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Novel Irreversible Agonists Acting at the A_1 Adenosine Receptor

Manuela Jörg[†,§], Alisa Glukhova,[‡,§], Alaa Abdul-Ridha,[‡] Elizabeth A. Vecchio,[‡] Anh T.N. Nguyen,[‡] Patrick M. Sexton,[‡] Paul J. White,[‡] Lauren T. May,[‡] Arthur Christopoulos,[‡,*] Peter J. Scammells[†,*]

[†]*Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences; Monash University, Parkville, VIC 3052, Australia;* [‡]*Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences Biology & Department of Pharmacology, Monash University, Parkville, VIC 3052, Australia*

[§]These authors contributed equally to this manuscript

ABSTRACT: The A_1 adenosine receptor (A_1AR) is an important G protein-coupled receptor that regulates a range of physiological functions. Herein we report the discovery of novel irreversible agonists acting at the A_1AR, which have the potential to serve as useful research tools for studying receptor structure and function. A series of novel adenosine derivatives bearing electrophilic substituents was synthesised and four compounds, **8b**, **15a**, **15b** and **15d**, were shown to posses similar potency and efficacy to the reference high efficacy agonist, NECA, in an assay of ERK1/2 phosphorylation assay. Insensitivity to antagonist addition in a real-time, label-free, xCELLigence assay was subsequently used to identify compounds that likely mediated their agonism through an irreversible interaction with the A_1AR. Of these compounds, **15b** and **15d** were more directly validated as irreversible agonists of the A_1AR using membrane-based [^{3}H]DPCPX and [^{35}S]GTPγS binding experiments.

■ INTRODUCTION

Adenosine receptors (ARs) are a family of G protein-coupled receptors (GPCRs) that are distributed broadly throughout the body and are linked to a number of physiological functions.[1, 2] The initiation of these functions occurs upon the activation of four different AR subtypes; namely the A_1, A_{2A}, A_{2B} and A_3ARs.[3, 4] The A_1AR is found in spinal cord, central nervous system, heart, and adipose tissue, and is associated with a wide range of effects including the ability to slow down heart rate, inhibit neurotransmitter release and inhibit lipolysis.[1, 5-8] The A_1AR has been recognized as a promising drug target for a variety of disorders, such as neuropathic pain[9, 10] and cardiac ischemia-reperfusion injury.[11, 12] Despite recent breakthroughs in GPCR structural biology,[13-17] and numerous crystal structures solved for the A_{2A}AR, there is surprisingly limited information available about the structural and functional features of the A_1AR.

Irreversible probes/ligands are valuable tools for studying GPCRs both *in vitro* and *in vivo*.[18-22] Of note, recent work has demonstrated advantages that covalent ligands could hold for GPCR structural biology studies, which is based on the irreversible probe's ability to form a stable and long living ligand-receptor complex with the targeted GPCR.[23-27] Irreversible agonists and antagonists targeting the A_1AR subtype have been reported in the past, and the chemical structures of the best-studied ligands are illustrated in Figure 1. The xanthine-based irreversible antagonists 1,3-dipropyl-8-isothiocyanatophenyl(aminothiocarbonyl(2-aminoethylaminocarbonyl(4-methyloxy (phenyl)))xanthine (DITC-XAC) and 8-cyclopentyl-3-(3-((4-fluorosulfonylbenzoyl)oxy)propyl)-1-propylxanthine (FSCPX), as well as variants thereof, have been part of extensive investigations.[28-30] Interrogation of linker attachment positions, irreversible groups, as well as a range of linker lengths and types, suggested that the 3- and 8-postions of the xanthine scaffold are suitable for the attachment of the irreversible group to maintain high affinity and selectivity for the A_1AR subtype.

