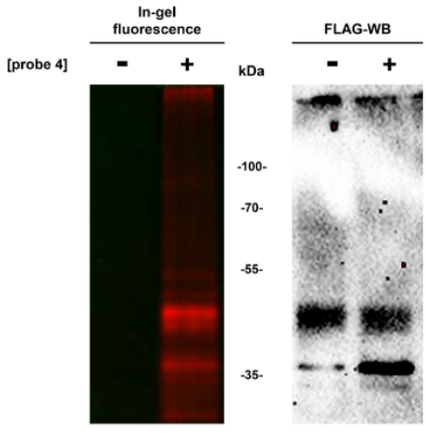


Figure 6. Affinity labeling of $hA_{2A}R$ in HEK293 cell membranes transiently expressing FLAG-tagged $hA_{2A}R$ using probe **4**. (a) cell membranes overexpressing FLAG-tagged $hA_{2A}R$ were incubated with either 1 µM probe **4** or vehicle (1% DMSO), then subjected to click chemistry ligation with Cy3-azide, followed by SDS-PAGE separation and in-gel fluorescence scanning (left). The blotted membranes were probed with anti-FLAG antibody, wherein bands corresponding to the $hA_{2A}R$ molecular weight (~50 kDa) are evident in all samples.

Thus, we utilized cell membranes transiently transfected with FLAG-$hA_{2A}R$-His, which have a relatively high level of receptor expression. Additionally, instead of premixing the copper sulfate and sodium ascorbate reagents, we slightly altered the click procedure by adding the copper sulfate last to achieve efficient and selective labeling of the A_{2A} receptors [35, 36].

Although we were able to decrease the strong background signals, a significant non-specific fluorescent dyes and labeled receptors, non-specific protein binding of the probe due to the inherently reactive warhead and the sensitivity of the used detection method. Hence, further technological refinement should help us in achieving better labeling of endogenously expressed GPCRs, e.g. in human tissues as has been shown recently on CB_2R [27]. The monitoring of endogenous GPCR expression and target engagement in human cells holds promise for future GPCRs studies.

3. Conclusion

Starting from a selective antagonist, ZM241385, we designed and synthesized a series of covalent ligands using the electrophilic nature of sulfonyl fluorides, eventually yielding probe **4**, the first affinity-based probe for the $hA_{2A}R$. We successfully demonstrated a concentration-dependent labelling of purified receptor by probe **4** via an experimental two-step labeling strategy, which could be inhibited by both reversible and irreversible competing ligands. Additionally, probe **4** displayed target selectivity in cell membranes overexpressing the $hA_{2A}R$, indicating that it may become a useful pharmacological tool to identify the $hA_{2A}R$ in living organisms for target validation or to assess receptor subtype distribution. In this strategy a probe depicts the native binding with less perturbation, which bridges the chemical biology study with molecular pharmacology to better investigate receptor-ligand interactions.

In future research, different tags may be introduced; for instance, a biotin-tag would allow for streptavidin-mediated receptor enrichment followed by LC/MS analysis. Similarly, the approach developed in this study may be applied to other GPCRs, such as the other adenosine receptor subtypes.

4. Experimental sections

4.1. Chemistry

All solvents and reagents were purchased from commercial sources and were of analytical grade. ^{1}H NMR spectra were recorded on a Bruker AV 400 liquid spectrometer (^{1}H NMR, 400 MHz) at ambient temperature. Chemical shifts are reported in parts per million (ppm) and are designated by δ. Coupling-constants are reported in Hz and are designated as *J*. Analytical purity of the final compounds was determined by high pressure liquid chromatography (HPLC) with a Phenomenex Gemini 3µ C18 110A column (50 × 4.6 mm, 3 µm), measuring UV absorbance at 254 nm. Sample preparation and HPLC method was as follows: 0.5 mg of compound was dissolved in 1 mL of a 1:1:1 mixture of CH_3CN/H_2O/tBuOH and eluted from the column within 15 min, with a three component system of H_2O/CH_3CN/1% TFA in H_2O, decreasing polarity of the solvent mixture in time from 80/10/10 to 0/90/10. All compounds showed a single peak at the designated retention time and are at least 95% pure. Liquid chromatography–mass spectrometry (LC–MS) analyses were performed using Thermo Finnigan Surveyor – LCQ Advantage Max LC-MS system and a Gemini C18 Phenomenex column (50 × 4.6 mm, 3 µm). The sample preparation was the same as for HPLC analysis. The elution method was set up as follows: 1–4 min isocratic system of H_2O/CH_3CN/1% TFA in H_2O, 80:10:10; from the 4th min, a gradient was applied from 80:10:10 to 0:90:10 within 9

