Development of Covalent Ligands for G Protein-Coupled Receptors: A Case for the Human Adenosine A3 Receptor

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Supporting Information

ABSTRACT: The development of covalent ligands for G protein-coupled receptors (GPCRs) is not a trivial process. Here, we report a streamlined workflow thereto from synthesis to validation, exemplified by the discovery of a covalent antagonist for the human adenosine A3 receptor (hA3AR). Based on the 1H,3H-pyrido[2,1-ff]purine-2,4-dione scaffold, a series of ligands bearing a fluorosulfonyl warhead and a varying linker was synthesized. This series was subjected to an affinity screen, revealing compound 17b as the most potent antagonist. In addition, a nonreactive methylsulfonyl derivative 19 was developed as a reversible control compound. A series of assays, comprising time-dependent affinity determination, washout experiments, and [35S]GTPγS binding assays, then validated 17b as the covalent antagonist. A combined in silico hA3AR-homology model and site-directed mutagenesis study was performed to demonstrate that amino acid residue Y2657.36 was the unique anchor point of the covalent interaction. This workflow might be applied to other GPCRs to guide the discovery of covalent ligands.

INTRODUCTION

The adenosine A3 receptor (A3AR) is one of four G protein-coupled receptor subtypes stimulated by adenosine. Different from the other subtypes (A1, A2A, and A2B) A3AR was identified by molecular biology studies prior to its pharmacological characterization. The initial studies indicated its important role in both physiological and pathophysiological conditions, such as cell proliferation, cell differentiation, neuroprotection, cardioprotection, and apoptosis. Nevertheless, the medical relevance of the human adenosine A3 receptor (hA3AR) is enigmatic due to its dichotomy in different therapeutic applications. In this regard, there is a continuing interest in the development of selective ligands of the hA3AR to investigate its pharmacological effects. For instance, selective A3AR antagonists have been applied for the treatment of glaucoma and respiratory tract inflammation such as asthma. In particular, a tricyclic xanthine derivative, 1-benzyl-8-methoxy-3-propyl-1H,3H-pyrido[2,1-ff]purine-2,4-dione (compound 1, Figure 1A), has been reported to exert high affinity for the hA3AR.

Initial efforts to study the structural biology of GPCRs suffered from numerous limitations, such as low expression, dynamic conformational states, and inherent instability. Covalent ligands, i.e., compounds that irreversibly bind to the receptor and possess a reactive moiety to target specific amino acid residues, helped to solve some of these obstacles. This is also the case for adenosine receptors. For example, the structure of the human adenosine A3 receptor, having the highest similarity to the hA3AR among all adenosine receptor subtypes (61% of sequence homology), has been elucidated by X-ray crystallography with a covalent antagonist DU172 (2) (Figure 1B). However, the application of covalent ligands in hA3AR studies has been limited to the characterization of the receptor type, far from providing a comprehensive study of receptor structure elucidation, pharmacological characteristics, and ligand–receptor binding description.

To this end, we devoted our efforts to the discovery of a well-defined covalent antagonist based on xanthine analogue 1 mentioned above. Inspired by the resemblance in the chemical...
structure between the potent hA3AR antagonist 1 and irreversible adenosine A1 receptor antagonist 2, we incorporated the reactive moiety, a fluorosulfonyl benzoyl group, connected to a spacer, at the N1 position of the scaffold. Using a structured approach to bring the reactive fluorosulfonyl group in close proximity to a nucleophilic amino acid residue, we diversified the type of linker, linker length, and position of the fluorosulfonyl substituent on the phenyl group, resulting in a series of analogues with a wide range of affinities. Our efforts led to the discovery of a best-in-class antagonist, 17b, which is bound to the hA3AR with an apparent affinity in the nanomolar range. To retain the chemical structure similarity, we replaced the warhead with a methylsulfonyl moiety to obtain a nonreactive derivative 19 as a reversible control compound. 17b was then validated to covalently bind and inactivate the hA3AR in an insurmountable manner. Molecular modeling suggested the fluorosulfonyl functionality of 17b in close proximity to Y2657.36, which was identified as the unique anchor point of the covalent interaction in a subsequent mutagenesis study. The confirmed binding mode between this novel covalent antagonist and hA3AR opens the door for exploring other ligand binding motifs and will benefit receptor stabilization and further structure elucidation of the hA3AR.

### RESULTS AND DISCUSSION

#### Design of Covalent hA3AR Antagonists

In previous studies, our research group disclosed several series of hA3AR antagonists based on the pyrido[2,1-f]purine-2,4-dione scaffold.6−8 Using compound 1, a nanomolar probe from the previous series, as the starting point, we further designed and synthesized compounds based on a previously suggested binding mode of the pyrido[2,1-f]purine-2,4-dione scaffold.7 When examining the suggested binding mode of this scaffold, we noted that this scaffold inserted into the binding pocket with a receptor interaction between TM3, TM6, and EL2. Two key H-bonds include the carbonyl-oxygen at the C4-position with residue N2506.55 and the methoxy substituent at the C8-position bonding to Q167EL2. Taking this into account, we reasoned that the only available space to incorporate the reactive warhead is limited to N1-position substituents. To explore the chemical space required to optimally position the warhead in close proximity to a nucleophilic amino acid residue, we examined various linker systems, connecting the warhead and the pyrido[2,1-f]purine-2,4-dione scaffold.

#### Scheme 1. Synthetic Route toward Scaffold 7<sup>a</sup>

![Scheme 1](image1)

*Reagents and conditions: (a) (i) Ac<sub>2</sub>O, 80 °C, 2 h; (ii) Et<sub>2</sub>O, room temperature (rt), 1 h; (iii) 3 M NaOH, 85 °C, 1 h; (iv) HCl (37%), 25%; (b) (i) NBS, MeCN, 80 °C; (ii) 4-methoxypyridine, 80 °C, 64%; (c) 1-bromopropane, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), MeCN, 70 °C, 73%; (d) Pd(OH)<sub>2</sub>/C, HCOONH<sub>4</sub>, EtOH, reflux, 40%.*

#### Scheme 2. Synthetic Route toward the Bromoalkyl Fluorosulfonylbenzoates 13a−c and 14a−c<sup>a</sup>

![Scheme 2](image2)

*Reagents and conditions: (a) 2 M KHF<sub>2</sub> solution, dioxane, rt, 1 h, 87−90%; (b) SOCl<sub>2</sub>, reflux; (c) corresponding bromoalkylalcohol, anhydrous dioxane, 100 °C, 18 h, 55−83%, (d) 11a−c or 12a−c, K<sub>2</sub>CO<sub>3</sub>, anhydrous DMF, 50 °C, 5−57%.*
Scheme 3. Synthetic Route toward the Amide-Linker Antagonists 17a−c, 18a−c, and 19

**Table 1. Apparent Affinities of Pyrido[2,1-f]purine-2,4-dione Derivatives 13−19**

| compound | n | X | R^1 | pKᵦ ± SEM or disp. at 10 μM (%) |
|----------|---|---|-----|---------------------------------|
| 13a      | 1 | O | 4-SO₂F | 6.7 ± 0.1                      |
| 13b      | 2 | O | 4-SO₂F | 7.7 ± 0.1                      |
| 13c      | 3 | O | 4-SO₂F | 7.5 ± 0.1                      |
| 14a      | 1 | O | 3-SO₂F | 6.4 ± 0.1                      |
| 14b      | 2 | O | 3-SO₂F | 7.0 ± 0.05                     |
| 14c      | 3 | O | 3-SO₂F | 7.1 ± 0.05                     |
| 17a      | 1 | NH | 4-SO₂F | 27%                            |
| 17b (LUF7602) | 2 | NH | 4-SO₂F | 8.0 ± 0.05                     |
| 17c      | 3 | NH | 4-SO₂F | 7.5 ± 0.05                     |
| 18a      | 1 | NH | 3-SO₂F | 18%                            |
| 18b      | 2 | NH | 3-SO₂F | 7.5 ± 0.01                     |
| 18c      | 3 | NH | 3-SO₂F | 6.8 ± 0.1                      |
| 19 (LUF7714) | 2 | NH | 4-SO₂Me | 6.3 ± 0.03                    |

**Reagents and conditions: (a) N-(bromoalkyl)phthalimide, K₂CO₃, DMF, 100 °C, 5−96%; (b) N₂H₄·H₂O, MeOH reflux, 86−90%; (c) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), corresponding acid (9a,b), CHCl₃ or CH₂Cl₂, rt; and (d) SOCl₂, K₂CO₃, dry DMF, 40 °C, 3−78%**