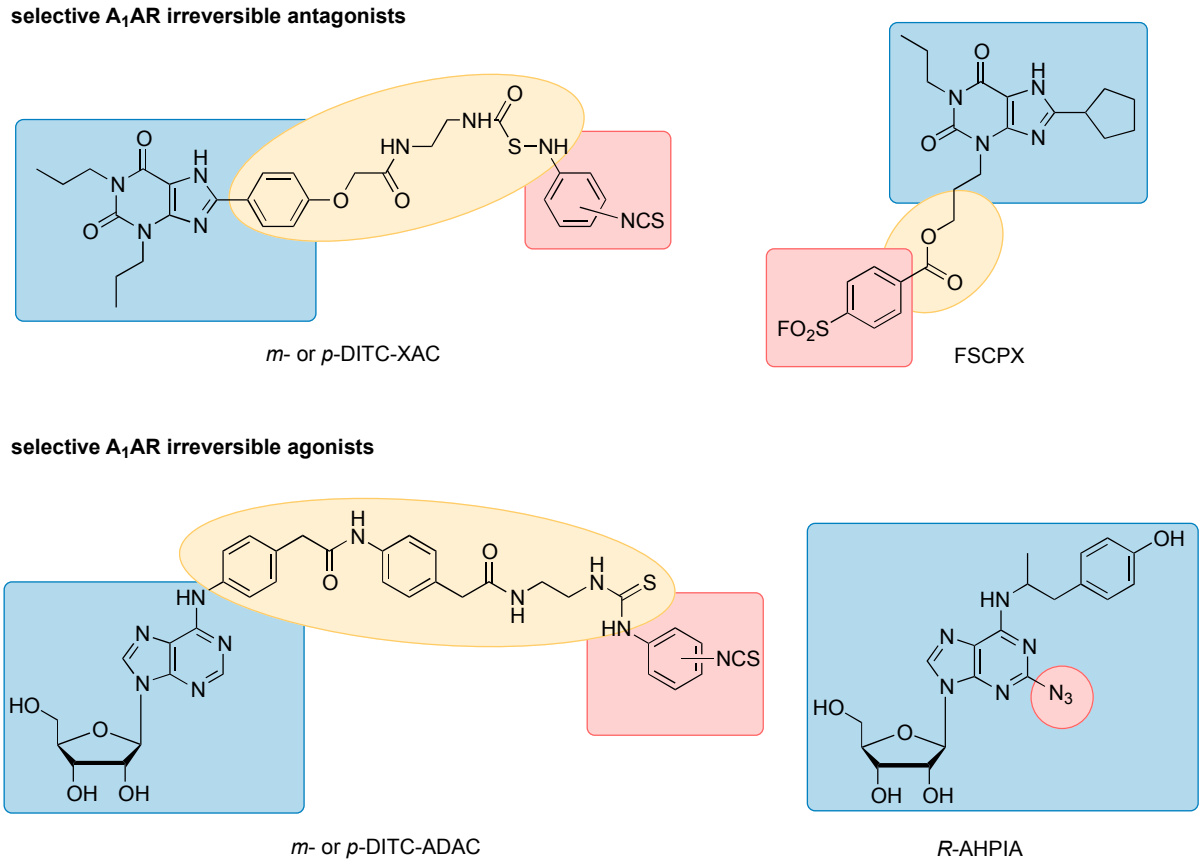


Figure 1. Chemical structures of irreversible xanthine-based A_1AR antagonists DITC-XAC and FSCPX as well as the adenosine-based agonists DITC-ADAC and *R*-AHPIA that haven been previously described in the literature (blue: pharmacophore; red: reactive group; orange: linker).

Although not as well-studied, some irreversible probes capable of activating the A_1AR have also been developed; namely, N^6[4-[[[4-[[[[2-[[[(*p*-(*m*)-isothiocyanatophenyl)amino]thiocarbonyl] amino]ethyl]amino]carbonyl]methyl]aniline]carbonyl]methyl]phenyl]adenosine (*p*- and *m*-DITC-ADAC)[31] and *R*-2-azido-N^6-*p*-hydroxyphenylisopropyladenoisine (*R*-AHPIA).[32, 33] Both probes are based on an adenosine scaffold; however, they are structurally and functionally different from each other. *R*-AHPIA forms its irreversible bond with the receptor upon photoactivation by UV light, due to the incorporation of a photoreactive azide group in the 2-postion of the adenosine scaffold. In contrast, *p*- and *m*-DITC-ADAC form a direct covalent bond with the receptor via a chemoreactive isothiocyanate group attached to a lengthy 2-(4-aminophenyl)acetic acid-based linker that is incorporated into the N^6-position of the adenosine scaffold. Both *p*- and *m*-DITC-

ADAC exhibited high (sub-nanomolar) affinity at the A_1AR, as well as, 200- to 600-fold selectivity over the A_2ARs.[31] In addition, both ligands were capable of activating the A_1AR to yield persistent cAMP responses in functional assays,[34] but limited progress has been made since these early studies, and it remains unknown as to whether such probes can also be used as useful tools to study A_1AR structural biology.

R-AHPIA possesses an aryl azide reactive group, which needs to be activated by photolysis to generate a covalent bond with the receptor. Even though *p*- and *m*-DITC-ADAC are known irreversible probes based on a chemoreactive isothiocyanate group, these probes have a relatively long and flexible linker. In view of the current interest in development of probes to facilitate GPCR structural biology studies, we have chosen to investigate the effects of different linkers in length and flexibility on the pharmacology of novel A_1AR irreversible agonists, as well as their effects on receptor thermostability. Specifically, we present the synthesis, characterization and pharmacological evaluation of 15 novel adenosine analogs containing a reactive functional group capable of forming a covalent bond with the receptor. A range of chemoreactive groups with different types of action were incorporated; namely bicyclo[2.2.2]octa-2,5-dienes (Michael acceptors), isothiocyanates and fluorosulfonylphenyl groups. The reactive groups were introduced via the N^6-position of the adenosine scaffold, since structure-activity relationship data[35-38] as well as the irreversible ligands *p*/*m*-DITC-ADAC[31] have shown that changes to this position are well tolerated and have no detrimental effect on the affinity and selectivity at the A_1AR. Since the formation of a covalent bond with chemoreactive irreversible probe is only possible by positioning the chemoreactive functionality in close proximity to a nucleophilic amino acid residue, three series of irreversible probes were synthesised incorporating linear linkers with 2-, 6- and 10-carbon in length. In addition, given that many high affinity A_1AR ligands have either an aromatic or another cyclic moiety at the N^6-position of the adenosine scaffold, an aromatic linker was also examined.[35, 37, 38]