min, followed by 1 min of equilibration at 0:90:10 and 1 min at 80:10:10. Thin-layer chromatography (TLC) was routinely performed to monitor the progress of reactions, using aluminum coated Merck silica gel F254 plates. Purification by column chromatography was achieved by use of Grace Davison Davisil silica column material (LC60A 30–200 micron). Solutions were concentrated using a Heidolph laborota W8 2000 efficient rotary evaporation apparatus and by a high vacuum on a Binder APT line Vacuum Drying Oven.

4-((4-((7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-5-yl)amino)butyl)carbamoyl)benzene sulfonyl fluoride (2) Previously synthesized N^5-(4-aminobutyl)-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazine-5,7-diamine **8** (TFA salt, 250 mg, 0.40 mmol, 1.0 eq) was suspended in acetonitrile (10 mL) and purged with N_2. Then DiPEA (0.42 ml, 2.4 mmol, 6.0 eq) was added after which 4-fluorosulfonylbenzoylchloride (134 mg, 0.60 mmol, 1.5 eq) was added last and the mixture was heated to 70 ºC for 7 h, and then stirred at room temperature for another 17 h. A flash column (MTBE + 1% AcOH → 90% MTBE + 10% EtOAc + 1% AcOH), a subsequent preparative TLC (1:1 MTBE:EtOAc + 1% MeOH) and an extraction using acetonitrile (10 mL) and petroleum ether (4 x 10 mL) afforded the product as a white solid (8 mg, 0.017 mmol, 4% yield). ^{1}H NMR (DMSO-d_6, 400 MHz) : δ 8.29–8.18 (m, 5H), 7.75 (s, 1H), 7.40 (br s, 2H), 7.05 (d, J = 3.2 Hz, 1H), 6.73 (m, 1H), 6.62 (s, 1H), 3.55–3.46 (m, 4H), 1.75–1.74 (m, 4H). HPLC: 96.5 %, RT 7.478 min. LC-MS: $[ESI+H]^+$: 475.20

4-((5-((7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-5-yl)amino)pentyl)carbamoyl) benzenesulfonylfluoride (3) N^5-(5-aminopentyl)-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazine-5,7-diamine **9** (TFA salt, 674 mg, 0.85 mmol, 1 eq) was suspended in acetonitrile (5 mL) . 4-Fluorosulfonylbenozylchloride (208 mg, 0.94 mmol, 1.1 eq) was added, along with DiPEA (0.8 mL, 5 mmol, 5.8 eq). The mixture was heated at 70 °C under N_2 atmosphere for 2.5 h. A flash column (DCM → 60% DCM, 40% EtOAc) with subsequent preparative TLC (100% EtOAc) was used to obtain the title compound as a colorless solid (9 mg, 0.018 mmol, 2% yield). ^{1}H NMR (C_3D_6O, 400 MHz): δ 8.27–8.16 (m, 4H), 7.73 (dd, J = 1.7, 0.8 Hz, 1H), 7.39 (s, 2H), 7.05 (d, J = 3.2 Hz, 1H), 6.80–6.63 (m, 1H), 6.62 (dd, J = 3.4, 1.8 Hz, 1H), 3.52–3.38 (m, 4H), 1.78–1.62 (m, 4H), 1.56–1.44 (m, 2H). HPLC: 100%, RT 7.637 min, LC-MS:$[ESI+H]^+$: 489.00

4-((5-((7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-5-yl)amino)pentyl)(prop-2-yn-1-yl)carbamoyl)benzenesulfonyl fluoride (4) *tert*-butyl (5-(4-(fluorosulfonyl)-*N*-(prop-2-yn-1-yl)benzamido)pentyl)carbamate **14** (586 mg, 1.38 mmol, 1 eq) was dissolved in DCM (10 mL). To this solution TFA (10 mL) was added. After 2 min the