**Ester Linker.** The fluorosulfonyl warhead is notorious for its reactivity, resulting in undesired side reactions or hydrolysis under several harsh reactions. So, we adopted a convergent synthetic strategy in which the fluorosulfonylphenyl linker unit was prepared separately and attached directly to the scaffold 7 at the N³ position. This approach offers flexibility to accommodate a variety of different linker lengths. The warhead was synthesized from commercially available chlorosulfonylbenzoic acids (8a and 8b) (Scheme 2), followed by a 2 M solution of potassium fluoride treatment to afford fluorosulfonylbenzoic acids (9a and 9b) in good yields. The next step converted the carboxylic acids to acid chlorides (10a and 10b) by excess thionyl chloride treatment. These acid chlorides are susceptible to hydrolysis and were thus used in the next step reaction without further purification. To incorporate the acyl chlorides with the corresponding bromoalkylalcohols, compounds 10a and 10b were heated to 100 °C with the addition of bromoalkylalcohols to afford the desired bromoalkyl fluorosulfonylbenzoates (11a−c and 12a−c) in decent yields. The final step was to couple the core to the corresponding bromoalkyl fluorosulfonylbenzoates. To preserve the functional fluorosulfonyl group, the reactions were carried out under mild conditions at low temperatures. Additionally,
experiments performed in duplicate. Affinity determined from the displacement of specific [3H]DPCPX binding on CHO cell membranes stably expressing human adenosine A1 receptors at 25 °C during 2 h of incubation. Affinity determined from the displacement of specific [3H]ZM241385 binding on HEK293 cell membranes stably expressing human adenosine A2A receptors at 25 °C during 2 h of incubation. Displacement at 1 μM concentration of specific [3H]PSB-603 binding on CHO cell membranes stably expressing human adenosine A3 receptors at 25 °C during 2 h of incubation. Displacement of specific [3H]PSB-11 binding on CHO cell membranes stably expressing the hA3AR at 25 °C during 0.5 h of incubation. Displacement of specific [3H]PSB-11 binding from CHO cell membranes stably expressing the hA3AR preincubated with an antagonist for 4 h at 25 °C, followed by a 0.5 h of co-incubation with [3H]PSB-11. P < 0.01** compared with the pKᵢ values in displacement experiments during 0.5 h of incubation time; Student’s test. Displacement of specific [3H]PSB-11 binding from CHO-K1 cell membranes transiently transfected with hA3AR-WT at 25 °C during 2 h of incubation. Displacement of specific [3H]PSB-11 binding from CHO-K1 cell membranes transiently transfected with hA3AR-Y265F at 25 °C during 2 h of incubation. P < 0.01* compared with the pIC₅₀ values in displacement experiments on hA3AR-WT. NS: no significant difference compared with the pIC₅₀ values in displacement experiments on hA3AR-WT membranes; Student’s test. For 17b, pKᵢ values are apparent affinity values as no dynamic equilibrium can be obtained.

Table 2. (Apparent) Affinities of 17b and 19 for All Adenosine Receptor Subtypes, hA₃AR-WT, and hA₃AR-Y265F<sup>7-36</sup>α

| cpd | hA₂AR<sup>b</sup> | hA₃AR<sup>c</sup> | hA₃AR<sup>d</sup> | hA₃AR<sup>e</sup> | hA₃AR-WT<sup>8</sup> | hA₃AR-Y265F<sup>7-36</sup><sup>δ</sup> |
|-----|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 17b | 6.1 ± 0.03 | 5.9 ± 0.09 | 0% (7, −7) | 6.9 ± 0.06 | 8.0 ± 0.01** | 7.8 ± 0.05 | 6.0 ± 0.3* |
| 19  | 4.8 ± 0.20 | 5.2 ± 0.20 | 0% (−10, −15) | 6.2 ± 0.03 | 6.1 ± 0.06<sup>NS</sup> | 5.9 ± 0.02 | 6.1 ± 0.1<sup>NS</sup> |

Values represent mean ± SEM of three separate experiments, each performed in duplicate, or percentage displacement at 1 μM of two separate experiments, each performed in duplicate.

excess DMF was removed by multiple washing steps, instead of vacuum removal at high temperatures. Six final products (13a–c and 14a–c) were obtained in acceptable yields.

Amide Linker. A similar synthetic approach was initially pursued to prepare analogues with an amide linker. However, the basicity and instability of bromoalkylamine caused complex side reactions with itself and with the warhead, ending up with an unacceptably low yield of amide-linked building blocks. An alternative synthetic route was devised, where 1-phthalimido-propyl bromide was attached directly to the N₃ position of the basicity and instability of bromoalkylamine caused complex side reactions with itself and with the warhead, ending up with an unacceptably low yield of amide-linked building blocks. An alternative synthetic route was devised, where 1-phthalimido-propyl bromide was attached directly to the N₃ position of the scaffold 6, to afford the substituted intermediates 15a–c (Scheme 3). Liberation of the amine took place by treatment with hydrazine monohydrate in methanol to obtain compound 16a–c in moderate yield. Then 16b and 16c were acylated with acyl chlorides 10a and 10b, respectively, to obtain 17c and 18b. However, impurities brought by the acylation reaction were not easily removed by column chromatography or preparative thin-layer chromatography (TLC). To overcome this, we used peptide coupling conditions with the corresponding benzoic acids (9a and 9b) to convert the free amine to the target compounds (17a,b, 18a, and 18c) in good yields (Scheme 3). A similar synthetic strategy was adapted to obtain reversible ligand 19 as a control compound.

Pharmacological Evaluation. Determination of the Apparent Affinity (Kᵢ) of Synthesized Ligands. To determine the binding affinity for the hA₃AR, all compounds were tested in a radioligand displacement binding assay in the presence of 10 nM [3H]PSB-11 at 25 °C according to previously reported procedures.<sup>5,19</sup> All compounds were able to concentration-dependently inhibit specific [3H]PSB-11 binding to the hA₃AR. As detailed in Table 1, all putative covalent compounds, except the two carbon linker compounds (13a, 14a, 17a, and 18a), displayed high affinities for the hA₃AR (Kᵢ < 100 nM). It should be mentioned that the putative covalent functionality, whereas a nonreactive control compound, chemically similar to the designed covalent ligand, is needed for the further pharmacological characterization. Therefore, we expressed the ligands’ affinity for the hA₃AR as “apparent Kᵢ”. Of note, 17b, bearing three carbon atoms with amide linkage and positioning the sulfonyl fluoride at the 4-position of the phenyl ring, interacted with the hA₃AR with comparable affinity (10 nM) as the parent compound 1. High affinity is desirable for covalent ligand design, as it allows sufficient receptor occupancy with the electrophilic warhead in proximity to a nucleophilic residue in the binding site over time, concomitant with putatively negligible or less interaction with off-targets. Thus, we chose compound 17b for further studies. However, featuring an electrophilic fluorosulfonyl functionality, 17b was no longer a close analogue of compound 1, whereas a nonreactive control compound, chemically similar to the designed covalent ligand, is needed for the further pharmacological characterization.

A nonsubstituted phenyl to replace the warhead might impress different steric and electronic characteristics of the ligand. To avoid this, we performed a conservative structural modification to replace the reactive warhead with an electron-withdrawing methysulfonyl group, yielding derivative 19 as a nonreactive control compound.

Figure 2. (A) Displacement of [3H]PSB-11 binding from the hA₁AR at 25 °C by 17b with and without preincubation of 4 h. (B) Displacement of [3H]PSB-11 binding from the hA₃AR at 25 °C by 19 with and without preincubation of 4 h. Data represent the mean ± SEM of three individual experiments performed in duplicate.
To better understand the time-dependent binding characteristics of these compounds, we carried out radioligand displacement assays under two different protocols. In detail, the CHO cell membranes overexpressing the hA3AR were either preincubated with the indicated compound for 4 h, followed by a 0.5 h co-incubation or only co-incubated for 0.5 h with the radioligand [3H]PSB-11. As detailed in Table 2, both compounds had comparable binding affinity in the low micromolar range (pKᵢ = 6.9 ± 0.06 for 17b and pKᵢ = 6.2 ± 0.03 for 19) at 0.5 h incubation time. However, compound 17b showed a significantly increased affinity (pKᵢ = 8.0 ± 0.01) when it was preincubated with the hA3AR, whereas the affinity of compound 19 did not change (pKᵢ = 6.1 ± 0.06). The effect of preincubation on the affinity of 17b and 19 is illustrated in Figure 2, i.e., the [3H]PSB-11 displacement curve was shifted to the left with an increased incubation time for compound 17b (Figure 2A), whereas no difference was observed for compound 19 (Figure 2B).

Presumably, this time-dependent binding affinity of compound 17b (i.e., resulting from an increased receptor occupancy over time) is a result of an increasing level of covalent binding. Similar results on other GPCRs, such as β₂ adrenergic receptor and A₂A adenosine receptor, showed that covalent bond formation generates an increased affinity over time. Meanwhile, control compound 19 showed no substantial pKᵢ shift in affinity at the two incubation times, indicating that a dynamic equilibrium was achieved at both incubation times. We can thus speculate that the possible covalent interaction between compound 17b and the receptor may be attributed to the presence of a reactive warhead.

Finally, we tested 17b and 19 for their affinity on the other adenosine receptor subtypes and learned that the two compounds were at least modestly selective for the hA3AR (Table 2).

Kinetic Characterization of the Covalent Ligand. Subsequently, the significant shift in apparent Kᵢ drove us to explore the binding kinetic profile of 17b at the hA3AR, specifically its dissociation rate and residence time (RT). Previously, the kᵢ (kᵢ = 0.281 ± 0.04 × 10⁵ M⁻¹ min⁻¹) and kᵦ (kᵦ = 0.3992 ± 0.02 min⁻¹) values of [3H]PSB-11 at 25 °C had been determined in our laboratory by traditional association and dissociation assays. Here, we performed a competition association assay to characterize the binding kinetics of 17b and 19 following previously reported procedures from our research group. Using the on- and off-rate constants from [3H]PSB, the kᵢ (kᵢ) and kᵦ (kᵦ) values for 17b were determined using the equations from the (equilibrium) Motulsky and Mahan model. 17b had a much slower association rate (kᵢ = 3.48 ± 0.22 × 10⁵ M⁻¹ min⁻¹) than the radioligand and a negligible dissociation rate (kᵦ = 1.38 ± 0.22 × 10⁻¹² min⁻¹), yielding an almost infinite residence time (RT = 7.63 ± 1.19 × 10¹¹ min), indicative of irreversible receptor binding by 17b. The inadequacy of the Motulsky–Mahan equations to fit this data is further evidence for the nonequilibrium features of the binding of 17b to the receptor. Compound 19 showed fast association and dissociation rate constants (Figure 3). Unfortunately, the data did not converge in the fitting procedure, possibly due to the low binding affinity of compound 19 (Kᵢ = 525 nM).

