Cryo-EM structures of adenosine receptor A3AR bound to selective agonists Postprint

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Abstract

The adenosine A3 receptor (A3AR) belongs to a subfamily of G protein-coupled receptors and is an important therapeutic target for conditions including inflammation and cancer. The clinical compounds CF101 and CF102 are potent and selective A3AR agonists, but the structural basis of their recognition was unknown. Here we present the cryogenic electron microscopy structures of the full-length human A3AR bound to CF101 and CF102 at 3.3-3.2 Å resolution in complex with heterotrimeric Gi protein. These agonists bind within the orthosteric pocket, with their adenine components engaging in conserved interactions while their substituted 3-iodobenzyl groups exhibit different orientations. Swapping extracellular loop 3 (ECL3) of A3AR onto other adenosine receptor subtypes enabled CF101/CF102 binding and receptor activaton, and mutations in key residues, including His3.37, Ser5.42 and Ser6.52 that form a unique subpocket in A3AR, abolished receptor activation, highlighting these structural elements are critical for ligand selectivity. Compared to inactive A2AAR, the A3AR structures reveal conserved mechanism of receptor activation, including an outward shift of TM6. These structures provide key insights into molecular recognition and signaling mechanisms of A3AR, which should aid rational design of subtype-selective ligands targeting this important class of adenosine receptors.

Full Text

Cryo-EM Structures of Adenosine Receptor A3AR Bound to Selective Agonists

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Abstract

The adenosine A3 receptor (A3AR) belongs to a subfamily of G protein-coupled receptors and represents an important therapeutic target for inflammatory conditions and cancer. The clinical compounds CF101 and CF102 are potent and selective A3AR agonists, yet the structural basis for their recognition remained unknown. Here we present cryogenic electron microscopy structures of full-length human A3AR bound to CF101 and CF102 at 3.3-3.2 Å resolution in complex with heterotrimeric Gi protein. These agonists bind within the orthosteric pocket, with their adenine components engaging in conserved interactions while their substituted 3-iodobenzyl groups adopt different orientations. Swapping extracellular loop 3 (ECL3) of A3AR onto other adenosine receptor subtypes enabled CF101/CF102 binding and receptor activation, and mutations in key residues—including His3.37, Ser5.42, and Ser6.52 that form a unique subpocket in A3AR—abolished receptor activation, highlighting these structural elements as critical determinants of ligand selectivity. Compared to inactive A2AAR, the A3AR structures reveal a conserved activation mechanism, including an outward shift of TM6. These structures provide key insights into the molecular recognition and signaling mechanisms of A3AR, which should aid rational design of subtype-selective ligands targeting this important class of adenosine receptors.

Introduction

The adenosine receptor subfamily of G protein-coupled receptors consists of four subtypes: A1, A2A, A2B, and A3 [1, 2]. These receptors are activated by the endogenous ligand adenosine to transduce downstream signals that mediate numerous physiological and pathological processes, including immunomodulation, energy balance, cardiac function, and neuroprotection [3-5]. A3AR is expressed in various tissues including the brain, heart, lungs, liver, kidneys, and

immune cells [6]. Through its signaling functions, A3AR participates in regulating cardiac function, vasodilation, inflammation inhibition, protection against ischemia-reperfusion injury, and suppression of oxidative stress. Additionally, A3AR is highly expressed in numerous tumor cell types, suggesting its potential as a therapeutic target for suppressing cancer cell proliferation [6-8].

While A2AAR and A2BAR activation predominantly elicits stimulatory G protein (Gs) signaling, A1AR and A3AR exhibit a preference for coupling to inhibitory G protein (Gi), leading to inhibition of adenylate cyclase and decreased intracellular cyclic AMP [2]. Based on the chemical structure of adenosine, numerous agonists and antagonists have been designed and tested against A3AR for disease indications such as cancer, inflammation, and pain [9]. Previous studies indicate that modifications at the N6 position on the purine ring and the 5' -N position on the ribose group within the adenosine framework yield potent A3AR agonists with high subtype selectivity [10-12]. CF101 and CF102 represent successful applications of this modification strategy, featuring similar nucleoside core structures that differ by only a single chloro-substituent, yet both demonstrate high affinity and selectivity for A3AR [13-15]. CF101 showed efficacy in Phase III trials for psoriasis and rheumatoid arthritis [6], while CF102 is currently in clinical trials for hepatocellular carcinoma and non-alcoholic steatohepatitis (NASH) [16, 17].