solvents were removed *in vacuo*. This crude intermediate was suspended in acetonitrile (10 mL), 2-(furan-2-yl)-5-(methylsulfonyl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-7-amine **5** (386 mg, 1.38 mmol, 1 eq) was added, along with DiPEA (2 mL, 11.0 mmol, 8 eq). The reaction mixture was heated at 70 °C for 2 h. Then the reaction mixture was concentrated and purified by a flash column (EtOAc) to yield a yellow solid (330 mg, 0.62 mmol, 45%). ^{1}H NMR (DMSO-*d*$_6$, 353K, 400 MHz,) δ 8.17 (d, *J* = 8.3 Hz, 2H), 7.93–7.73 (m, 5H), 7.12 (t, *J* = 5.3 Hz, 1H), 7.03 (d, *J* = 3.3 Hz, 1H), 6.64 (dd, *J* = 3.0, 1.5 Hz, 1H), 4.17 (s, 2H), 3.41 (s, 2H), 3.29 (d, *J* = 6.2 Hz, 2H), 3.14 (s, 1H), 1.73–1.61 (m, 2H), 1.61–1.44 (m, 2H), 1.40–1.23 (m, 2H) ppm. HPLC: 95.772%, RT: 8.117 min, MS: [ESI+H]$^{+}$: 527.20

***tert*-butyl(4-((7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-5-yl)amino)butyl)carbamate (6)** 2-(Furan-2-yl)-5-(methylsulfonyl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-7-amine **5** (435 mg, 1.55 mmol, 1.0 eq), synthesized as previously reported,[13] was suspended in acetonitrile to yield a 0.1 M solution. *tert*-butyl (4-aminopropyl) carbamate (0.33 mL, 1.71 mmol, 1.1 eq) was added, followed by the addition of N,N-diisopropylethylamine (1.08 mL, 6.21 mmol, 4 eq). The mixture was heated at 85 °C for 29 hours and stirred at r.t. for another 18 hours. A flash column (DCM:EtOAc, 0% → 90% EtOAc) was used to purify the crude mixture. This gave a yellowish solid (444 mg, 1.14 mmol, 74% yield). ^{1}H NMR (DMSO-*d*$_6$, 400 MHz): δ 8.49–7.92 (m, 2H), 7.86 (s, 1H), 7.51 (t, *J* = 5.9 Hz, rotamer 1, 0.3H), 7.44 (t, *J* = 5.7 Hz, rotamer 2, 0.7H), 7.07–7.00 (m, 1H), 6.87–6.76 (m, 1H), 6.67 (dd, *J* = 3.2, 1.7 Hz, 1H), 3.29–3.19 (m, 2H), 2.92 (d, *J* = 6.5 Hz, 2H), 1.48 (d, *J* = 7.2 Hz, 2H), 1.44–1.29 (m, 11H).

***tert*-butyl (5-((7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-5-yl)amino)pentyl)carbamate (7)** 2-(Furan-2-yl)-5-(methylsulfonyl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-7-amine **5** (280 mg, 1.0 mmol, 1.0 eq) and commercially available *tert*-butyl (5-aminopentyl)carbamate (0.2 mL, 1.0 mmol, 1.1 eq) were put in a microwave tube and dissolved in acetonitrile (1.5 mL). DIPEA (0.3 mL, 1.7 mmol) was added, the tube was charged with a stirring bar, sealed and heated at 70 °C for 1.5 h. After 1.5 h HPLC analysis indicated full conversion. The mixture was concentrated, EtOAc (50 mL) and HCl (1M in H_2O, 50 mL) were added for extraction. The organic layer was washed with H_2O (50 mL) and brine (50 mL). After drying over $MgSO_4$ the solvent was removed *in vacuo* to give the title compound as a yellow foam (186 mg, 0.46 mmol, 46% yield). ^{1}H NMR (DMSO-*d*$_6$, 400 MHz,) δ 8.48–7.96 (m, 2H), 7.86 (s, 1H), 7.48 (t, *J* = 5.1 Hz, rotamer, 0.38H), 7.41 (t, *J* = 5.7 Hz, rotamer, 0.62H), 7.10–7.01 (m, 1H), 6.77 (t, *J* = 5.0 Hz, 1H), 6.67 (dd, *J* = 3.0, 1.7 Hz, 1H), 3.28–3.17 (m, 2H), 2.90 (d, *J* = 6.6 Hz, 2H), 1.57–1.44 (m, 2H), 1.44–1.21 (m, 13H).