As detailed in Figure 3, the control curve represented the association curve of radioligand [3H]PSB-11 alone, approaching equilibrium over time. Compound 19 equally associated with and dissociated from the receptor and reached equilibrium within 30 min, evidenced by the same curve shape as the control curve. Of note, 17b’s behavior caused an initial “overshoot” of the competition association curve, followed by a linear decline over time indicating that no equilibrium was reached. The shape of 17b’s kinetic curve is a quintessential example for the irreversible interaction, similar to the reported covalent ligands’ behavior for the adenosine A₂A receptor and mGlu2 receptor.

Wash-Resistant Interaction between 17b and hA3AR. Inspired by the negligible dissociation of compound 17b from the hA3AR, we performed a “washout” experiment to ascertain the irreversible binding between the ligand and the receptor. A protocol previously reported by our laboratory was adapted. We first exposed hA3AR cell membranes to 17b or 19 both at 10-fold Kᵢ for 2 h, and without washing the samples were supplemented with [3H]PSB-11 to assess the competitive binding capacity of the receptor (“control group” in Figure 4).

For washed samples, hA3AR cell membranes were subjected to four-cycle washing steps to remove unbound ligand following the preincubation (“4x wash group” in Figure 4), after which the membranes were exposed to [3H]PSB-11 to determine the remaining binding capacity. In the absence of the ligand (labeled “+ vehicle” in Figure 4), we normalized membranes’ binding ability to 100%. Following preincubation with 17b, membranes containing the hA3AR lost most of the ability to bind to the radioligand (11.3 ± 1.2% binding remaining). Furthermore, after the preincubation, membranes were washed by cycles of centrifugation in an attempt to regenerate binding characteristics.
capacity. However, washing steps failed to restore hA3AR binding of [3H]PSB-11 (8.7 ± 3.8%). This was in contrast to preincubation of the hA3AR-expressing membranes with ligand \(19\), in which binding function was completely restored from 19.8 ± 4.7 to 97.6 ± 4.5% following four washing steps. This result indicates that \(19\) is a reversible ligand which can be rapidly washed off the membranes, whereas \(17b\) forms a wash-resistant bond between the ligand and the receptor. Similar experiments on other GPCRs, such as adenosine A1 and A2A receptors and the metabotropic glutamate receptor 2 (mGluR2), demonstrated that the covalent interaction between the ligand and the receptor resulted in a wash-resistant bond formation.

**Insurmountable Antagonism Caused by Covalent Interaction.** To further evaluate the effect of irreversible inhibition by covalent ligand \(17b\) on receptor function, we performed a membrane functional assay using \([35S]GTP\gamma S\), which is a typical readout for the activation of receptor-coupled G\(_{i/o}\) proteins. Pretreatment of the hA3AR with increasing concentrations of ligand \(17b\), prior to the stimulation with hA3AR agonist 1-\[2-chloro-6-[[\{3-iophenyl\}methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-\(\beta\)-ribofuranuronamide (2-Cl-IB-MECA), produced rightward shifts of agonist concentration–response curves with a concomitant decline in maximal stimulation (Figure 5A). Therefore, the covalent ligand \(17b\) generated insurmountable antagonism in the preincubation experiment. In contrast, pretreatment of the hA3AR with \(19\), followed by 2-Cl-IB-MECA agonist exposure resulted in surmountable antagonism (Figure 5B), i.e., shifting dose–response curves to the right with no alteration of its maximum effect. The extent of the shifts was used to construct a Schild plot as previously described, which would have a slope of unity if the interaction is competitive and the \(pA_2\) value corresponds to the \(pK_i\) value of the antagonist. The slope for \(19\) was found to be 1.1 ± 0.1 and the compound’s \(pA_2\) value was 5.9 ± 0.1, comparable with its \(pK_i\) value (6.3 ± 0.03), suggesting that \(19\) competed with 2-Cl-IB-MECA for the same receptor binding site. To unravel the molecular mechanism responsible for the insurmountable antagonism of \(17b\), we also co-incubated either \(17b\) or \(19\) with the hA3AR in the presence of 2-Cl-IB-MECA. Both ligands produced a rightward shift of the agonist’s concentration–response curve (Figure 5C,D) with no suppression of maximal response, indicative of surmountable

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**Figure 5.** Effects of \(17b\) and \(19\) on hA3AR activation as measured by \([35S]GTP\gamma S\) binding. (A, B) Compound \(17b\) (A) or \(19\) (B) was preincubated with the hA3AR stably expressed on CHO cell membranes (25 °C) for 60 min prior to the addition of 2-Cl-IB-MECA at a concentration ranging from 0.1 nM to 10 μM for 30 min. (C, D) Compound \(17b\) (C) or \(19\) (D) were co-incubated with 2-Cl-IB-MECA, at a concentration ranging from 0.1 nM to 10 μM, for 30 min. The agonist curves were generated in the presence of increasing concentrations of antagonists, such as 0.3-, 1-, 3-, and 10-fold \(K_i\) values, respectively. Data are from three independent experiments performed in duplicate, normalized according to the maximal response (100%) produced by 10 μM 2-Cl-IB-MECA alone. The shift in agonist EC\(_{50}\) values was determined to perform Schild analyses.

**Table 3.** Functional Analysis of hA3AR Antagonism from \([35S]GTP\gamma S\) Binding Assays

| compound | preincubation | co-incubation |
|----------|--------------|---------------|
|          | p\(A_2\)     | Schild slope  | p\(A_2\)     | Schild slope  | mode of antagonism |
| \(17b\)  | NA           | 7.4 ± 0.1     | 5.9 ± 0.1     | 1.1 ± 0.1     | competitive insurmountable |
| \(19\)   | 5.9 ± 0.1    | 6.2 ± 0.1     | 5.9 ± 0.1     | 1.0 ± 0.1     | competitive surmountable |

*Values represent mean ± SEM of three separate experiments each performed in duplicate.*
antagonism. The Schild plot showed that both antagonists inhibited receptor activation in a competitive manner, with their Schild-slopes close to unity (1.1 ± 0.1 for 17b, 1.0 ± 0.1 for 19, Table 3). In addition, 19’s pA₂ value was in agreement with that from the preincubation experiments (6.2 ± 0.1, Table 3), and the pA₂ value of 17b was also comparable with its pKᵢ value (7.4 ± 0.1 vs 8.0 ± 0.05). Taken together, both ligands fully competed with 2-ChI-B-MECA bound to the hA₃AR.

Notably, it is likely that the insurmountable behavior relates to the covalent binding of 17b due to an irreversible blockade that reduces the total receptor population available.

**Binding Model for 17b in the hA₃AR Receptor-Binding Pocket.** To examine the interaction between receptor residues possibly involved in covalent binding, we docked 17b into a ligand optimized homology model on the basis of the A₂A receptor crystal structure (PDB: 4EIY27), as described.

**Figure 6.** Proposed binding mode of compound 17b (green carbon sticks) in a homology model (violet ribbons) of the hA₃AR. The hA₃AR homology model was based on the high-resolution antagonist-bound crystal structure of the adenosine A₂A receptor (PDB: 4EIY27). Atom color code: red = oxygen, blue = nitrogen, white = hydrogen, yellow = sulfur, cyan = fluorine. Hydrogen bonds between the ligand and receptor are indicated by yellow dashed lines. Residue Y265.36 is in the proximity of the fluorosulfonyl warhead.