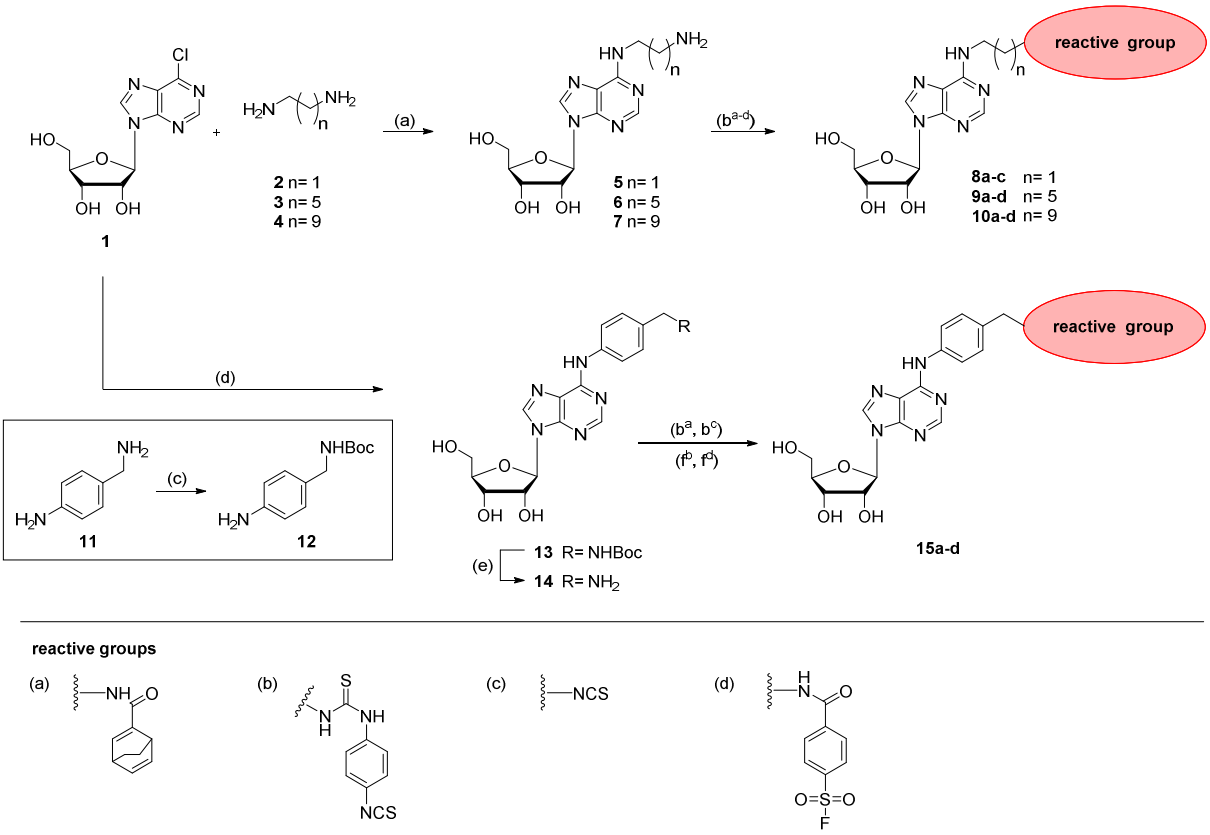
■ RESULTS AND DISCUSSION

Chemistry. The synthesis of the putative irreversible A_1AR agonists was based on a two-step procedure; the attachment of a linker unit to the adenosine scaffold, followed by the introduction of a chemoreactive group to the linker unit (Scheme 1). The linear linkers were introduced via a substitution reaction of the respective diaminoalkane **2-4** with 6-chloropurine riboside (**1**) in ethanol and triethylamine. The resulting intermediates **5-7** were obtained in good yields (68-90%) after 3 hours at reflux. In the case of the 4-aminobenzylamine linker (**11**), the more nucleophilic aliphatic amino group was first protected with a Boc group under standard conditions. Next, the Boc-protected linker **12** was attached to the adenosine scaffold in 88% yield using the previously described conditions for the substitution reaction with 6-chloropurine riboside (**13**). The lower nucleophilicity of this aryl amine contributed to the increased reaction time of 26 h compared to 3 h for the alkyl linkers. The Boc protecting group was removed with trifluoroacetic acid in dichloromethane to furnish **14**, noting that it was important to avoid hydrochloric acid since it initiated cleavage of the sugar moiety from the purine unit.