Adenosine receptors mediate many important functions, and their broad expression pattern makes subtype selectivity of ligands critical for minimizing potential side effects [18, 19]. Elucidating the structural basis of selective ligand binding to A3AR could significantly improve the drug design process. However, no A3AR structure has been reported to date. In this study, we present the cryo-EM structures of A3AR bound to Gi in the presence of CF101 and CF102, which reveals the molecular basis of ligand recognition and the ligand-induced activation mechanism of A3AR. Our work provides important insights for designing effective A3AR-targeted therapies and, more broadly, for the adenosine receptor subfamily.

Results and Discussion

Overall Structures of the Complexes

CF101 and CF102 are A3AR agonists containing modifications to the ribose and adenine moieties that confer potent binding to A3AR (Fig. 1a [Figure 1: see original paper]). Specifically, both compounds feature a 5' -N-methylcarboxamide substitution on the ribose group and an N6-(3-iodobenzyl) substitution on the adenine base (Fig. 1a). These modifications result in significantly higher potency at A3AR compared to the endogenous agonist adenosine. We verified the selectivity of these nucleoside-derived compounds for A3AR versus other adenosine receptor subtypes (A1/A2A/A2BAR) using NanoBiT association assays (Fig. 1b-d). While adenosine activated all four subtypes with similar micro-

molar potencies, CF101 and CF102 displayed strong potency of approximately 3 nM at A3AR but showed weak or negligible responses at other adenosine receptor subtypes.

We employed a NanoBiT tether strategy to stabilize the full-length A3AR-G protein complexes, an approach that has been widely used for many GPCR structural studies [20-22] (Supplementary Fig. 1). The A3AR construct used in this study contained an N-terminal thermostabilized apocytochrome b562RIL (BRIL) fusion to enhance expression and was co-expressed with G protein subunits and scFv16, an antibody fragment that further stabilizes the receptor-G protein complex. For the CF101-A3AR-Gi complex, data from 20,779 movies comprising 271,323 particles were used to determine the structure at 3.29 Å resolution (Supplementary Fig. 2 [Figure 2: see original paper], Supplementary Table 1). For the CF102-A3AR-Gi complex, data from 13,581 movies yielding 283,561 particles were used to determine the structure at 3.19 Å resolution (Supplementary Fig. 3 [Figure 3: see original paper], Supplementary Table 1).

The structures of the CF101/CF102-A3AR-Gi complexes revealed that the ligands occupy the orthosteric binding pocket, with their core structures clearly modeled into the cryo-EM density at the center of the receptor transmembrane helices (TMs) (Fig. 1e-h). The structures showed the canonical seven-transmembrane architecture for A3AR, with the intracellular domains occupied by the $ 5helixofG iforGicoupling.Thedensitymapsenabledmodelingofmostregions, exceptforA3ARN-terminusresiduesM1 - L8, thethirdintracellularloopN211 - Y222, C - terminusV301 - E318, andthealpha - helicaldomainofG $i. The extracellular loop M151-S165 was also less defined, though the backbone could be established (Supplementary Fig. 4 [Figure 4: see original paper]). Aside from these regions, the models were well-resolved. Overall, the two agonist-bound complexes were highly similar, with a root mean square deviation (RMSD) of 0.593 Å for the whole receptor.

Binding Mode of CF101/CF102 in the A3AR Orthosteric Site

The A3AR agonists CF101 and CF102 bind at a conserved orthosteric pocket formed by ECL2, TM3, TM5, TM6, and TM7, similar to the endogenous ligand adenosine bound to other adenosine receptor subtypes (Fig. 2a-b). However, the orientations of the modified 3-iodobenzyl moieties differ between CF101 and CF102. The adenine core mediates conserved receptor interactions commonly observed in other adenosine receptors [21, 23, 24]. Notably, the adenine pyrimidine forms π-stacking interactions with F45.52, and the F45.52A mutation greatly impaired the ability of CF101/CF102 to induce receptor activation (Fig. 2c-f, Supplementary Table 2). Additionally, the ribose and 3-iodophenyl groups form hydrogen bonds with polar side chains at positions 3.36, 6.55, and 7.43, which are key for recognition of nucleoside ligands by all adenosine receptors (Fig. 2c-f, Supplementary Table 2).