**Figure 7.** (A, B) Displacement of specific [³H]PSB-11 binding from transiently transfected hA₃AR-WT and hA₃AR-Y265F7.36 at 25 °C by compound 17b (A) and 19 (B) during incubation of 2 h. (C) hA₃AR-Y265F7.36 cell membranes were pretreated with vehicle (vehicle) or 10 × IC₅₀ of compound 17b for 2 h followed by no washing (control) or four-cycle washing treatment (4× wash) before being exposed to [³H]PSB-11. Data represent the mean ± SEM of three individual experiments performed in duplicate, normalized to the vehicle (set at 100%). NS: no significant difference between groups; ***Significant difference between groups (P < 0.001); Student’s t-test.
Previously,7 as detailed in Figure 6, the core structure of compound 17b interacted with the TM3, TM6, and EL2 regions. Additionally, the carbonyl-oxygen at the C6-position participated in H-bond formation with residue N250.65 and the methoxyl moiety at the C8-position functioned as H-bond acceptor with Q167EL2. Interestingly, the latter is a unique residue in the hA3AR, as it is not conserved in other subtypes of adenosine receptors. Due to the flexibility of the three carbon linkers, the tyrosine residue Y265.36 is in close proximity of the ligand, and could therefore interact with the 4-fluorosulfonylbenzoic warhead to form a covalent sulfonyl amide. Similarly, the same residue Y271.36 located within the human adenosine A3 receptor has also been reported to covalently interact with the fluorosulfonyl warhead of compound 2.13 Comparison of the binding modes of compound 2 and ligand 17b in an A1/A2 receptor overlay showed that key interactions between ligands and binding sites are preserved, such as a hydrogen bond with K.65 (Figure S1). Y265.36 as an Anchor Point for the Covalent Bond. Based on the docking study, we postulated that Y265.36 is the anchor point for covalent bond formation. To investigate our hypothesis this tyrosine was mutated to phenylalanine (hA3AR-Y265F.7). First, we performed standard [3H]PSB-11 displacement assays to investigate the binding affinity of 17b and 19 using CHO-K1 cell membranes transiently transfected with either wild-type (hA3AR-WT) or mutant receptors (hA3AR-Y265F.36). As shown in Table 2 and Figure 7, the affinity of control compound 19 on hA3AR-Y265F.36 (pIC50 = 6.09 ± 0.11) was similar to the affinity to hA3AR-WT (pIC50 = 5.95 ± 0.03), indicating that the mutation has no impact on the binding affinity of the reversible ligand. In marked contrast, 17b’s affinity was decreased nearly 43-fold relative to the WT, from an IC50 value of 27 to 1072 nM, indicative of the loss of irreversible interaction. Moreover, there were no marked affinity differences on hA3AR-Y265F.36 between 17b and 19. This suggests that the chemically dissimilar ligands 17b (reactive) and 19 (nonreactive) exhibit a similar binding interaction with hA3AR-Y265F.36. We thus speculate that the amino acid in position 7.36 plays a prominent role in the covalent bond formation between the fluorosulfonyl warhead and the receptor. To support this idea, we repeated the washout assay on hA3AR-Y265F.36. Membranes treated with 17b at 10-fold IC50 inhibited the specific [3H]PSB-11 binding to 7.2 ± 0.6%. After extensive washing, hA3AR-Y265F.36 showed a complete recovery of [3H]PSB-11 binding to 91 ± 2% (Figure 7C). This full recovery for mutant hA3AR-Y265F.36 is in sharp contrast to the findings in the wild-type washout assay (Figure 4), indicating that Y265F.36 completely prevented the wash-resistant bond formation. In other words, Y265.36 is the unique amino acid residue involved in the covalent attachment of 17b’s fluorosulfonyl group within the hA3AR binding pocket. A similar approach was also adopted to pinpoint the anchor point between covalent probes and other subtypes of GPCRs, such as the adenosine A3 receptor, mGlu2 receptor,21 and cannabinoid CB1 receptor.28

17b can be a useful structural biology tool as it would be expected to stabilize the 7TM domain in its inactive state, thereby potentially facilitating crystallization of the receptor material. This could be highly valuable for the structure elucidation of the hA3AR, which up to now remains unreported. Furthermore, understanding the precise molecular interactions between the ligand and the receptor may stimulate the more rational design of novel ligands. Such ligands may have improved receptor subtype selectivity, fewer undesirable side effects, and enhanced potency and efficacy, leading to potentially attractive therapeutic agents that produce their effects by modulating the functionality of the adenosine system. Given that GPCR-targeted covalent drugs went through clinical success across various indications,29 our covalent compound 17b may serve as a probe to explore the problematic translation of hA3AR ligands into the clinical utility in certain disease states such as eye disorder glaucoma, in which an increased A3 adenosine receptor mRNA and protein levels have been detected.

CONCLUSIONS

By introducing a reactive sulfonyl fluoride warhead onto the 1-benzyl-3-propyl-1H,3H-pyrido [2,1-f]purine-2,4-dione scaffold, we designed and synthesized a series of novel covalent hA3AR antagonists. Compound 17b acted as the most potent antagonist, with a time-dependent apparent affinity in the low nanomolar range. Meanwhile, we removed the warhead and inserted a methylsulfonyl moiety into the scaffold, to obtain ligand 19 as a reversible control compound. Ligand 17b was then validated as a covalent antagonist through its wash-resistant nature and insurmountable antagonism in [35S]GTPγS binding assays. In silico homology-docking suggested that Y265.36 is responsible for the covalent interaction. Site-directed mutagenesis showed that removal of the nucleophilic tyrosine phenolic hydroxyl group resulted in the complete loss of covalent binding, validating that Y265.36 is the only anchor point of reactive covalent ligand 17b. The results contribute to a better understanding of pharmacological behaviors caused by covalent interaction with GPCRs. In the end, we developed a structured approach to quickly obtain a well-defined covalent ligand. Besides, we envisioned that a methylsulfonyl replacement would be suitable for providing a nonreactive sulfonyl-bearing control compound. The rational design of covalent probes may have further value in receptor structure elucidation or in new technologies such as affinity-based protein profiling13,30 with the perspective of imaging or structurally probing GPCRs.

EXPERIMENTAL SECTION

Chemistry. All solvents and reagents were purchased from commercial sources and were of analytical grade. Demineralized water is simply referred to as H2O, and was used in all cases unless stated otherwise (i.e., brine). 1H were recorded on a Bruker AV 400 liquid spectrometer (1H NMR, 400 MHz) at ambient temperature and 13C NMR spectra were recorded on a Bruker AV 600 liquid spectrometer (13C NMR, 125 MHz) at indicated temperature. Chemical shifts are reported in parts per million (ppm), using residual solvent as the internal reference in all cases. The values are given in δ scale. Coupling-constants are reported in Hz and are designated as J. Analytical purity of the final compounds was determined by high-performance liquid chromatography (HPLC) with a Phenomenex Gemini 3 μm C18 110 Å column (50 × 4.6 mm, 3 μm), measuring UV absorbance at 254 nm. Sample preparation and the HPLC method were as follows: 0.3−1.0 mg of compound was dissolved in 1 mL of a 1:1:1 mixture of MeCN/H2O/BuOH and eluted from the column within 15 min at a flow rate of 1.3 mL·min−1 with a three-component system of H2O/MeCN/1% trifluoroacetyl (TFA) in H2O. The elution method was set up as follows: 1−4 min isocratic system of H2O/MeCN/1% TFA in H2O, 80:10 from the 4th min, a gradient was applied from 80:10 to 0:90:10 within 9 min, followed by 1 min of equilibration at 0:90:10 and 1 min at...
80:10:10. All final 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 a Thermo Finnigan Surveyor–LCQ Advantage Max LC–MS system and a Gemini C18 Phenomenex column (50 × 4.6 mm, 5 μm). High-resolution mass spectrometry (HRMS) analyses were performed using a Thermo Scientific LTQ Orbitrap XL Hybrid Ion Trap–Orbitrap Mass Spectrometer. The sample preparation was the same as for HPLC and HRMS analyses. The compounds were eluted from the column within 15 min after injection, with a three-component system of H₂O/MeCN/0.2% TFA in H₂O, decreasing polarity of the solvent mixture in time from 80:10:10 to 0:90:10. 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 using the Grace Davison Davsil silica column material (LC60A 30–200 μm). Solutions were concentrated using a Heidolph Laborota W8200 efficient rotary evaporation apparatus. All reactions in the synthetic routes were performed under a nitrogen atmosphere unless stated otherwise. The procedure for a series of similar compounds is given as a general procedure for all within that series, annotated by the numbers of the compounds.

1-Benzyl-8-methoxy-3-propyl-1H,3H-pyrrolo[2,1-f]purine-2,4-dione (1).§ To a stirred suspension of 6 (6.0 g, 19 mmol, 1.0 equiv) in MeCN (120 mL) were added 1-bromopropane (5.6 mL, 57 mmol, 3.0 equiv) and DBU (50 mL, 57 mmol, 3.0 equiv). This mixture was stirred at 80 °C overnight. After the completion of the reaction was observed by TLC (5% MeOH in CH₂Cl₂), 4-MeOH (200 mL) was added to the mixture. The precipitate was filtered off, washed with acetonitrile and MeOH, yielding product 4 (2.0 g, 1.7 mmol, 55%) compound was used without further purification.

4-Bromobutyl-3-(fluorosulfonyl)benzoate (9a,b). To a solution of chlorosulfonylbenzoic acid (9a,b) (2.2 g, 10 mmol, 1.0 equiv) in dioxane (25 mL) was added a solution of HF/KF (15 mL, 2.0 M, 3.0 equiv). The mixture was stirred at room temperature. After 1 h, the reaction mixture was diluted with EtOAc (80 mL). The organic phase was washed with H₂O (50 mL), dried over MgSO₄, filtered, and concentrated in vacuo.

3-(Fluorosulfonyl)benzoic Acid (9a). White solid (1.9 g, 8.7 mmol, 87%). § H NMR (400 MHz, DMSO-d₆): δ 8.47–8.44 (m, 2H), 8.4 (d, J = 8.0 Hz, 1H), 7.94 (s, J = 7.6 Hz, 1H).

Bromoethyl-4-(fluorosulfonyl)benzoate (11a). Colorless oil (0.088 g, 0.28 mmol, 23%) 1H NMR (400 MHz, DMSO-d₆): δ 8.31 (d, J = 8.2 Hz, 2H), 8.11 (d, J = 8.5 Hz, 2H), 4.69 (t, J = 5.9 Hz, 2H), 3.67 (t, J = 5.9 Hz, 2H).