N^5-(4-aminobutyl)-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazine-5,7-diamine (8) TFA (4.3 mL, 57 mmol, 50 eq) was added to the suspension of Boc-protected amine **6** (444 mg, 1.14 mmol, 1 eq) in DCM (equal volume as TFA). solvents were removed under reduced pressure after completion of the reaction (5 min). This gave the product as brown oil (899 mg, 1.13 mmol, quantitative yield). Products were confirmed by ^{1}H NMR first and then stored under N_2 until used. ^{1}H NMR (DMSO-*d*$_6$, 400 MHz): δ 8.49–8.05 (m, 3H (R-NH_3^+), 7.88 (dd, *J* = 1.7, 0.7 Hz, 1H), 7.62 (br s, 3H (R-NH_3^+)), 7.52 (t, *J* = 6.0 Hz, 1H), 7.09–7.02 (m, 1H), 6.68 (dd, *J* = 3.4, 1.7 Hz, 1H), 3.35–3.23 (m, 2H), 2.87–2.76 (m, 2H), 1.63–1.50 (m, 4H).

N^5-(5-aminopentyl)-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazine-5,7-diamine (9) TFA (3 mL, 40 mmol, 50 eq) was added to the suspension of Boc protected amine **7** (324 mg, 0.8 mmol, 1 eq) in DCM. Once the reaction was completed, the solvent was removed and the mixture was co-evaporated twice with water and dried using high vacuum. This gave a brown oil (556 mg, 0.8 mmol, quantitative yield) as a TFA salt. The crude product was used without further purification.

***tert*-butyl (5-hydroxypentyl) carbamate (11)** 5-amino-1-pentanol **10** (4.2 mL, 38.8 mmol) was dissolved in DCM (20 mL). Di-*tert*-butyl dicarbonate (8.4 g, 38.8 mmol) was slowly added as a solid. The reaction was left stirring at r.t. for 18 h, then the solvent was removed to give a yellow oil (8.83 g, quantitative yield, some *t*-BuOH left). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.57 (s, 1H), 3.65 (t, *J* = 6.5 Hz, 2H), 3.13 (t, *J* = 6.5 Hz, 2H), 1.67 – 1.35 (m, 15H (under water peak)).

***tert*-butyl (5-bromopentyl) carbamate (12)** *tert*-butyl (5-hydroxypentyl) carbamate **11** (8.83 g, 38.8 mmol, 1eq) and PPh_3 (15.3 g, 58.2 mmol, 1.5 eq) were dissolved in THF (120 mL). A solution of CBr_4 (19.3 g, 58.2 mmol, 1.5 eq) in THF (40 mL), was added over 2 h using a syringe pump. After 3 h at room temperature the reaction mixture was filtered and the filtrate was concentrated. This crude product was dissolved in DCM (~5 mL) and purified by flash column chromatography (100% PE → 90% PE + 10% EtOAc). This gave the product as a colorless oil (9.31 g, 35.0 mmol, 90% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.54 (s, 1H), 3.41 (t, *J* = 6.7 Hz, 2H), 3.13 (m, 2H), 1.97–1.80 (m, 2H), 1.58–1.36 (m, 13H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 40.5, 33.8, 32.5, 29.4, 28.6, 25.5.

***tert*-butyl (5-(prop-2-yn-1-ylamino) pentyl) carbamate (13)** Propargylamine (1 mL, 15 mmol, 3 eq) was dissolved in acetonitrile (10 mL). To this stirred solution a solution of *tert*-butyl (5-bromopentyl) carbamate **12** (798 mg, 3 mmol, 1 eq) and DiPEA (1 mL, 6 mmol, 2 eq) in acetonitrile (18 mL) was added using a syringe pump. Afterwards the solvent was removed and the product purified by flash column chromatography (EtOAc). This gave a yellowish oil

(331 mg, 1.38 mmol, 46% yield) with EtOAc as an impurity. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.54 (s, 1H), 3.44 (d, *J* = 2.0 Hz, 2H), 3.12 (q, *J* = 6.4 Hz, 2H), 2.70 (t, *J* = 7.1 Hz, 2H), 2.22 (t, *J* = 2.2 Hz, 1H), 1.55–1.34 (m, 15H) ppm.