The ligand binding pocket is primarily composed of hydrophobic residues at po-

sitions 3.33, 5.38, 5.47, 6.48, 6.51, and 7.39, which form hydrophobic contacts important for CF101/CF102 potency (Fig. 2c-f, Supplementary Table 2). Alanine mutations at these positions severely reduced the agonists' ability to induce receptor activation. His3.37 and Ser5.42 participate in van der Waals contacts with the bound ligands, and their alanine mutations also affected activity (Fig. 2c-f, Supplementary Table 2). The side chains of M1745.35 and L2647.35 in the receptor form hydrophobic interactions with the 3-iodophenyl group extending from the N6 position of the adenosine base in CF101. In contrast, the corresponding group in CF102 is surrounded by V169ECL2 and L2647.35 from the receptor. Alanine mutations at these residues did not significantly affect compound potency at A3AR (Supplementary Fig. 6a [Figure 6: see original paper], Supplementary Table 2), suggesting that the 3-iodophenyl substituents may exist in alternative states within the receptor extracellular domains. This demonstrates that the N6 position can accommodate various substituted groups through distinct conformations in the A3AR pocket.

Moreover, CF102 is a 2-chloro derivative of CF101 (Fig. 1a). In the CF102-bound A3AR structure, Y151.35 forms hydrophobic contact with the 2-chloro group of CF102, while also forming a π-π interaction with Y2657.36 in TM7. The Y151.35A mutation in A3AR abolished agonist activity for both CF102 and CF101 (Fig. 2c-f, Supplementary Table 2). According to previous reports, modifications at the 2-position of the adenosine structure tend to be well-tolerated in A3AR binding [14], whether the substituent is small or large, even including macrocyclic groups linked to the N6 moiety [25]. Elucidation of these subtle structural variations in ligand-receptor interactions thus provides molecular insight into the conformational adaptability and binding poses governing molecular recognition at A3AR.

Role of ECL3 in Adenosine Receptors

CF101 and CF102 exhibit high selectivity for A3AR over other subtypes. Sequence analysis of adenosine receptors reveals strong conservation within the transmembrane helices, while the extracellular loops diverge among subtypes (Supplementary Fig. 5 [Figure 5: see original paper]). Although ECL1 is relatively distant from the orthosteric site and F16845.52 in ECL2 provides key π-π interactions with agonists in all adenosine receptors, A3AR possesses a shorter ECL3 than other subtypes (Fig. 3a). This shorter ECL3 may rigidify A3AR to minimize conformational changes required for specific ligand binding.

To assess the role of ECL3 in A3AR, we engineered chimeric receptors by grafting ECL3 from A3AR onto the backbones of other adenosine receptors. These chimeric receptors gained the ability to bind CF101 and CF102 with increased efficacy or potency (Fig. 3b-c, Supplementary Table 3). These findings suggest that ECL3 serves as a structural factor mediating the selective recognition of CF101 and CF102 by A3AR. According to reported structure-activity relationships of ligands at A3AR, numerous N6-substituted adenosine derivatives have been synthesized, with studies showing that either too small or too bulky groups

at the N6 position reduce potency or affinity at A3AR [13, 14]. The N6 position on adenosine projects outward into the binding pocket of A3AR and is in close spatial proximity to the ECL3 region, making the ECL3 loop an important consideration in structure-activity studies of N6-modified adenosine derivatives targeting adenosine receptors. Delineating these subtle structural variations provides molecular insight into the selectivity of structurally analogous ligands for adenosine receptors.

Binding Pocket Residues Across Adenosine Receptors

A3AR shares the lowest sequence identity with other subtypes among adenosine receptors. Sequence analysis reveals that A3AR may confer selectivity through different residue types in the orthosteric binding pocket (Fig. 4a), including positions 3.32 (L/V/V/V in A3/A1/A2A/A2BAR), 3.37 (H/Q/Q/Q), 5.42 (S/N/N/N), 5.47 (I/V/V/V), 6.52 (S/H/H/H), and 6.58 (I/T/T/T) (Fig. 4b, Supplementary Fig. 7 [Figure 7: see original paper]). We mutated these residues in A3AR to the corresponding residues of other receptor subtypes to assess their impact on CF101 and CF102 activity (Fig. 4c-d).

The leucine at position 3.32 in A3AR, versus valine in other subtypes, did not affect CF101/CF102 activity when mutated to valine, consistent with their similar hydrophobic properties (Fig. 4c-d, Supplementary Fig. 7, Supplementary Table 2). Likewise, mutating isoleucine at positions 5.47 and 6.58 in A3AR to the valine and threonine found in other subtypes only slightly impacted activation (Fig. 4c-d, Supplementary Table 2), suggesting that the slightly shorter side chains in valine and threonine do not impair binding.