Bromopropyl-4-(fluorosulfonyl)benzoate (11b). White solid (2.0 g, 6.2 mmol, 50%) 1H NMR (400 MHz, CDCl₃): δ 8.27 (d, J = 8.4 Hz, 2H), 8.09 (d, J = 8.4 Hz, 2H), 4.54 (t, J = 6.0 Hz, 2H), 3.54 (d, J = 6.4 Hz, 2H), 2.35 (m, 2H).

Bromobutyl-4-(fluorosulfonyl)benzoate (11c). White solid (0.30 g, 0.89 mmol, 45%) compound was used without further purification.

Bromoethyl-3-(fluorosulfonyl)benzoate (12a). Colorless oil (0.51 g, 1.7 mmol, 55%) 1H NMR (400 MHz, CDCl₃): δ 8.65 (s, 1H), 8.47 (d, J = 7.6 Hz, 1H), 8.25–8.20 (m, 1H), 7.78 (t, J = 8.0 Hz, 1H), 4.71 (t, J = 6.0 Hz, 2H), 3.68 (t, J = 6.0 Hz, 2H).

Bromopropyl-3-(fluorosulfonyl)benzoate (12b). Colorless oil (0.12 g, 0.38 mmol, 23%) 1H NMR (400 MHz, CDCl₃): δ 8.65 (t, J = 1.6 Hz, 1H), 8.44 (d, J = 7.8 Hz, 1H), 8.21 (d, J = 8.0 Hz, 1H), 7.76 (t, J = 7.9 Hz, 1H), 4.55 (t, J = 6.1 Hz, 1H), 3.55 (t, J = 6.4 Hz, 1H), 2.37 (m, J = 6.3 Hz, 1H).

Bromobutyl-3-(fluorosulfonyl)benzoate (12c). Colorless Oil (0.84 g, 2.5 mmol, 83%) 1H NMR (400 MHz, CDCl₃): δ 8.65 (s, 1H), 8.45 (d, J = 8.0 Hz, 1H), 8.21 (d, J = 8.0 Hz, 1H), 7.78 (t, J = 7.6 Hz, 1H), 4.44 (t, J = 6.0 Hz, 2H), 3.50 (t, J = 6.4 Hz, 2H), 2.11–1.85 (m, 4H).

General Procedure for the Synthesis of 13a–c and 14a–c.

The synthesis of these compounds was adapted from the conditions previously described by Priege et al. The scaffolds 8-methoxy-3-propyl-1H,3H-pyrrolo[2,1-f]purine-2,4-dione 7 (1.0 equiv) and K₂CO₃ (1.6 equiv) were suspended in anhydrous DMF. The mixture was added dropwise to a stirred solution of the corresponding bromoaryl (fluorosulfonyl)benzoates (11a–c or 12a–c) (1.0 equiv) in anhydrous DMF (4 mL). The reaction was stirred at 50 °C overnight. After the conversion was observed by TLC, an excess amount of CH₂Cl₂ was added. Then the mixture was washed with 1 M HCl (aq), water, and brine. The organic layer was dried over MgSO₄, filtered, and concentrated in vacuo. The crude product was observed by TLC (5% MeOH in CH₂Cl₂). The reaction was filtered over Celite and the residue was extracted with hot DMF. Purification of the crude product was performed by column chromatography using 2–10% MeOH in CH₂Cl₂ to obtain 5 as a white solid (0.30 g, 12 mmol, 40%). § H NMR (400 MHz, DMSO-d₆): δ 11.31 (br s, 1H), 8.70 (d, J = 7.2 Hz, 1H), 7.38–7.16 (m, 6H), 6.90 (dd, J = 7.4, 2.2 Hz, 1H), 5.18 (s, 2H), 3.89 (s, 3H).

8-Methoxy-3-propyl-1H,3H-pyrrolo[2,1-f]purine-2,4-dione (7). To a mixture of intermediate 6 (1.1 g, 30 mmol, 1.0 equiv), Pd(OH)₂/C (2.0 g, 14 mmol, 1.0 equiv), and ammonium formate (0.20 g, 3.0 mmol, 1.0 equiv) was added EtOH (250 mL). During the reaction, five portions of ammonium formate (0.20 g, 3.0 mmol, 1.0 equiv) was added, after which completion of the reaction was observed.
purified by column chromatography, followed by prep TLC to further purify the compound if necessary.

2-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]-purine-1(2H)-yl)ethyl 4-(fluorosulfonyl)benzoate (13a). Prepared from 11a and purified by column chromatography (1% CH$_2$OH in CH$_2$Cl$_2$) to give the desired product as a white solid (0.038 g, 0.27 mmol, 52%).

3-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]-purine-1(2H)-yl)propyl 4-(fluorosulfonyl)benzoate (13b). Prepared from 11b and purified by column chromatography (1% CH$_2$OH in CH$_2$Cl$_2$) to give the desired product as a white solid (0.096 g, 0.19 mmol, 76%).

4-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]-purine-1(2H)-yl)propyl 4-(fluorosulfonyl)benzoate (13c). Prepared from 11c and purified by column chromatography (2% CH$_3$OH in CH$_2$Cl$_2$) to give the desired product as a white solid (0.010 g, 0.019 mmol, 52%).

5-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]-purine-1(2H)-yl)butyl 4-(fluorosulfonyl)benzoate (13d). Prepared from 11d and without purification to give the desired product as a white solid (0.030 g, 0.056 mmol, 34%).

General Procedure for the Synthesis of 1-(2-(1,3-Dioxoisindolin-2-yl)alkyl)-8-methoxy-3-propyl-1H,3H-pyrido[2,1-f]-purine-2,4-dione (15c). Prepared from 15a and purified by column chromatography to give the desired product as a white solid (0.13 g, 0.76 mmol, 96%).

1H NMR (400 MHz, CDCl$_3$): δ 8.82 (d, J = 7.2 Hz, 1H), 8.12 (d, J = 7.6 Hz, 1H), 8.14−8.09 (m, 1H), 7.66 (t, J = 7.8 Hz, 2H), 6.84 (d, J = 2.4 Hz, 1H), 6.74 (d, J = 7.6 Hz, 2H), 6.48 (t, J = 4.8 Hz, 2H), 6.45 (t, J = 4.8 Hz, 2H), 4.04−3.97 (m, 2H), 3.90 (s, 3H), 2.05−1.90 (m, 4H), 1.77−1.68 (m, 2H), 0.99 (t, J = 7.6 Hz, 3H). MS: [ESI + H$^+$]: 533.1. HPLC: 9.40 min

2-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]-purine-1(2H)-yl)ethyl 3-(hydroxy)benzoate (14a). Prepared from 12a and without purification to give the desired product as a white solid (0.19 g, 0.36 mmol, 57%).

5-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]-purine-1(2H)-yl)butyl 3-(hydroxy)benzoate (14b). Prepared from 12b and purified by column chromatography (1% CH$_2$OH in CH$_2$Cl$_2$) to give the desired product as a white solid (0.035 g, 0.068 mmol, 34%).

1H NMR (400 MHz, CDCl$_3$): δ 8.74 (d, J = 7.6 Hz, 1H), 8.65 (s, 1H), 8.36 (d, J = 8.0 Hz, 1H), 8.18 (d, J = 8.0 Hz, 1H), 7.73 (t, J = 8.0 Hz, 1H), 6.86 (d, J = 2.0 Hz, 1H), 6.72 (dd, J = 7.2, 2.4 Hz, 1H), 4.51 (t, J = 6.0 Hz, 2H), 4.41 (t, J = 6.0 Hz, 2H), 3.99 (t, J = 7.6 Hz, 2H), 3.01 (s, 3H), 2.39−2.35 (pentet, J = 6.0 Hz, 2H), 1.70 (sextet, J = 7.6 Hz, 2H). 0.98 (t, J = 7.6 Hz, 3H). MS: [ESI + H$^+$]: 519.1. HPLC: 8.14 min

4-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]-purine-1(2H)-yl)butyl 3-(hydroxy)benzoate (14c). Prepared from 12c and purified by column chromatography (first 30% DCM in EtOAc). Further purification by another column (4% methyl tert-butyl ether/petroleum ether) gives the desired product as a white solid (0.20 g, 0.37 mmol, 38%).

5-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]-purine-1(2H)-yl)butyl 3-(hydroxy)benzoate (14d). Prepared from 12d and purified by column chromatography (1% DCM in EtOAc). Further purification by another column (5% DCM in MeOH) gives the desired product as a white solid (0.20 g, 0.37 mmol, 38%).

General Procedure for the Synthesis of 1-(2-(1,3-Dioxoisindolin-2-yl)alkyl)-8-methoxy-3-propyl-1H,3H-pyrido[2,1-f]-purine-2,4-dione (15a−c). To a mixture of the core (7) (0.8 mmol, 1 equiv), N-(bromomethyl)phthalimide (1.2 mmol, 1.5 equiv), and K$_2$CO$_3$ (1.2 mmol, 1.5 equiv) was added anhydrous DMF (8 mL). The mixture was refluxed at 100°C. After completion of the reaction, monitored by TLC (1% MeOH in CH$_2$Cl$_2$), the mixture was concentrated in vacuo and diluted with EtOAc (30 mL). The organic layer was washed with H$_2$O (3 × 30 mL) and brine (15 mL), and dried over MgSO$_4$. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography using 1% MeOH as an eluent to give 15a−c as solids.