***tert*-butyl (5-(4-(fluorosulfonyl)-*N*-(prop-2-yn-1-yl)benzamido)pentyl)carbamate (14)** *tert*-butyl (5-(prop-2-yn-1-ylamino)pentyl)carbamate **13** (664 mg, 1.38 mmol, 1 eq) was dissolved in acetonitrile (10 mL), 4-fluorosulfonyl benzoylchloride (338 mg, 1.52 mmol, 1.1 eq) was added and followed by the addition of DiPEA (0.75 mL, 4.14 mmol, 3 eq). Once the reaction was completed, the solvent was removed and the crude mixture purified by flash column chromatography (DCM + 5% MTBE → DCM + 7.5% MTBE). This yielded a yellow oil (586 mg, 1.52 mmol, quantitative yield). ^{1}H NMR (DMSO-d_6, 332K, 400 MHz) δ 8.19 (d, *J* = 8.0 Hz, 2H), 7.77 (d, *J* = 7.9 Hz, 2H), 6.37 (brs, 1H), 4.16 (brs, 2H), 3.37 (brs, 2H), 2.90 (m, 2H), 1.61 (m, 2H), 1.44–1.29 (m, 9H), 1.29–1.14 (m, 4H).

4.2. Biology

The radioligand [^{3}H]ZM241385 with a specific activity of 50 Ci × mmol^{-1} was purchased from ARC Inc. (St. Louis, MO). Unlabeled ZM241385 was a kind gift from Dr. S.M. Poucher (Astra Zeneca, Macclesfield, UK). 5'-N-ethylcarboxamidoadenosine (NECA) was purchased from Sigma-Aldrich (Steinheim, Germany). Adenosine deaminase (ADA) was purchased from Sigma-Aldrich Chemie N.V. Bicinchoninic acid (BCA) and BCA protein assay reagent were obtained from Pierce Chemical Company (Rockford, IL, USA). Human embryonic kidney (HEK) 293 cells stably expressing the hA_{2A} receptor (hA_{2A}R-WT) were kindly provided by Dr J Wang (Biogen/IDEC, Cambridge, MA, USA). The purified hA_{2A} receptor material was kindly provided by Dr. Niek Dekker and Dr. Euan Gordon (AstraZeneca). All other chemicals were of analytical grade and obtained from standard commercial sources.

Cell culture, transfection and membrane preparation. We followed the procedures reported previously.[13, 37] Briefly, HEK293 cells were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% newborn calf serum, 50 μg mL^{-1} streptomycin, and 50 IU mL^{-1} penicillin at 37°C and 7% CO_2 atmosphere. Cells were subcultured twice a week at a ratio of 1:20 on 10 cm ø culture plates. The cells were transfected with pcDNA3.1(-) plasmid containing the hA_{2A}R with N terminal FLAG and C-terminal His tags (FLAG-hA_{2A}R-His^4) using the calcium phosphate precipitation method (1 μg plasmid DNA), followed by a 48-hour incubation, as previously described.[38] Stably transfected hA_{2A}R-WT cells were grown in the same medium but with the addition of G-418 (500 mg ml^{-1}). For both transiently transfected cells and stably hA_{2A}R-WT cells were

detached from the plates by scraping them into PBS and centrifuged to remove PBS buffer. The pellets were resuspended in ice-cold Tris-HCl buffer (50 mM, pH 7.4) and then homogenized. The cell membrane suspensions were centrifuged at 100,000 × g at 4°C for 20 minutes, after which the procedure was repeated one more time. After this, the same Tris-HCl buffer was used to resuspend the pellet, and adenosine deaminase was added to break down endogenous adenosine. HEK293 cells stably expressing $hA_{2A}R$ were grown as monolayers in same culture medium and detached from plates by same treatment for membrane preparation. For both membranes were stored in 250 µL aliquots at -80°C until further use. Membrane protein concentrations were measured using the BCA method.[39]

*[^{3}H]ZM241383 radioligand displacement assay***.** Radioligand displacement experiments were performed as previously described.[13] $hA_{2A}R$-WT cell membrane aliquots stably expressing $hA_{2A}R$ containing 10 µg of protein were incubated in a total volume of 100 µL of assay buffer to obtain an assay window of approximately 3000 DPM of receptor-specific radioligand binding. Nonspecific binding was determined in the presence of 100 µM NECA and represented less than 10% of the total binding. Briefly, to each tube were added 25 µL cell membranes (10 µg of protein), 25 µL of radioligand [^{3}H]ZM241383, 25 µL of assay buffer [25 mM Tris-HCl, pH 7.4 at 25°C, supplemented with 5 mM $MgCl_2$ and 0.1% (w/v) CHAPS and 25 µL of the indicated compounds in increasing concentrations in the same assay buffer. The mixture was incubated at 25ºC for 60 min to reach equilibrium. Incubations were terminated by rapid vacuum filtration to separate the bound and free radioligand through 96-well GF/B filter plates using a Perkin Elmer Filtermate-harvester (PerkinElmer, Groningen, Netherlands). Filters were subsequently washed three times with 2 mL of ice-cold buffer (25 mM Tris-HCl, pH 7.4, supplemented with 5 mM $MgCl_2$). The filter-bound radioactivity was determined by scintillation spectrometry using a P-E 1450 Microbeta Wallac Trilux scintillation counter (PerkinElmer).