The side chains of H3.37 and S5.42 in A3AR form a hydrogen bond that cannot be formed by the corresponding Q3.37 and N5.42 residues in other adenosine receptor subtypes (Fig. 4e-i). The H3.37Q mutation showed limited impact on CF101/CF102 activity, whereas the S5.42N mutation almost completely abolished the ability of CF101/CF102 to induce receptor activation (Fig. 4c-d, Supplementary Table 2). Additionally, mutating S6.52 to histidine (H) also severely decreased CF101 and CF102 activity, likely due to unfavorable steric or electronic properties of the longer histidine side chain (Fig. 4c-d, Supplementary Fig. 7, Supplementary Table 2). In contrast to other adenosine receptor subtypes, H3.37, S5.42, and S6.52 form a distinctive subpocket in A3AR to accommodate the 5' -N-methylcarboxamide from the ribose (Fig. 4e-i, Supplementary Fig. 8 [Figure 8: see original paper]). These results implicate this microdomain as a structural determinant for stabilizing CF101 and CF102 in A3AR versus other subtypes. Together, these findings demonstrate that minor sequence changes in the receptors can impact their conformations, thereby affecting ligand binding specificity.

Activation Mechanism of Adenosine Receptor A3AR

Structural comparisons between active, agonist-bound A3AR complexes and an inactive, antagonist-bound A2AAR structure (PDB ID: 4EIY) [26] reveal hallmark conformational changes associated with GPCR activation [27, 28]. The A3AR structures exhibit an outward movement of TM6 compared to inactive A2AAR, shifting 11.6 Å based on measurements of Glu6.30 Cα atoms, analogous to movements observed in other activated class A GPCRs upon G protein coupling (Fig. 5a). Additional rearrangements associated with activation include inward movements of TM1 and TM7 and an upward shift of TM3 in A3AR relative to inactive A2AAR (Fig. 5b-d). The A3AR agonists CF101 and CF102 dock deeper into the orthosteric pocket compared to the A2AAR antagonist ZM-241385, enabling engagement of conserved GPCR activation motifs such as the "toggle switch" on W6.48 and transmission switch motifs D3.49R3.50Y3.51 and N7.49P7.50xxY7.53 (Fig. 5e-h). These microswitches trigger conformational changes that propagate from the ligand binding pocket to the cytoplasmic G protein coupling interface. This series of structural transitions leads to receptor rearrangements that enable G protein coupling and activation. By providing near-atomic resolution of A3AR activation mechanisms, these findings reveal fundamental insights into the relationship between ligand recognition in the receptor and activation of downstream signaling.

G Protein Coupling of Adenosine Receptors

All adenosine receptors exhibit differential G protein coupling preferences that correlate with distinct conformational orientations of the associated G proteins [21, 23, 24]. A1AR and A3AR preferentially couple to inhibitory Gi proteins, while A2AAR and A2BAR primarily couple to stimulatory Gs proteins to mediate intracellular signaling cascades (Fig. 6a). Structural comparisons reveal conformational differences between Gi- and Gs-coupled adenosine receptor complexes. Specifically, the TM6 helix of A1AR/A3AR-Gi complexes shows a 3.1 Å inward shift to accommodate Gi binding compared to A2AAR/A2BAR-Gs complexes (Fig. 6b). Additionally, the $ 5*helix of G ssubunits displays an* 8.6*displacement relative to its orientation in Gi complexes based on measureme*
atom (Fig. 6c). The αN helix of Gαi exhibits a 3.3 Å tilt compared to Gαs when measuring the GαHN.38 Cα atom (Fig. 6d). Overall, the structural arrangements of A1AR/A3AR-Gi complexes closely resemble each other, similar to the consistency observed between A2AAR/A2BAR-Gs complexes. These findings reveal that Gi-coupled adenosine receptors adopt conserved G protein-binding conformations that differ distinctly from those of Gs-coupled adenosine receptors. Elucidation of these structural differences governing adenosine receptor-G protein interactions provides fundamental insights into the molecular determinants of G protein coupling specificity.

Conclusion

In conclusion, we have determined the cryo-EM structures of A3AR bound to selective agonists CF101 and CF102 in complex with heterotrimeric Gi protein. Despite conserved binding of the core adenosine moiety, the structures revealed differences in the orientations of the N6-substituted groups in CF101 and CF102. Through mutagenesis studies, we identified ECL3 and key pocket residues His3.37, Ser5.42, and Ser6.52 as critical determinants of selectivity over other adenosine receptor subtypes. Comparison to an inactive A2AAR structure provided insight into the conformational changes associated with A3AR activation and G protein coupling. By elucidating the molecular mechanisms governing ligand recognition, signaling, and subtype selectivity, these A3AR structures significantly advance our fundamental understanding of this important drug target. The findings pave the way for structure-guided design of improved therapeutics targeting adenosine receptors for the treatment of cancer, inflammation, and other diseases.