The chemoreactive bicyclo[2.2.2]octa-2,5-diene and 4-fluorosulfonylphenyl moieties were introduced using standard peptide bond coupling conditions (BOP and triethylamine). The bicyclo[2.2.2]octa-2,5-diene (**8a-10a**, **15a**) analogs were obtained in reasonable yields ranging between 11-42%, whereas the reactions with 4-(fluorosulfonyl)benzoic acid resulted in the formation of several by-products which made the purification extremely challenging. Accordingly, compounds **9d-10d** and **15d** were only isolated in very low yield (1-10%). The 4-fluorosulfonylphenyl product with the diaminoethyl linker could not be isolated with high purity (>95%) due to interference from other side products. It is worth mentioning that the introduction of chemoreactive groups with acid halides instead of carboxylic acids failed due to side reactions on the adenosine scaffold. Nevertheless, by simply changing the base from triethylamine to 2,6-lutidine we were able significantly improve the yield of **15d** from 1% to 68%, as no side products were formed under this conditions and purification by column chromatography was not necessary.

To afford the 1-(4-isothiocyanatophenyl)thiourea (**8b-10b**, **15b**) and isothiocyanate (**8c-10c**, **15c**) analogs, either 1,4-phenylene diisothiocyanate or *N*,*N*'-thiocarbonyldiimidazole was reacted with the amine linker of intermediates **5-7** and **14** in dichloromethane at room temperature. In case of the isothiocyanate analogs **8c-10c** and **15c** it was important to use only one equivalent of the reagent to avoid the formation of further side products, while for the 1-(4-isothiocyanatophenyl)thiourea analogs (**8b-10b**, **15b**), an excess of reagent (3 equivalents) was used without the earlier described implications. The yields of the synthesized isothiocyanates were generally very modest, especially the two analogs with the 4-aminobenzylamine linker (**15b** and **15c**) which were obtained in extremely poor yields, possibly due to the low solubility of intermediate **14** in dichloromethane. The synthesis of **15b** was optimized by using DMF as the solvent to improve the conversion of the starting material to product as well as 1.1 equivalent of reagent and a reaction concentration of 10 mg of starting material/mL of solvent to minimize the formation of side products, including the dimer product. Preparative RP-HPLC was used for purification avoiding silica gel column chromatography (normal phase), which contributed to the degradation of **15b**. The optimized protocol allowed the isolation of **15b** in 17% yield (previously 1%).

Scheme 1. Synthesis of the adenosine A_1 receptor irreversible agonists[a]



[a]**Reagents and conditions:** (a) respective diaminoalkane, Et_3N, EtOH, reflux, 3 h, 68-90%; (b^a) bicyclo[2.2.2]octa-2,5-diene-2-carboxylic acid, BOP reagent, Et_3N, DMF, rt, 20 h, 11-42%; (b^b) 1,4-phenylene diisothiocyanate, CH_2Cl_2, rt, 5-22 h, 18-35%; (b^c) *N*,*N*'-thiocarbonyldiimidazole, CH_2Cl_2, rt, 43 h, 3-35%; (b^d) 4-(fluorosulfonyl)benzoic acid, BOP reagent, Et_3N, DMF, rt, 20 h, 8-10%; (c) Boc anhydride, THF, rt, 5 h, 94%; (d) **12**, Et_3N, EtOH, reflux, 26 h, 88%; (e) TFA, CH_2Cl_2, rt, 2 h, followed by 1 M NaOH, 70%; (f^b) 1,4-phenylene diisothiocyanate, DMF, rt, 2 h, 17%; (f^d) 4-(fluorosulfonyl)benzoic acid, BOP reagent, 2,6-lutidine, DMF, rt, 1 h, 68% .

Pharmacology. To assess the biological activity of the newly synthesised compounds, assays of ERK1/2 phosphorylation (pERK1/2), a downstream signalling pathway linked to receptor activation, were performed in FlpInCHO cells stably expressing the human A_1AR.[39] No effects on pERK1/2 were noted for any of the compounds in parental FlpInCHO cells not transfected with the A_1AR (data not shown). Concentration-response curves were constructed at the peak-response time, identified from time-course experiments as 5 min of agonist exposure. Analysis of

concentration-response curves allowed for the estimation of agonist potency and maximal pERK1/2 stimulated in the presence of each agonist (Table 1 and Figure 2). The maximal agonist response was not significantly different from that of NECA for any of the compounds tested ($p>0.05$, one-way ANOVA, Dunnett's post hoc test), indicating that these ligands acted as full agonists of the A_1AR in the pERK1/2 assay. Despite most compounds being essentially equally effective, greater variations were noted in their potencies. Only four compounds, **8b**, **15a**, **15b** and **15d**, exhibited low-nanomolar potencies that were not significantly different to NECA (Table 1 and Figure 2), while the potencies of all other compounds were significantly lower ($p<0.05$, one-way ANOVA, Dunnett's post hoc test). Interestingly, **8b**, **15a**, **15b** and **15d** all possess a rigid linker composed of either an aromatic group (**15a**, **15b** and **15d**) or a short carbon chain (**8b**) between the adenosine moiety and the irreversible reactive group. As such, results from the current series of compounds suggest compact chemical structures are beneficial for generating high potency A_1AR agonists.