Heterologous displacement binding of probe 4 and ZM241385 to hA2AR-WT cell membranes. To assess the irreversible binding level, cell membranes stably expressing $hA_{2A}R$ were incubated with either 50 mM Tris-HCl (pH = 7.4) or two concentrations (0.3 IC_{50} and IC_{50}) of probe 4 or ZM241385 for 3h at 25ºC on a Eppendorf Thermomixer. Subsequently, the mixture was centrifuged at 16,100 × g at 4ºC for 5 minutes and the supernatant was removed, followed by a resuspension of the pellet in 1 mL assay buffer and spun again for 5 min at 16,100 × g at 4ºC. This washing procedure was repeated three times. 50 µL aliquots of these pretreated membranes were incubated with 25 µL of radioligand [^{3}H]ZM241383 and 25 µL of

a concentration range (100 pM-1 μM) of unlabeled ZM241385 for 1h at 25 ºC. Incubation was terminated as described under [^{3}H]ZM241385 radioligand displacement assay.

Expression and purification of wild type hA$_{2A}$R. The gene coding for hA$_{2A}$R (residues 1-316) was synthesised by Genscript and cloned into pPICZb with an N-terminal α-factor signal sequence from Saccharomyces cerevisiae (MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNS TNNGLLFINTTIASIAAKEEGVSLEKRLVPRGS), followed by hA$_{2A}$R and a C-terminus biotinylation domain from Propionibacterium shermanii (TSEFENLYFQGQFGGGTGG APAPAAGGAGAGKAGEGEIPAPLAGTVSKILVKEGDTVKAGQTVLVLEAMKMETEI NA PTDGKVEKVLVKERDAVQGGQGLIKI) for enhanced expression[40] and a decaHis tag (GHHHHHHHHHHGS).

The receptor was expressed in Pichia pastoris SMD1168 at 3 L scale in a fermentor essentially as described,[41] except that dissolved oxygen was maintained at 25%, and 2.5% DMSO and 10 mM theophylline were included in the fermentation media. Approximately 200 grams of wet cells were harvested per litter. 200 g cells were resuspended using a Turax in 600 mL ice-cold lysis buffer (50 mM HEPES pH 7.4, 200 mM NaCl, Complete EDTA free protease inhibitor tablets (Roche) at 1/50 mL). Cells were lyed by a single passage through a Constant Cell system at 30 kpsi with extensive cooling. Cell debris was removed by centrifugation at 1000 × g for 10 min at 4°C. Membranes were collected by ultracentrifugation at 100,000 × g for 45 minutes at 4°C. Membrane pellet was resuspended in buffer to a total protein concentration of 20 mg mL^{-1} (final volume of 180 mL) and stored at -80°C.

Membranes (20 mL) were resuspended in 200 mL solubilization buffer (25 mM HEPES, pH7.4, 300 mM NaCl, 20% glycerol, 1% DDM/0.1% CHS, Complete tablets (1/50 mL), 200 μM theophylline). The suspension was incubated for 2 hours at 4˚C on a rolling table, prior to centrifugation for 30 min at 100,000 × g to remove unsolubilized material. Imidazole was added to a final concentration of 15 mM and the clarified solution was loaded on a 5 mL HisTrap crude column at 2.5 mL min^{-1}. The column was washed with 100 mL buffer A (25 mM HEPES, 25 mM imidazole pH 7.4, 300 mM NaCl, 10% glycerol, 0.05% DDM/0.0005% CHS, 100 μM theophylline) to which imidazole was added to final concentration of 25 mM to reduce nonspecific binding, followed by step wise washes with increasing concentrations of imidazole in this buffer (50 mM and 75 mM) and hA$_{2A}$R was eluted in 25 mM HEPES pH