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Author Contributions

H.C. designed the expression constructs and purified the protein complexes under supervision of H.E.X. Y.X. and H.C. prepared the grids and performed cryo-EM data processing and model building with help from J.L. H.E.X., Y.J., and H.C. analyzed the structures. S.G. and Z.X. performed functional studies with help from J.S. under supervision of X.X. H.C. prepared the figures and manuscript. Y.X. and S.G. contributed to manuscript preparation. H.E.X. and H.C. wrote the manuscript with input from all authors.

Data Availability

The atomic coordinates of the CF101/CF102-A3AR-Gi complexes have been deposited in the Protein Data Bank (http://www.rcsb.org) with accession codes xxxx and xxxx, respectively. The corresponding cryo-EM density maps have been deposited in the Electron Microscopy Data Bank (https://www.ebi.ac.uk/pdbe/emdb/) with accession codes EMD-xxxxx and EMD-xxxx, respectively.

Competing Interests

The authors declare no competing interests.

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Figure Legends

Fig. 1. Cryo-EM structures of CF101-A3AR-Gi and CF102-A3AR-Gi complexes. (a) Chemical structures of adenosine, CF101, and CF102, highlighting modifications at the 5' -N-methylcarboxamide in the ribose group and N6 and C2 positions of the adenosine group. Atom numbering is indicated in blue. CF101, also named IB-MECA and N6-(3-iodobenzyl)adenosine-5' -N-methyluronamide. CF102, also named Cl-IB-MECA and 2-chloro-N6-(3-iodobenzyl)adenosine-5'-N-methyluronamide. (b-d) NanoBiT association assays monitoring ligand activity on adenosine receptors for adenosine (b), CF101 (c), and CF102 (d) from three independent experiments in triplicate (n=3). (e, f) Cryo-EM map (e) and model (f) of the CF101-A3AR-Gi complex, with inset showing CF101 density. The density map in the inset is shown at 0.232 threshold. (g, h) Cryo-EM map (g) and model (h) of the CF102-A3AR-Gi complex, with inset showing CF102 density. The density map in the inset is shown at 0.17 threshold. Subunits are colored as indicated.

Fig. 2. The orthosteric binding pocket. (a, b) Detailed interactions between A3AR and CF101 (a) or CF102 (b) viewed from the membrane plane. Residues involved in ligand interaction are colored blue and pink in the two complexes, respectively. Black dashed lines indicate hydrogen bonds. (c-f) Dose-response curves of A3AR mutants induced by CF101 (upper panels, c, e) or CF102 (lower panels, d, f) using NanoBiT assay.

Fig. 3. Swapping ECL3 increases CF101/CF102 potency at adenosine A1/A2A/A2B receptor subtypes. (a) Superposition of ECL3 in adenosine receptors shows A3AR has a shorter ECL3. Other TMs were omitted. (b, c) Effects of CF101 and CF102 were tested on A1AR, A2AAR, and A2BAR and their corresponding mutants containing the swapped ECL3 from A3AR using NanoBiT assays.

Fig. 4. Key residues in the A3AR binding pocket. (a) Sequence alignment of residues in the orthosteric binding pocket among adenosine receptors. Conserved residues are colored blue and marked with stars. Unique residues in A3AR, different from other adenosine receptor subtypes, are colored orange, while other subtypes at corresponding positions are colored green. All residues are annotated based on the GPCR Ballesteros-Weinstein numbering

scheme. (b) Superposition of adenosine receptors; unique residues in A3AR compared to other adenosine receptors are shown as yellow balls. (c-d) Effects of CF101/CF102 on A3AR mutants containing swapped residues from other adenosine receptors by NanoBiT assay. (e-i) Binding cavities of adenosine receptors generated in PyMOL, depicted as gray surfaces. In A3AR, His3.37, Ser5.42, and Ser6.52 form a subpocket, whereas these positions are conserved as Gln3.37, Asn5.42, and His6.52 in other adenosine receptor subtypes, respectively (His, H; Ser, S; Gln, Q; Asn, N). Dashed lines in h and i show hydrogen bonds between His3.37 and Ser5.42.

Fig. 5. A3AR activation mechanism. (a-b) Superposition of active A3AR-CF101/CF102 complexes (blue/pink) with inactive A2AAR-ZM241385 complex (gray, PDB ID 4EIY). (c, d) Comparison of extracellular (c) and cytoplasmic (d) views of active A3AR and inactive A2AAR. (e-g) Conformational changes in conserved motifs, including toggle switch, PIF, DRY, and NPxxY, upon CF101/CF102 binding to A3AR relative to inactive A2AAR-ZM241385. Arrows indicate movement directions.