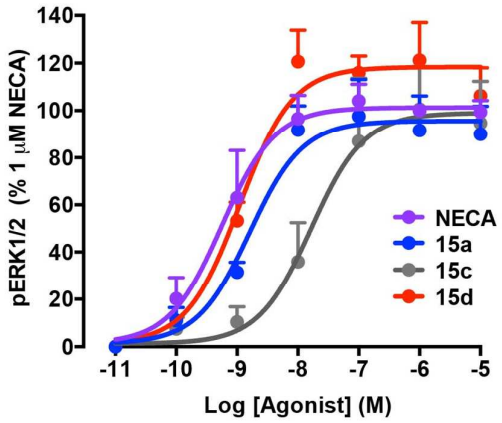


Figure 2. Functional pERK1/2 concentration-response curves to test compounds **15a**, **15c**, **15d** and NECA in A_1AR expressing FlpInCHO cells. Data points represent the mean and standard error of three independent experiments performed in duplicate.

Table 1: Effect of test compounds on ERK1/2 phosphorylation in human A_1AR-expressing FlpIn CHO cells

Compound	pEC_{50}	Maximal response (% 1 μM NECA)
NECA	9.3 ± 0.4	103 ± 2
8a	NA	NA
8b	8.5 ± 0.1	82 ± 18
8c	6.8 ± 0.1****	82 ± 9
9a	7.8 ± 0.1****	93 ± 17
9b	7.7 ± 0.1****	95 ± 5
9c	8.2 ± 0.1**	82± 13
9d	6.7 ± 0.2****	75 ± 12
10a	7.6 ± 0.4****	49 ± 23
10b	7.6 ± 0.1****	87 ± 16
10c	7.1 ± 0.2****	84 ± 21
10d	7.1 ± 0.2****	119 ± 13
15a	8.8 ± 0.1	96 ± 14
15b	8.8 ± 0.1	113 ± 3
15c	7.7 ± 0.3****	101 ± 18
15d	9.0 ± 0.1	119 ± 12

NA - not applicable; values represent the mean ± SEM from three independent experiments performed in duplicate.

** Significantly different ($p \leq 0.001$) compared to pEC_{50} or maximum response of NECA (One-way ANOVA, Dunnett's post-hoc test)

**** Significantly different ($p \leq 0.0001$) compared to pEC_{50} or maximum response of NECA (One-way ANOVA, Dunnett's post-hoc test)

The pERK1/2 assay identified all novel compounds to behave as agonists at the A_1AR, however these experiments could not differentiate between reversible or irreversible receptor activators. To address this, we next used a label-free xCELLigence assay capable of real-time monitoring of A_1AR-mediated changes in cellular impedance over prolonged periods of time,[40] including the ability to determine whether activation of the receptor can be reversed by subsequent addition of an A_1AR antagonist. A_1AR expressing FlpInCHO cells were plated on an E-plate™ one day before the experiment and the change in impedance assessed for 1.5 h upon exposure to 1 μM compound (or buffer as a control). The change in impedance in the presence of 1 μM agonist or vehicle was normalised within each experiment to the initial peak NECA response (5 min). The overall profile

of the compound response over time was compared to that of the reference agonist, NECA (Figure 3A). Similarly to NECA, each test compound stimulated a positive change in impedance relative to the buffer control, further supporting the suggestion that these compounds promote A_1AR activation. Of note, only **10c** and **15b**–**15d** promoted a maximal change in cellular impedance that was not significantly different from that of NECA (one-sample t-test; Figure 3B). These results suggest that within this assay, the remaining compounds were lower potency and/or partial agonists relative to NECA.

To probe for potential irreversibility of the agonistic effect of the test compounds, the sensitivity of the cellular impedance response to the subsequent addition of a high concentration of A_1AR selective antagonist was assessed. After the cells were incubated with the compounds for 1.5 h, the potent and selective A_1AR antagonist, SLV320 (**16**)[41] was added at 1 μM. Competition of **16** with reversible ligands would be expected to result in a change (reduction) in impedance, whereas cells incubated with irreversible compounds would not be expected to show a significant change in impedance upon antagonist exposure. As expected, a significant decrease in cellular impedance was observed upon antagonist addition to cells pre-incubated with NECA (Figure 3C). Because the maximal drop in NECA impedance values was observed 16 min after adding **16**, all cellular responses were normalised to those of NECA observed 16 min after antagonist addition. As shown in Figure 3D, cells that had been exposed to **8b** or **15b**–**15d** were significantly resistant to reversal of their effect on cellular impedance upon addition of **16** ($p<0.05$, one-sample t-test), consistent with an irreversible mode of receptor activation. Compounds containing a 4-fluorosulphonylbenzoyl moiety or an aryl isothocyanate (similar to that of DITC-ADAC) appeared to be the most successful in showing resistance to reversal by **16**. Collectively, **15b** and **15d** emerged as our most prominent compounds, because they were most potent and efficacious in the pERK1/2 assay and could not be inhibited by **16** in the real time xCELLigence assay, suggesting irreversible binding to the A_1AR.