The mixture was stirred at 100°C. After completion of the reaction, monitored by TLC (1% MeOH in CH$_2$Cl$_2$), the mixture was concentrated in vacuo and diluted with EtOAc (30 mL). The organic layer was washed with H$_2$O (3 × 30 mL) and brine (15 mL), and dried over MgSO$_4$. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography using 1% MeOH as an eluent to give 15a−c as solids.
3.47 (q, 2H), 1.00 (t, obtained as a colorless oil. Subsequently, amine removal of solvent and other volatiles under vacuum, 10a column chromatography using CH2Cl2 with 1% methanol as the eluent to give 17a as a white solid (0.20 g, 0.80 mmol, 1.5 equiv) was dissolved in CH2Cl2 (4 mL). To this stirring solution was added acid EDC (0.12 g, 0.60 mmol, 1.2 equiv) was dissolved in CHCl3 (8 mL) and added dropwise via an automatic syringe at a rate of 0.2 mL min−1. The reaction was stirred at room temperature and monitored by TLC (4% MeOH in CH2Cl2). After completion, the solvent was removed under vacuo and the residue was dissolved in CHCl3 (4 mL). To this stirring solution amine was added (16b) (0.25 g, 0.76 mmol, 1.0 equiv) at room temperature. The reaction was stirred for 2 h and monitored by TLC (3% MeOH in CH2Cl3). After completion, the solvent was removed in vacuo and the residue was dissolved in CHCl3 (40 mL). The organic layer was washed with 1 M HCl (40 mL) and twice with H2O (2 × 40 mL), dried over MgSO4, and concentrated in vacuo. The product was purified by column chromatography using 2% MeOH in CH2Cl2 to afford the title compound as a white solid (0.26 g, 0.50 mmol, 66%). 1H NMR (400 MHz, CDCl3): δ 8.66 (d, J = 7.2 Hz, 1H), 8.38 (t, J = 5.6 Hz, 1H), 8.25 (d, J = 8.4 Hz, 2H), 8.16 (d, J = 8.4 Hz, 2H), 6.85 (d, J = 2.4 Hz, 1H), 6.81 (d, J = J = 7.2, 2.4 Hz, 1H), 4.35 (s, J = 6.0 Hz, 2H), 4.05 (t, J = J = 7.6 Hz, 2H), 3.92 (s, 3H), 3.47 (q, J = 6.4 Hz, 2H), 2.19–2.13 (m, 2H), 1.73 (sextet, J = J = 7.6 Hz, 2H), 1.00 (t, J = J = 7.6 Hz, 3H). 13C NMR (600 MHz, DMSO-d6) δ 164.0, 160.9, 153.4, 150.6, 150.5, 149.1, 141.3, 133.3 (d, δ = 96 Hz), 128.5, 127.9, 127.3, 107.0, 99.8, 95.4, 55.7, 41.5, 40.6, 36.8, 27.1, 20.5, 10.6. MS: [ESI + H]+: 518.1. HPLC: 8.21 min.

3-(4-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]purin-1(2H)-yl)butyl)carbamoyl)benzenesulfonyl Fluoride (17c). Acid 9a (0.11 g, 0.53 mmol, 1.5 equiv) was dissolved in an excess of thiouyl chloride (20 mL) at 75°C under nitrogen for 3 h. After removal of solvent and other volatiles under vacuum, 10a was obtained as a colorless oil. Subsequently, amine 16c (0.12 g, 0.35 mmol, 1.0 equiv), K2CO3 (0.073 g, 0.53 mmol, 1.5 equiv), and dry MeOH (20 mL) and stirred for 3 h at 75°C. 1H NMR (400 MHz, CDCl3): δ 8.88 (d, J = 7.2 Hz, 1H), 8.68 (s, 1H), 8.55–8.50 (m, 2H), 8.20 (d, J = 8.0 Hz, 1H), 7.82 (t, J = J = 8 Hz, 2H), 7.00 (d, J = J = 2.4 Hz, 1H), 6.80 (dd, J = J = 7.2, 2.4 Hz, 1H), 4.33 (t, J = 6.0 Hz, 2H), 4.05 (t, J = J = 7.6 Hz, 2H), 3.94 (s, 3H), 3.47 (q, J = 6.0 Hz, 2H), 2.17–2.12 (m, 2H), 1.74 (sextet, J = J = 7.6 Hz, 2H), 1.00 (t, J = J = 7.6 Hz, 3H). MS: [ESI + H]+: 518.1. HPLC: 8.28 min.

3-(4-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]purin-1(2H)-yl)butyl)carbamoyl)benzenesulfonyl Fluoride (18c). EDC (0.13 g, 0.69 mmol, 1.2 equiv) was dissolved in CH2Cl2 (3 mL). Acid 9b (0.13 g, 0.63 mmol, 1.1 equiv) was added to this solution and the mixture was stirred. Amine 16c (0.20 g, 0.57 mmol, 1.1 equiv) was dissolved in CHCl3 (8 mL) and added dropwise via an automatic syringe at a rate of 0.2 mL min−1 to the stirring solution. After 3 h at room temperature, the reaction was completed and the mixture was concentrated in vacuo. The residue was dissolved in CHCl3 (40 mL) and washed with 1 M HCl (40 mL) and twice with H2O (2 × 40 mL). The organic layer was dried over MgSO4 and concentrated in vacuo. Purification by column chromatography (CH2Cl3/acetone = 3:2) gave 18c as a white solid (0.14 g, 0.26 mmol, 47%). 1H NMR (400 MHz, CDCl3): δ 8.84 (d, J = 7.2 Hz, 1H), 8.54 (s, 1H), 8.36 (d, J = 7.6 Hz, 1H), 8.12 (d, J = J = 7.6 Hz, 1H), 7.72 (t, J = J = 7.6 Hz, 1H), 7.62 (br s, 1H), 6.84 (s, 1H), 6.80–6.70 (m, 1H), 4.27 (t, J = 7.2 Hz, 2H), 4.04 (t, J = 8.0 Hz, 2H), 3.89 (s, 3H), 3.74–3.60 (m, 6H, 2H), 2.07–1.92 (m, 2H), 1.85–1.64 (m, 4H), 0.98 (t, J = J = 7.2 Hz, 3H). MS: [ESI + H]+: 532.3. HPLC: 8.21 min.

N-(3-(8-Methoxy-2,4-dioxo-3-propyl-3,4-dihydropyrido[2,1-f]purin-1(2H)-yl)propyl)-4-(methylsulfonyl)benzamide (19). To a solution of EDC (0.061 g, 0.32 mmol, 1.2 equiv) in CHCl3 (5 mL) was subsequently added dropwise via an automatic syringe at a rate of 0.15 mL min−1. The reaction was stirred at room temperature and monitored by TLC (4% MeOH in CHCl3). After 3 h, the reaction was completed and CHCl3 (50 mL) was added. The organic layer was washed with 1 M HCl (60 mL), H2O (60 mL), and brine (60 mL), dried over MgSO4 and concentrated under vacuum. The product was purified by column chromatography using 2% MeOH in CH2Cl2 to afford the title compound (0.075 g, 0.14 mmol, 54%). 1H NMR (400 MHz, CDCl3): δ 8.86 (d, J = 7.2 Hz, 1H), 8.36 (t, J = 5.6 Hz, 1H), 8.19 (d, J = 8.4 Hz, 2H), 8.09 (d, J = 8.4 Hz, 2H), 6.90–6.71 (m, 2H), 4.45–4.28 (m, 2H), 4.13–3.99 (m, 2H), 3.91 (s, 3H), 3.55–3.41 (m, 2H), 3.11 (s, 2H), 2.27–2.09 (m, 2H), 1.83–1.61 (m, 2H), 1.00 (t, J = 7.4 Hz, 3H). 13C NMR (600 MHz, DMSO-d6, 318 K): δ 164.7, 161.1, 153.5, 150.7, 150.6, 149.3, 142.8, 138.9, 127.9, 127.5, 126.7, 107.4, 99.9, 95.4, 56.0, 43.2, 41.6, 40.8, 36.8, 27.4, 20.7, 10.9. MS: [ESI + H]+: 514.2. HMRM-ESI: [M + H]+ calculated: 518.1760 found: 518.1791, C13H16O6N5S. HPLC: 6.89 min.

Computational Studies. All calculations were performed using the Schrodinger Suite. 31 Since compound 17b shares high similarity with the ligands on which we previously published, 7 the same homology model based on the high-resolution antagonist-bound crystal structure of the adenosine A2b receptor (PDB: 4EY2) 27 was used for the docking studies performed here. Based on these proposed structures...
docking poses, we used induced fit docking with core constraints on the pyridopurinedione to dock the different ligands.

**Biology.** [3H]8-Ethyl-4-methyl-2-phenyl-(8R)-4,5,7,8-tetrahydro-1H-imidazo[2,1-i]-purin-5-one ([3H]PSB-11, specific activity 56 Ci mmol⁻¹) was a gift from Prof. C. E. Müller (University of Bonn, Germany). Unlabeled PSB-11, 1-deoxy-1-[6-3- and 2-chloro-N6-(3-iodobenzyl)adenosine-5'-N-methyluranoamide] (2-Cl-IB-MECA) were purchased from Tocris (Abingdon, U.K.). 5'-N-Ethyl-carboxamidoadenosine (NECA) was purchased from Sigma-Aldrich (Steinheim, Germany). Adenosine deaminase was purchased from Boehringer Mannheim (Mannheim, Germany). Bicinchoninic acid (BCA) and BCA protein assay reagents were purchased from Pierce Chemical Company (Rockford, IL). Chinese hamster ovary (CHO) cells stably expressing the human A₁ adenosine receptor (CHOHaA₁) were a gift from Dr. K.-N. Klotz (University of Würzburg, Germany). All other chemicals were obtained from standard commercial sources and were of analytical grade.