Fig. 6. G protein coupling of adenosine receptors. (a) Comparison of G protein conformations in A1/A3AR-Gi and A2A/A2BAR-Gs complexes. (b) Conformational comparison of TM6 in adenosine receptors, referencing toggle switch W6.48 in TM6 of receptor. (c-d) Conformational comparison of $ $5 helix and αN helix in adenosine receptor-G protein complexes. Arrows indicate movement directions.

Methods

Construct Design

The full-length gene encoding human A3AR was synthesized (Synbio) and subcloned into a pFastBac vector using the CloneExpress II one-step cloning kit (Vazyme Biotech). A hemagglutinin signal peptide and thermostabilized apocytochrome b562RIL (BRIL) were fused at the N-terminus of A3AR to enhance receptor expression. To improve complex stability, a NanoBiT tethering approach was employed where an LgBiT domain was fused to the C-terminus of the receptor [20]. A dual maltose-binding protein was linked after LgBiT through a tobacco etch virus protease site (TEV site) for subsequent cleavage. A dominant-negative mutant of bovine Gαi containing G203A/A326S mutations [29] was generated to stabilize the heterotrimeric G$\alpha i\beta\gamma$ protein. Rat G$ $1 was fused with HiBiT at its C-terminus for structural complementation with LgBiT to form a NanoBiT complex. The single-chain variable fragment scFv16 was used to bind the G i γ protein for stabilization [30]. Gαi, G$ $1-HiBiT, G $, and scFv16 were each cloned into pFastBac vectors (Supplementary Fig. 1a).

Protein Expression and Purification

Recombinant A3AR, Gαi, G$ 1 - HiBiT, G $, and scFv16 were co-expressed in Trichoplusia ni High Five insect cells using the Bac-to-Bac baculovirus expression system. High Five cells were co-infected with baculovirus at a density of 3.5 $\times$ 10^6 cells per milliliter. Forty-eight hours post-infection, cells were harvested and stored at -80 °C until use.

For purification of the CF101-A3AR-Gi complex, cell pellets were thawed and resuspended in Buffer A (100 mM NaCl, 20 mM HEPES, pH 7.5) supplemented with protease inhibitor cocktail (TargetMol). Cells were lysed by Dounce homogenization followed by centrifugation to remove insoluble material. The pellets were resuspended in Buffer B (100 mM NaCl, 10% (v/v) glycerol, 20 mM HEPES, pH 7.5) supplemented with 10 mM $MgCl_2$, 5 mM $CaCl_2$, 0.2 mM tris-(2-carboxyethyl)phosphine (TCEP, Hampton Research), and protease inhibitor cocktail. The complex was formed by rotating at room temperature for 1 hour after addition of 25 mU/mL apyrase and 10 M CF101. Following incubation, the sample was solubilized in 0.5% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace) and 0.1% (w/v) cholesteryl hemisuccinate (CHS, Anatrace) for 3 hours at 4 °C. The supernatant was clarified by centrifugation at 100,000 $\times$ g for 40 minutes, then incubated with dextrin beads 6FF (Smart-Lifescience) for 3 hours at 4 °C. The beads were loaded onto a gravity column and washed with 20 column volumes of Buffer C (100 mM NaCl, 2 mM $MgCl_2$, 10 M CF101, 0.2 mM TCEP, 0.01% (w/v) LMNG, 0.002% (w/v) CHS, 20 mM HEPES, pH 7.5). The complex was eluted with Buffer C supplemented with 10 mM maltose and concentrated using a 100 kDa molecular weight cut-off concentrator.

TEV protease was added to the concentrated protein at 4 °C overnight to cleave the dual maltose-binding protein from the fusion protein. After digestion, the sample was loaded onto a Superdex 200 Increase 10/300 GL column (GE Healthcare) equilibrated with Buffer D (100 mM NaCl, 2 mM $MgCl_2$, 10 M CF101, 0.1 mM TCEP, 0.00075% (w/v) LMNG, 0.00025% (w/v) glyco-diosgenin, 0.0002% (w/v) CHS, 20 mM HEPES, pH 7.5). The desired fractions were pooled and concentrated to 5–8 mg/mL for cryo-EM sample preparation.

Cryo-EM Data Collection

Cryo-EM grids were prepared using a Vitrobot Mark IV plunger (FEI) set to 8 °C and 100% humidity. Three microliters of the CF101-A3AR-Gi complex were applied to glow-discharged Quantifoil R1.2/1.3 holey carbon grids. The sample was incubated for 10 seconds on the grids before blotting for 3.5 seconds (double-sided, blot force 1) and flash-frozen in liquid ethane. Identical conditions were used for the CF102-A3AR-Gi complex sample.