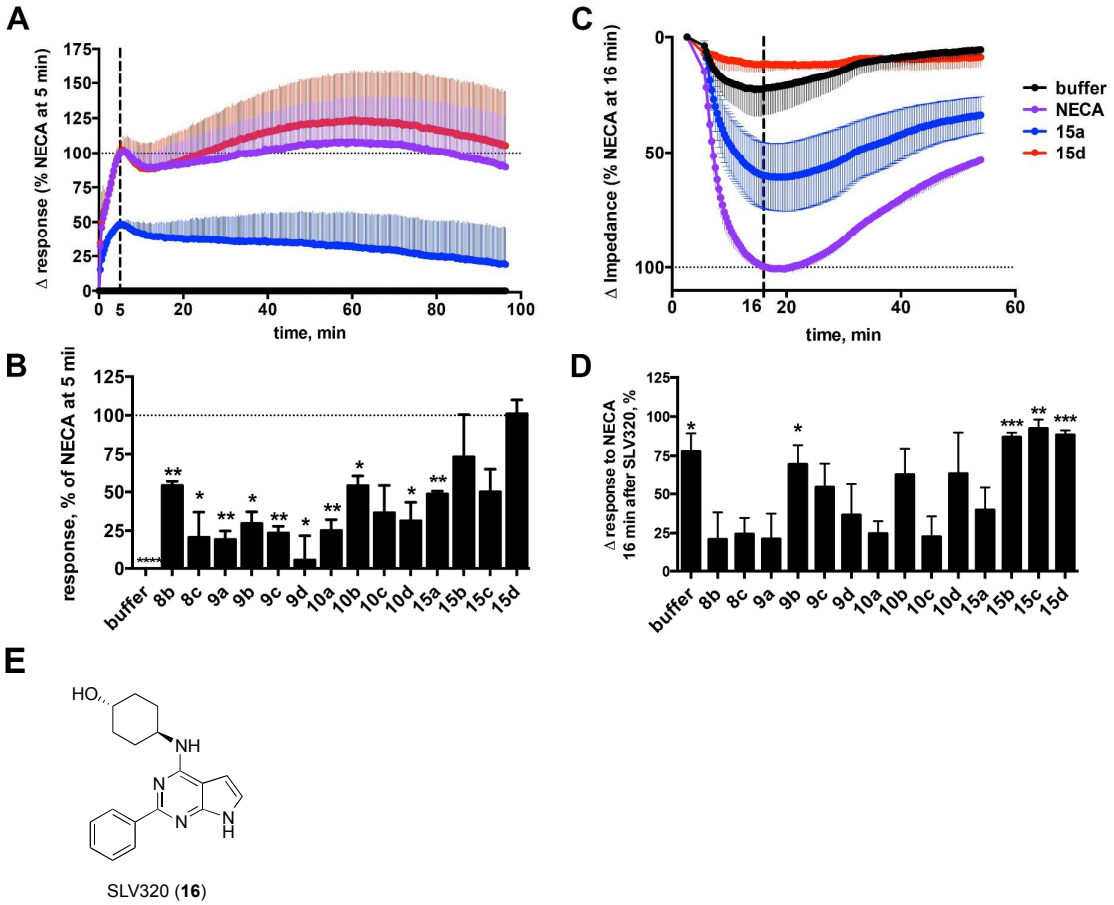


Figure 3. Test for irreversible binding using a real-time xCELLigence assay. (A) Changes in cellular impedance over time caused by 1 μM of **15a**, **15d** or NECA. Data points were normalised to the NECA response observed at 5 min. (B) Normalised cell responses 5 min after ligand addition. (C) Changes in impedance over time caused by the addition of **16** (1 μM) to the cells pre-treated with indicated compounds for 100 min. Data points were normalised to NECA response 16 min after **16** addition (indicated by the dashed line). (D) Normalised cell responses 16 min after **16** addition following pre-treatment with ligands from (B). Values represent the mean ± SEM from three independent experiments performed in duplicate.* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$,**** $p \leq 0.0001$ (one-sample t-test, comparing means to 100%). (E) Chemical structure of **16**.