**Cell Culture and Membrane Preparation.** Chinese hamster ovary (CHO) cells, stably expressing the human A₁ adenosine receptor (CHOHaA₁), were cultured and membranes were prepared and stored as previously reported. Protein determination was performed based on the bicinchoninic acid (BCA) method.

**Y265F** Site-Directed Mutagenesis. The single tyrosine mutation introduced in TM7 of the hA₁AR was performed with the QuickChange II Site-Directed Mutagenesis system (Stratagene, Heerlen, The Netherlands). The wild-type pcDNA3.1-hA₁AR plasmid DNA with N-terminal 3 x HA-tag was used as a template for polymerase chain reaction (PCR) mutagenesis. Mutant primers for directional PCR product cloning were designed using the online Quickchange primer design program (Agilent Technologies, Santa Clara, CA) and obtained from Eurogentec (Maastricht, The Netherlands). Forward primer used for this procedure was 5'-cagcttcgctttgcctgcgccaggaagctg-3' and the reverse primer was 5'-aggagtagcttacagcagacagacagtgctg-3'. All DNA sequences were verified by Sanger sequencing at LGTC (Leiden, The Netherlands).

**Transient Expression of Wild Type (WT) and Mutant Receptors in CHO-K1 Cells.** CHO-K1 cells were seeded into 150 mm culture dishes to achieve 60% confluence in the presence of 10 mL culture medium consisting of Dulbecco’s modified Eagle’s medium/F12 (1:1) supplemented with 10% (v/v) newborn calf serum, streptomycin (50 µg mL⁻¹), and penicillin (50 IU mL⁻¹). The cells were transfected approximately 24 h later with plasmid DNA (20 µg of DNA/dish) by the PEI method⁶ (PEI/DNA = 3:1) and left for 24 h. Subsequently, the medium was removed and fresh medium containing 5 mM sodium butyrate was added (to enhance the receptor expression level)⁶ and cells were grown for an additional 24 h at 37 °C and 5% CO₂. Membrane preparation followed the procedure described above for the CHO cell membranes stably expressing the hA₁AR.⁷ ³³

**Radioligand Displacement Assay.** Radioligand displacement experiments were performed as in previously published methods. ³³ Membrane aliquots containing 15 µg of protein were incubated in a total volume of 100 µL assay buffer (50 mM Tris–HCl, 5 mM MgCl₂, supplemented with 0.1% 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4) at 25 °C for 120 min. Displacement experiments were performed using six concentrations of competing antagonist in the presence of ~10 nM [³H]PSB-11. Nonspecific binding was determined in the presence of 100 µM NECA and represented less than 10% of total binding. Incubation was terminated by rapid filtration performed on 96-well GF/B filter plates (PerkinElmer, Groningen, the Netherlands) in a PerkinElmer Filtermate-harvester (PerkinElmer, Groningen, the Netherlands). After the filter plate was dried at 55 °C for 30 min, the filter-bound radioactivity was determined by scintillation spectrometry using a 2450 MicroBeta² Plate Counter (PerkinElmer, Boston, MA).

**Radioligand Competition Association Assay.** The competition association assay was performed by incubation of ~10 nM [³H]PSB-11 in the absence or presence of the competing hA₁AR antagonist at its IC₅₀ concentration with membrane aliquots. The amount of receptor-bound radioligand was determined at different time points up to 240 min. Incubations were terminated and samples were obtained as described under the Radioligand Displacement Assay.

**[³S]GTPyS Binding Assay.** The assays were started by adding 15 µg of homogenized CHOHaA₁ membranes in an ice-cold assay buffer to a total volume of 80 µL containing 50 mM Tris–HCl buffer, 5 mM MgCl₂, 1 mM EDTA, 0.05% bovine serum albumin and 1 mM dithiothreitol, 100 mM NaCl, pH 7.4, supplemented with 1 µm GDP and 5 µg saponin. The assays were performed in a 96-well plate format, where stock solutions of the compounds were added to a total volume of 100 µL using an HP D300 Digital Dispenser (Tecan, Männedorf, Switzerland). The final concentration of dimethyl sulfoxide (DMSO) per assay point was ≤0.1%. The basal level of [³S]GTPyS binding was determined in the absence of the ligand, whereas the maximal level of [³S]GTPyS binding was determined in the presence of 10 µM 2-Cl-IB-MECA. For the insurmountability experiments, membrane preparations were preincubated with or without antagonists (0.1-, 1-, 3-, 10-fold Kᵢ values) for 60 min at 25 °C, prior to the addition of 2-Cl-IB-MECA (10 µM to 0.1 nM) and 20 µL [³S]GTPyS (final concentration ~0.3 nM), after which incubation continued for another 30 min at 25 °C. For the surmountability (control) experiments, antagonists (1-, 3-, 10-fold Kᵢ values) and 2-Cl-IB-MECA (10 µM to 0.1 nM) were co-incubated with [³S]GTPyS for 30 min at 25 °C. For all experiments, incubations were terminated and samples were obtained as described under the Radioligand Displacement Assay, using GF/B filters (Whatman International, Maidstone, U.K.).

**Data Analysis.** All experimental data were analyzed using the nonlinear regression curve fitting program GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA). Data from the radioligand displacement assays were fit to one-site binding mode, and the obtained IC₅₀ values were converted into Kᵢ values using the Cheng–Prusoff equation to determine the affinity of the ligands. ³⁷ The observed association rate constants (kₐ) derived from both assays were obtained by fitting association data using one-phase exponential association. The dissociation rate constants were obtained by fitting dissociation data to a one phase exponential decay model. The kₐ values were converted into association rate constants (kₐ) using the equation kₐ = (kₐ - kₐ)/[L], where [L] is the amount of radioligand used for the association experiments. Association and dissociation rate constants for unlabeled compounds were calculated by fitting the data into the competition association model using “kinetics of competitive binding”. ²²

\[
K_A = k_1[L] \times 10^{-9} + k_2 \\
K_S = k_3[H] \times 10^{-9} + k_4 \\
S = \sqrt{(K_A - K_B)^{2} + 4 \times k_1 \times k_3 \times L \times I} \times 10^{-15} \\
K_F = 0.5(K_A + K_B + S) \\
K_S = 0.5(K_A + K_B - S) \\
Q = \frac{B_{max} \times k_2 \times L \times I}{K_F - K_S} \\
Y = Q \times \left( \frac{k_4 - (k_3 - k_2)}{K_F \times K_S} + \frac{k_2 - k_3}{e^{-(k_4 \times X)}} \right) \\
\frac{1}{k_4} \times \frac{k_2}{k_3} \times \frac{k_3}{k_2} \\
\text{where } X \text{ is the time (min), } Y \text{ is the specific [³H]PSB-11 binding (DPM), } k_1 \text{ and } k_4 \text{ are the } k_{on} \text{ (min⁻¹) and } k_{off} \text{ (min⁻¹) of [³H]PSB-11, respectively, } B_{max} \text{ is the total binding (DPM), } I \text{ is the radioligand concentration (nM), and } L \text{ is the concentration of the unlabeled competitor (nM). Association and dissociation rate constants for [³H]PSB-11 (k_1 = 0.281 ± 0.04 × 10^6 M⁻¹ min⁻¹ and k_2 = 0.3992 ± 0.02 min⁻¹) were obtained from Xia et al.}
that, the $k_1$, $k_2$, and $B_{max}$ were calculated, where $k_1$ represents the $k_{on}$ (M$^{-1}$ min$^{-1}$) of the unlabeled ligand, $k_2$ stands for the $k_{off}$ (min$^{-1}$) of the unlabeled ligand and $B_{max}$ equals the total binding (DPM). All competition association data were globally fitted. The residence time (RT, in min) was calculated using the equation RT = 1/$k_{off}$, as $k_{off}$ values are expressed in min$^{-1}$. [8] $K_1$ and $K_2$ binding curves were analyzed by nonlinear regression using "log (agonist) vs response--variable slope" to obtain potency, inhibitory potency, or efficacy values of agonists and antagonists (EC$50$ and $E_{max}$ respectively). In the (in)surmountability assays, Schild $E_{max}$ shift equations were used to obtain Schild-slopes and $pA_2$ values. All experimental values obtained are means of three independent experiments performed in duplicate.