7.4, 300 mM NaCl, 10% glycerol, 0.05% DDM/0.0005%CHS, 300 mM imidazole, 100 µM theophylline. Fractions were analyzed on SDS-PAGE and those containing $hA_{2A}R$ were pooled and concentrated to 2.5 mL using a 50 kDa filter. High imidazol is harmful to $hA_{2A}R$ and the buffer was changed to buffer A on a PD10 G25 column. Eluted fraction was further concentrated to 0.5 mL and loaded on a Superdex-200 10/30 column running in in 25mM NaPi pH7.2, 100 mM NaCl, 10 µM LMNG, 500 µM Caffeine. Fractions were analyzed on SDS-PAGE. $hA_{2A}R$ eluted as single peak at expected position for the detergent-protein complex (around 80kDa). Fractions were pooled and concentrated on a 50 kDa filter to final volume of 0.4 mL and stored at -80°C. Protein concentration was determined using absorbance measurement against buffer A (Abs280(0.1%) =1.05). Final concentration was 7 mg mL^{-1} with a total of ~2mg $hA_{2A}R$.

Affinity-based protein labeling assay on purified $hA_{2A}R$ with probe 4. For purified $hA_{2A}R$, both affinity labeling and click reactions were performed on ice, unless indicated otherwise. Purified $hA_{2A}R$ was diluted to a concentration of 0.1 mg mL^{-1} in assay buffer (25 mM HEPES pH 7.5, 100 mM NaCl and 10 µM LMNG). 38 µL samples were incubated with 2 µL probe **4** at indicated concentrations or vehicle control (1% DMSO) for 1 h. To initiate the click reaction, 5.6 mM $CuSO_4$ (2.5 µL / reaction, from a 100 mM stock solution in water) was mixed vigorously with 33 mM sodium ascorbate (1.5 µL / reaction, freshly made as a 1 M stock solution in water) to obtain a yellow mixture, followed by the immediate addition of 1.1 mM THPTA (0.5 µL / reaction, from a 100 mM stock solution in water) and 4.4 µM fluorescent tag Cy3-azide (0.5 µL / reaction, from a 400 µM stock solution in DMSO). The reaction mixtures were incubated for 1h and quenched with 15 µL 4xSDS loading buffer. Proteins in the mixture were separated by SDS-PAGE on 10% polyacrylamide gels. In-gel fluorescence was detected with a ChemiDoc MP system (605/50 filter). Proteins were transferred from gel to a PVDF membrane by Trans-Blot®Turbo (BioRad). Then the membrane was washed in 20 mL TBS for 10 min on a roller bench, followed by a three times wash with TBST (PBS with 0.1% Tween-20). Afterwards, the membrane was blocked in 5% (w/v) non-fat milk for 1h at room temperature and probed with rabbit-anti-His antibody (Rockland)(1:1000 [v/v] dilution in blocking buffer) overnight at 4 °C, washed three times again with TBST and incubated with goat-anti-rabbit IgG-HRP (1:5000 in 5% milk in TBST; Santa Cruz) for 1 h at room temperature. After two wash cycles in TBST and one in TBS, the blot was developed in the dark using a 10 mL luminal solution, with 100 µL ECL enhancer and 3 µL H_2O_2. Chemiluminescence was visualized with ChemiDoc XRS (BioRad).

Competitive labeling assays in purified hA$_{2A}$ AR by probe 4. Prior to the two-step labeling experiment purified hA$_{2A}$R was diluted to a concentration of 0.1 mg mL^{-1} in assay buffer and incubated with 10 µM compound **1**, ZM241385 or vehicle control (1% DMSO) for 1h on ice, followed by labeling with 1 µM probe **4** for 0.5 h on ice. Samples were then subjected to the click chemistry procedure using the protocol described above.