For the CF101-A3AR-Gi complex, three datasets comprising 20,779 movies were collected on a Titan Krios equipped with a Gatan K3 direct electron detection device at 300 kV with a magnification of 105,000×, corresponding to a pixel size of 0.824 Å. Image acquisition was performed with EPU Software (FEI Eind-

hoven, Netherlands). A total of 36 frames were collected, accumulating to a total dose of 50 $e^-/Å^2$ over a 2.5-second exposure.

For the CF102-A3AR-Gi complex dataset, two datasets totaling 13,581 movies were collected on a Titan Krios equipped with a Gatan K3 detector at 300 kV with a magnification of 105,000× and pixel size of 0.824 Å, using EPU Software (FEI Eindhoven, Netherlands). Thirty-six frames were collected over a 2.5-second exposure to a dose of 50 $e^-/Å^2$.

Image Processing

MotionCor2 was used to perform frame-based motion correction to generate drift-corrected micrographs for further processing, and CTFFIND4 provided estimation of contrast transfer function (CTF) parameters [31, 32].

For the CF101-A3AR-Gi dataset, the previously resolved structure of BAY 60-6583-A2BAR-Gs [21] was used as a reference for automatic particle picking in Relion 3.0 [33]. Particle picking and extraction yielded 4,550,294 particles after 2D classification clearance, which were imported into cryoSPARC [34]. Four rounds of 2D classification selected 1,267,837 particles, followed by two rounds of 3D heterogeneous refinement yielding 982,833 particles. After two additional rounds of 2D classification and two rounds of heterogeneous refinement, 271,323 particles were refined to a structure at 3.29 Å global resolution using non-uniform refinement (Supplementary Fig. 2).

For the CF102-A3AR-Gi complex dataset, the BAY 60-6583-A2BAR-Gs structure [21] was again used for reference-based particle picking. A total of 4,090,959 and 4,833,382 particles were autopicked and extracted from Dataset 1 and Dataset 2, respectively. For Dataset 1, two rounds of 2D classification were used to select 1,070,085 particles. Masked 3D classification on the receptor portion was used to separate 175,747 particles that resulted in clearer density for A3AR. For Dataset 2, two rounds of 2D classification and two rounds of 3D classification were performed to select 246,392 particles. After clearance, the remaining particles from both datasets were combined and subjected to alignment-free 3D classification. A total of 283,561 particles remained and were transferred to cryoSPARC [34]. One round of heterogeneous refinement yielded a final set of 102,581 particles that were refined to a structure at 3.19 Å global resolution using non-uniform refinement (Supplementary Fig. 3).

Model Building

An A3AR structure predicted by AlphaFold2 was used as the starting reference model for receptor building [35]. Structures of Gαi, Gβ, Gγ, and scFv16 derived from PDB entry 7EZH [36] were rigid-body fit into the density. All models were fitted into the EM density map using UCSF Chimera [37] followed by iterative rounds of manual adjustment and automated rebuilding in COOT [38] and PHENIX [39], respectively. The model was finalized by rebuilding in ISOLDE [40] followed by refinement in PHENIX with torsion-angle restraints

to the input model. Final model statistics were validated using Comprehensive validation (cryo-EM) in PHENIX and are provided in the supplementary information (Supplementary Table 1). All structural figures were prepared using Chimera [37], Chimera X [41], and PyMOL (Schrödinger, LLC).

NanoBiT Assay

To monitor G protein interaction with A1AR, A2AAR, A2BAR, or A3AR upon agonist stimulation, a NanoLuc-based NanoBiT enzyme complementation assay was used (Promega). The C-terminus of A1AR, A2AAR, or A2BAR was fused to SmBiT, while LgBiT was fused to the N-terminus of G proteins. The C-terminus of A3AR was fused with LgBiT, and the SmBiT element was fused to the N-terminus of G proteins. HEK293 cells were seeded at 4×10^4 cells/well in 96-well plates and co-transfected with AR-SmBit and LgBiT-G protein plasmids at a 1:1 mass ratio. After 24 hours, cells were replaced with 40 L fresh culture medium without fetal bovine serum. Ten microliters of Nano-Glo Live Cell reagent were added following the manufacturer' s protocol (Promega, N2011), and incubated at 37 °C, 5% CO_2 for 5 minutes. Another 25 L of culture medium containing various compound concentrations were added and incubated at room temperature for 10 minutes before measuring bioluminescence using an EnVision multiplate reader (PerkinElmer).