**REFERENCES**

1. Fredholm, B. B.; IJzerman, A. P.; Jacobson, K. A.; Klotz, K. N.; Linden, J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol. Rev. 2001, 53, 527–552.
2. Ali, H.; Cunhamelo, J. R.; Saul, W. F.; Beaven, M. A. Activation of phospholipase-C via adenosine receptors provides synergistic signals for secretion in antigen-stimulated Rbl-2h3 cells - evidence for a novel adenosine receptor. J. Biol. Chem. 1990, 265, 745–753.
3. Borea, P. A.; Varani, K.; Vincenzi, F.; Baraldi, P. G.; Tabrizi, M. A.; Merighi, S.; Gessi, S. The A1 adenosine receptor: history and perspectives. Pharmacol. Rev. 2015, 67, 74–102.
4. Yang, H.; Avila, M. Y.; Peterson-Tantorno, K.; Coca-Prados, M.; Stone, R. A.; Jacobson, K. A.; Civan, M. M. The cross-species A1 adenosine-receptor antagonist MRS 1292 inhibits adenosine-triggered human nonpigmented ciliary epithelial cell fluid release and reduces mouse intraocular pressure. Curr. Eye Res. 2005, 30, 747–754.
5. Brown, R. A.; Spina, D.; Page, C. P. Adenosine receptors and asthma. Br. J. Pharmacol. 2008, 153, 5446–456.
6. Priego, E. M.; Perez–Perez, M. J.; von Frijtag Drabbe Kuenzel, J. K.; de Vries, H.; IJzerman, A. P.; Camarasa, M. J.; Martin-Santamaría, S. Selective human adenosine A1 antagonists based on pyrido[2,1-f]purine-2,4-diones: novel features of hA1 antagonist binding. ChemMedChem 2008, 3, 111–119.
7. Xia, L.; Burger, W. A. G.; van Veldhoven, J. P. D.; Kuiper, B. J.; van Duijl, T. T.; Lenselink, E. B.; Paasman, E.; Heitman, L. H.; IJzerman, A. P. Structure-affinity relationships and structure-kinetics relationships of pyrido[2,1-f]purine-2,4-dione derivatives as human adenosine A1 receptor antagonists. J. Med. Chem. 2017, 60, 7555–7568.
8. Priego, E. M.; Kuenzel, J. V.; IJzerman, A. P.; Camarasa, M. J.; Perez–Perez, M. J. Pyrido[2,1-f]purine-2,4-dione derivatives as a novel class of highly potent human A1 adenosine receptor antagonists. J. Med. Chem. 2002, 45, 3337–3344.
9. Weichert, D.; Greiner, P. Covalent molecular probes for class A G protein-coupled receptors: advances and applications. ACS Chem. Biol. 2015, 10, 1376–1386.
10. Morrison, E. M.; Goodson, S. J.; Edbrooke, M. R.; Harris, C. A. Cloning and characterisation of the human adenosine A2 receptor gene. FEBS Lett. 1996, 384, 243–246.
11. Glukhova, A.; Thal, D. M.; Nguyen, A. T.; Vecchio, E. A.; Jorg, M.; Scammells, P. J.; May, L. T.; Sexton, P. M.; Christopoulos, A. Structure of the adenosine A2A receptor reveals the basis for subtype selectivity. Cell 2017, 168, 867–877.
12. Li, A. H.; Chang, L.; Ji, X. D.; Melman, N.; Jacobson, K. A. Functionalized congeners of 1,4-dihydropyridines as antagonist molecular probes for A2 adenosine receptors. Bioconjugate Chem. 1999, 10, 667–677.
13. Baraldi, P. G.; Cacciari, B.; Moro, S.; Romagnoli, R.; Ji, X. D.; Jacobson, K. A.; Gessi, S.; Borea, P. A.; Spalluto, G.; Flavosulfonyl- and bis-(beta-chloroethyl)amino-phenylamino functionalized pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine derivatives: irreversible antagonists at the human A2A adenosine receptor and molecular modeling studies. J. Med. Chem. 2001, 44, 2735–2742.
14. Ji, X. D.; Gallorodriguez, C.; Jacobson, K. A. Selective agonist affinity label for A2 adenosine receptors. Biochem. Biophys. Res. Commun. 1994, 203, 570–576.
15. Yang, X.; Michels, T. J. M.; de Jong, C.; Soehoudt, M.; Dekker, N.; Gordon, E.; van der Stelt, M.; Heitman, L. H.; van der Es, D.; IJzerman, A. P. An affinity-based probe for the human adenosine A2A receptor. J. Med. Chem. 2018, 61, 7892–7901.
(16) Picone, R. P.; Fournier, D. J.; Makriyannis, A. Ligand based structural studies of the CB1 cannabinoid receptor. J. Pept. Res. 2002, 60, 348–356.

(17) Narayanan, A.; Jones, L. H. Sulfonyl fluorides as privileged warheads in chemical biology. Chem. Sci. 2015, 6, 2650–2659.

(18) Grimster, N. P.; Connelly, S.; Baranaczk, A.; Dong, J. J.; Krasnova, L. B.; Sharpless, K. B.; Powers, E. T.; Wilson, I. A.; Kelly, J. W. Aromatic sulfonyl fluorides covalently kinetically stabilize transphyrin to prevent amyloidogenesis while affording a fluorescent conjugate. J. Am. Chem. Soc. 2013, 135, 5656–5668.

(19) Müller, C. E.; Diekmann, M.; Thorand, M.; Ozola, V. [H]-8-Ethyl-4-methyl-2-phenyl-(8R,4S,7,8-tetrahydro-1H-imidazo[2,1-f]-purin-5-one ([3H]PSB-11), a novel high-affinity antagonist radio-ligand for human A1 adenosine receptors. Bioorg. Med. Chem. Lett. 2002, 12, 501–503.

(20) Weichert, D.; Kruse, A. C.; Manglik, A.; Hiller, C.; Zhang, C.; Hubner, H.; Kobilka, B. K.; Gmeiner, P. Covalent agonists for studying G protein-coupled receptor activation. Proc. Natl. Acad. Sci. U.S.A. 2014, 111, 10744–10748.

(21) Yang, X.; Dong, G.; Michaels, T. J. M.; Lenselink, E. B.; Heitman, L.; Louvel, J.; IJzerman, A. P. A covalent antagonist for the human adenosine A2A receptor. Purinergic Signalling 2017, 13, 191–201.

(22) Motulsky, H. J.; Mahan, L. C. The kinetics of competitive radioligand binding predicted by the law of mass-action. Mol. Pharmacol. 1984, 25, 1–9.

(23) Doormbos, M. L. J.; Wang, X.; Vermond, S. C.; Peeters, L.; Perez-Benito, L.; Trabanco, A. A.; Lavreysen, H.; Cid, J. M.; Heitman, L. H.; Tresadern, G.; IJzerman, A. P. Covalent allosteric probe for the metabotropic glutamate receptor 2: design, synthesis, and pharmacological characterization. J. Med. Chem. 2018, 233–273.

(24) Jörg, M.; Gluhkova, A.; Abdul-Rida, A.; Vecchio, E. A.; Nguyen, A. T. N.; Sexton, P. M.; White, P. J.; May, L. T.; Christopoulos, A.; Scammells, P. J. Novel irreversible agonists acting at the A1 adenosine receptor. J. Med. Chem. 2016, 59, 11182–11194.

(25) van Muijlwijk-Koezen, J. E.; Timmerman, H.; van der Sluis, R. P.; van de Stolpe, A. C.; Menge, W. M. P.; Beukers, M. W.; van der Graaf, P. H.; de Groot, M.; IJzerman, A. P. Synthesis and use of FSCPX, an irreversible adenosine A1 antagonist, as a ‘receptor knockdown’ tool. Bioorg. Med. Chem. Lett. 2001, 11, 815–818.

(26) Strange, P. G. Use of the GTP gamma S ([35S]GTP gamma S and Eu-GTP gamma S) binding assay for analysis of ligand potency and efficacy at G protein-coupled receptors. Br. J. Pharmacol. 2010, 161, 1238–1249.

(27) Liu, W.; Chun, E.; Thompson, A. A.; Chabukov, P.; Xu, F.; Katritch, V.; Han, G. W.; Roth, C. B.; Heitman, L. H.; IJzerman, A. P.; Cherezov, V.; Stevens, R. C. Structural basis for allosteric regulation of GPCRs by sodium ions. Science 2012, 337, 232–236.

(28) Picone, R. P.; Khandolkar, A. D.; Xu, W.; Ayotte, L. A.; Thakur, G. A.; Hurst, D. P.; Abood, M. E.; Reggio, P. H.; Fournier, D. J.; Makriyannis, A. (–)-7′-Isotiocyanato-11-hydroxy-1′,3′-dimethylheptyltetrahydrocannabinol (AM841), a high-affinity electrophilic ligand, interacts covalently with a cysteine in helix six and activates the CB1 cannabinoid receptor. Mol. Pharmacol. 2005, 68, 1623–1635.

(29) Singh, J.; Petter, R. C.; Baillie, T. A.; Whitty, A. The resurgence of covalent drugs. Nat. Rev. Drug Discovery 2011, 10, 307–317.

(30) Soethoudt, M.; Stolze, S. C.; Westphal, M. V.; van Stralen, L.; Martella, A.; van Rooden, E. J.; Guba, W.; Varga, Z. V.; Deng, H.; van Kasteren, S. I.; Grether, U.; IJzerman, A. P.; Pacher, P.; Carreira, E. M.; Overkleeft, H. S.; Ioan-Facsinay, A.; Heitman, L. H.; van der Stelt, M. Selective photoaffinity probe that enables assessment of cannabinoid CB1 receptor expression and ligand engagement in human cells. J. Am. Chem. Soc. 2018, 140, 6067–6075.

(31) Goebel, U.; Siepe, M.; Schwer, C.; Schlensak, C.; Loop, T. Sevoflurane-induced preconditioning in the isolated mouse heart is mediated by A1- and A2A-adenosine receptors. Eur. J. Anaesthesiol. 2010, 27, 72.