Affinity-based protein labeling of membranes transiently overexpressing FLAG-HA-hA$_{2A}$R-His. hA$_{2A}$R -FLAG-His membranes were diluted to a concentration of 1 mg mL^{-1} in 50 mM Tris-HCl (pH = 7.4 at 25 ºC). Either 2 µL probe **4** at indicated concentrations (0.1 µM, 0.3 µM, 1 µM and 3 µM) or vehicle control (1% DMSO) was added to 38 µL samples for 1 h incubation at room temperature. Then all samples were subjected to the click chemistry conjugation reaction. The click reagents were added in the following sequence: 4.4 µM fluorescent Cy3-azide (0.5 µL / reaction, 400 µM stock in DMSO) was added to the mixture followed by 33 mM sodium ascorbate (1.5 µL / reaction, freshly made in 1M stock in water) and 1.1 mM THPTA (0.5 µL / reaction, 100 mM stock in water). Finally, 5.6 mM $CuSO_4$ (2.5 µL / reaction, 100 mM stock in water) was added to start and run the cycloaddition reaction for 1 h at room temperature. Then, the reaction was quenched with 15 µL 4xSDS loading buffer and protein material denatured for 30 min at 37 ºC. Proteins (60 µL sample) were separated by SDS-PAGE on 10% polyacrylamide gels. In-gel fluorescence was detected with the ChemiDoc MP system (605/50 filter). Proteins were transferred from gel to a PVDF membrane by Trans-Blot®Turbo (BioRad). Then the membrane was washed in 20 mL TBS for 10 min on a roller bench, followed by a three times wash with TBST (PBS with 0.1% Tween-20). Then the membrane was blocked in 5% (w/v) non-fat milk and incubated with mouse-anti-FLAG (Sigma) (1:5000 [v/v] dilution in blocking buffer) as primary antibody. Thereafter, the membrane was washed in TBST three times and incubated with goat-anti-mouse HRP (Sigma) (1:5000 [v/v] dilution in blocking buffer) as secondary antibody. After two wash cycles in TBST and one in TBS, the blot was developed in the dark using a 10 mL luminal solution, with 100 µL ECL enhancer and 3 µL H_2O_2. Chemiluminescence was imaged using a ChemiDoc XRS (BioRad).

Abbreviations used

ADA, Adenosine deaminase; BCA, Bicinchoninic acid; CHAPS,3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; Cy3-azide, sulfonated Cyanine 3 dye azide; DiPEA, Di-isopropylethylamine; ECL, enhanced chemiluminescence; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LMNG, Lauryl Maltose Neopentyl Glycol; MTBE, Methyl

tert-butyl ether; NaPi, Sodium Phosphate Buffer; NECA, 5'-N-ethylcarboxamidoadenosine; TBS, Tris-buffered saline; TBST, Tris Buffered Saline with 0.05% Tween; THPTA, Tris(3-hydroxypropyl triazolylmethyl)amine; TFA, Trifluoroacetic acid; PVDF, Polyvinylidene difluoride; ZM241385, 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol.

Supporting Information

Table S1. Apparent affinities of 4 at the human A_1 and A_3 adenosine receptor subtypes. Data are expressed as pK_i values (means ± SEM) of three separate experiments each performed in duplicate.

Compound	pK_i		
	hA_1[a]	hA_{2A}[b]	hA_3[c]
4	7.72 ± 0.05	8.82 ± 0.02	7.22 ± 0.01

[a]Affinity determined from displacement of specific [^{3}H]DPCPX binding on CHO cell membranes stably expressing human adenosine A_1 receptors at 25°C during 3h incubation; [b] Affinity determined from displacement of specific [^{3}H]ZM241385 binding from the $hA_{2A}R$ at 25°C during 3h incubation; [c]Affinity determined from displacement of specific [^{3}H]PSB-11 binding on CHO cell membranes stably expressing human adenosine A_3 receptors at 25°C during 3h incubation.

Table S2. Affinities of ZM241385 on $hA_{2A}R$ preincubated with different concentrations of the indicated compounds[a]

Preincubated Compound	pK_i IC_{50}	pK_i $0.3IC_{50}$	pK_i control
4[b]	9.01 ± 0.05[ns]	8.84 ± 0.04[ns]	9.03 ± 0.10
ZM241385[c]	8.94 ± 0.07[ns]	8.84 ± 0.03[ns]	8.77 ± 0.04

[a] Data are expressed as means ± SEM of three separate experiments each performed in duplicate. ns indicates a non-significant difference with $p>0.05$ when compared with the pK_i values in control groups; One-way ANOVA test. [b] Affinity of ZM241385, expressed as pK_i value, determined from displacement of specific [^{3}H]ZM241385 binding from the $hA_{2A}R$ cell membranes preincubated with compound **4** at indicated concentrations for 3h at 25°C and then treated with a three-cycle washing step.[c] Affinity of ZM241385, expressed as pK_i value, determined from displacement of specific [^{3}H]ZM241385 binding from the $hA_{2A}R$ cell membranes preincubated with ZM241385 at indicated concentrations for 3h at 25°C and then treated with a three-cycle washing step.

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