Cell-Surface Expression Assay

Wild-type A1AR, A2AAR, A2BAR, or A3AR genes were subcloned into pcDNA3.0 vector with an N-terminal human influenza hemagglutinin tag (HA-tag). HEK293 cells were seeded at 4×10^4 cells/well in 96-well plates and transfected with wild-type or mutant adenosine receptor constructs. After 24 hours, cells were washed with PBS buffer, fixed with 4% (w/v) paraformaldehyde for 15 minutes, and blocked with 2% (w/v) bovine serum albumin (BSA) for 1 hour. Cells were then incubated with polyclonal anti-HA antibody (Sigma, H6908) overnight at 4 °C, followed by 1 hour with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Cell Signaling, 7074S) at room temperature. After washing, 50 L tetramethylbenzidine (Sigma, T0440) was added for 30 minutes before stopping the reaction with 25 L TMB substrate stop solution (Beyotime, P0215). Absorbance at 450 nm was measured on a FlexStation III microplate reader (Molecular Devices).

Statistical Analysis

All functional study data were analyzed in Prism 8 (GraphPad) and presented as means ± S.E.M. from at least three independent experiments. Concentration-response curves were evaluated with a three-parameter logistic equation. EC_{50} values were calculated using the sigmoid three-parameter equation. Significance was determined by one-way ANOVA followed by multiple comparisons test, with $^*P < 0.05$ vs. wild-type (WT) considered statistically significant.

Supplementary Information

Supplementary Fig. 1. Expression and purification of the A3AR-Gi complex. (a) Schematic diagrams of A3AR and G$ 1*expressionconstructsusingtheNanoBiTtetheringapproach*, *with*A3. fused to LgBiT and HiBiT, respectively. (b) Size-exclusion chromatography profile of the CF101-A3AR-Gi complex. (c) SDS-PAGE of the peak fraction indicated by the arrow in (b). (d) Size-exclusion chromatography profile of the CF102-A3AR-Gi complex. (e) SDS-PAGE of the peak fraction indicated by the arrow in (d).

Supplementary Fig. 2. Cryo-EM data processing of the CF101-A3AR-Gi complex. (a) Representative image from the cryo-EM dataset. Scale bar, 50 nm. (b) Representative 2D average classification classes. Scale bar, 10 nm. (c) Flow chart of cryo-EM data processing. (d) FSC curves. (e) Local resolution map.

Supplementary Fig. 3. Cryo-EM data processing of the CF102-A3AR-Gi complex. (a) Representative image from the cryo-EM dataset. Scale bar, 50 nm. (b) Representative 2D average classification classes. Scale bar, 10 nm. (c) Flow chart of cryo-EM data processing. (d) FSC curves. (e) Local resolution map.

Supplementary Fig. 4. Representative regions of cryo-EM density maps shown for each transmembrane helix (TM) of A3AR and the $ $5 and αN helices of Gαi.

Supplementary Fig. 5. Sequence alignment of adenosine receptors. The alignment was generated with Jalview [42] and depicts the N-/C-termini, transmembrane helices (TMs), extracellular loops (ECLs), and intracellular loops (ICLs). Residues lining the orthosteric binding site are highlighted with red circles and annotated with GPCR Ballesteros-Weinstein numbering. The C-termini of the adenosine receptors were omitted.

Supplementary Fig. 6. Effects of CF101 or CF102 on A3AR mutants. These residues in A3AR form hydrophobic interactions with the 3-iodophenyl group present in CF101 and CF102.

Supplementary Fig. 7. Orthosteric binding pockets among adenosine receptors. Highlighted positions indicate where unique residues occur in A3AR compared to other adenosine receptors. Receptor names and associated colors are shown above the models. Side chains in A3AR are depicted as bold sticks, while corresponding side chains in other adenosine receptors are shown as thick sticks.

Supplementary Fig. 8. Binding cavities in adenosine receptors. (a-e) Binding cavities of adenosine receptors depicted as gray surfaces, with bound ligands

shown as sticks. Receptor names and associated PDB codes [21, 24, 43] are indicated below each model. The unique subpocket in A3AR is circled in red boxes.

Supplementary Table 1. Cryo-EM data collection, model refinement, and validation statistics for A3AR-CF101-Gi and A3AR-CF102-Gi complexes.

Supplementary Table 2. Cell-surface expression of A3AR and its mutants in CF101- and CF102-induced NanoBiT assays.

Supplementary Table 3. Cell-surface expression of A1AR/A2AAR/A2BAR and their respective mutants in CF101- and CF102-induced NanoBiT assays.

Note: Figure translations are in progress. See original paper for figures.