To more directly validate the likelihood of an irreversible mode of binding to, and activation of, the A_1AR by **15b** and **15d**, we performed additional biochemical assays. First, these compounds were tested for their ability to covalently bind A_1ARs using equilibrium radioligand binding assays. A_1AR-FlpInCHO cell membranes were incubated with the compounds of interest for 1 h at 37°C, and, after extensive washing, the specific binding of the orthosteric antagonist, [^{3}H]DPCPX, was determined (Figure 4A). As expected, following our washing protocol, membranes pre-treated with

NECA bound [^{3}H]DPCPX with a similar B_{max} as membranes pre-incubated with buffer (108 ± 11% and 93 ± 10%, respectively; Figure 4A). In contrast, pre-treatment of membranes, followed by extensive washout, with the irreversible A_1AR antagonist, FSCPX,[42] prevented subsequent [^{3}H]DPCPX binding (2 ± 5% of the B_{max} for membranes alone). Importantly, both **15b** and **15d** behaved similarly to FSCPX in preventing [^{3}H]DPCPX binding after the same pre-treatment and washout protocol (to 8 ± 4% and 16 ± 5%, respectively), indicating that these compounds remained associated with the A_1AR after the extensive membrane washing procedure.

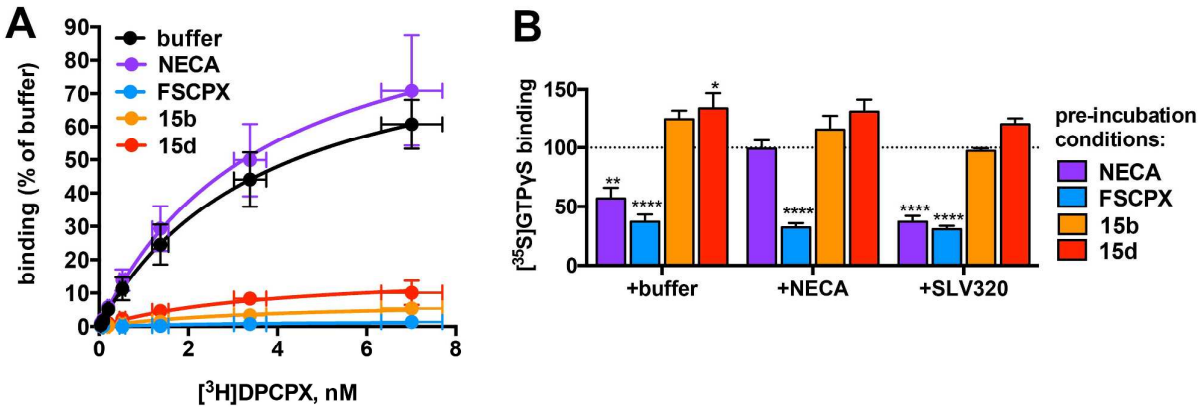


Figure 4. Radioligand (A) and [^{35}S]GTPγS binding assays (B) on extensively washed membranes following pre-incubation with 10 μM test compounds. In (B) membranes were stimulated with buffer or 10 μM NECA or **16** following pre-incubation and washing procedure. Data points were normalised to [^{3}H]DPCPX B_{max} (A) or 10 μM NECA-induced [^{35}S]GTPγS binding (B) for membranes pre-incubated with buffer alone, then washed. Values represent the mean ± SEM from three independent experiments performed in duplicates. ** $p \leq 0.001$, **** $p \leq 0.0001$ (One-way ANOVA, Dunnett's post hoc, compare to [^{35}S]GTPγS binding of membranes treated with NECA following NECA pre-incubation).

Second, because formation of an irreversible bond could sometimes convert a compound exhibiting agonist properties into an antagonist, e.g., as previously reported for acetylcholine mustard at muscarinic acetylcholine receptors,[43] we examined if **15b** and **15d** remained agonists after the formation of a covalent bond, and, as such, promoted persistent A_1AR-mediated guanine nucleotide exchange activity. To test this, we monitored the binding of [^{35}S]GTPγS to activated G proteins using the same A_1AR-FlpInCHO cell membranes as used in the [^{3}H]DPCPX binding assay.

After membranes were pre-treated with test compounds and washed extensively, they were stimulated with reaction buffer, NECA, or **16** (Figure 4B) and [^{35}S]GTPγS incorporation was measured. The amount of incorporated [^{35}S]GTPγS was normalised to the response of control membranes pre-incubated with vehicle, subjected to the same washing protocol and then stimulated with NECA (defined as 100%). As expected, membranes pre-incubated with NECA followed by washout responded to stimulation in the same way as vehicle-treated membranes. The basal response, measured in the absence of drug (41 ± 3% and 57 ± 9%, for the control and NECA pre-treatments, respectively), could still be further increased by subsequent addition of 10 μM NECA, but not 10 μM compound **16** (99 ± 7% and 37 ± 5%, respectively). Membranes pre-treated with FSCPX showed similar basal levels of [^{35}S]GTPγS binding compared to vehicle controls (37 ± 6%), but were completely unresponsive to NECA stimulation (32 ± 4%), as expected for an irreversible antagonist occupying the A_1AR orthosteric site. In contrast, the basal level of [^{35}S]GTPγS binding in membranes pre-treated with **15b** or **15d**, followed by washout, was similar to that of control samples exposed to 10 μM NECA (124 ± 7 and 134 ± 13 %, respectively), indicating persistent and maximal activation. Moreover, neither addition of NECA or **16** had any effect on this persistent activity in **15b** or **15d** pre-treated membranes, indicating that the persistent effect is likely due to irreversible binding.

Interestingly, evidence gathered from multiple GPCR crystallography studies over recent years suggests a strong correlation between the ability of a ligand to stabilize a receptor and the ability to form well diffracting crystals.[44] In addition, receptor stabilisation by different classes of ligand could be very beneficial in assisting the discovery of conformation-specific antibodies.[23, 45] Thus, to determine if our best compounds, **15b** and **15d**, enhanced A_1AR thermo-stability, we utilized a ThermoFluor assay[46, 47] using purified A_1ARs; as shown in the Supporting Information, the purified receptor retained its native pharmacological properties. In the absence of drugs, the A_1AR was extremely unstable, as evidenced by high fluorescence at the start of the melting curve and a shallow slope of the transition (Figure 5A). All tested compounds stabilised the A_1AR to some

degree (Figure 5B). For instance, NECA modestly increased apo-A_1AR melting temperature (T_m) by 4° (35.2 ± 1.6 and 38.7 ± 0.4°C, respectively), as did **8b**, **9b**, **9d**, **10c** and **15a**. However, only three compounds, **15b**–**15d** significantly increased the receptor T_m substantially above the effect of NECA ($p<0.05$, one-way ANOVA, Dunnett's post hoc test), to 46.6 ± 1.2, 42.7 ± 0.3 and 46.7 ± 0.7°C, respectively, highlighting the importance of an aromatic linker between the adenosine moiety and an irreversible group - not only for achieving high agonist potency, but also for receptor stabilisation; only FSCPX yielded a greater degree of thermo-stabilization. While only the binding of G proteins or G protein-mimicking nanobodies can achieve close to full activation of the receptor, recent studies of the β_2 adrenergic receptor activation using molecular dynamics simulations, ^{19}F-fluorine NMR and double electron-electron resonance spectroscopy have revealed that there are multiple intermediate steps, and that these can indeed be stabilized by pseudo-irreversible ligands.[48, 49] Thus, it is reasonable to suggest that, similar to the β_2 adrenergic receptor, binding of our irreversible agonists, **15b** or **15d**, to the A_1AR increases the population of active-like intermediate states, which explains the increase in observed thermostability and identifies these compounds as useful tools for future structural biology studies of this important GPCR.

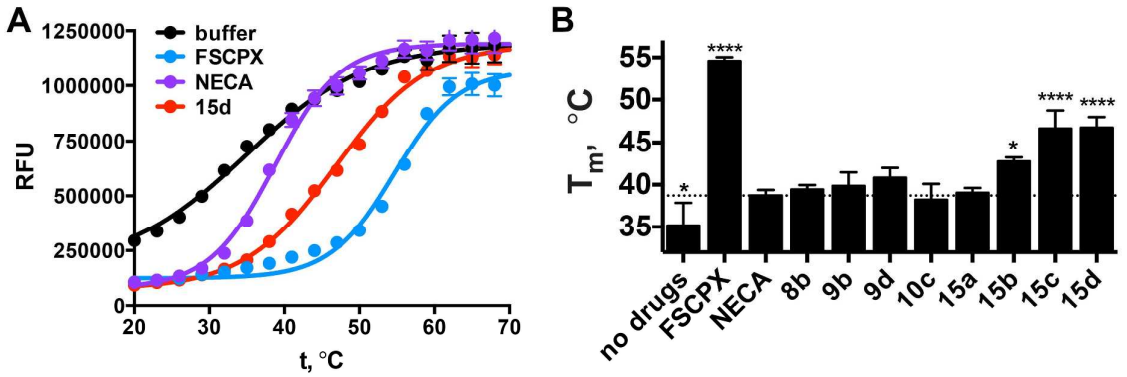


Figure 5. Test compounds induce A_1AR stabilisation in a ThermoFluor assay. (A) A_1AR melting curves were monitored by the increase in CPM dye fluorescence due to receptor unfolding. 10 μM of NECA and test compounds increased receptor melting temperature (T_m), producing a right-shift of the melting curves. (B) T_m values of A_1AR in the presence of 10 μM of test compounds. Data points represent the mean ± SEM from three independent experiments performed in duplicates. $^{*}p\leq0.05$, $^{****}p\leq0.0001$ (One-way ANOVA, Dunnett's post hoc test, compared to T_m in the presence

of NECA).

Finally, albeit not the major focus of the current study, we selected the three key irreversible agonists that improved the thermostability of the A_1AR and assessed their activity at the other AR subtypes (A_{2A}, A_{2B} and A_3). For this purpose, we utilized a common downstream pathway linked to activation of all AR subtypes, namely cAMP accumulation, which is inhibited by the A_1 and A_3ARs, but stimulated by the A_{2A} and A_{2B} ARs[3]. As shown in Figure 6, all three compounds were full agonists in this assay when compared to the reference agonist, NECA; no responses to any compound were noted in parental FlpInCHO cells that did not express any AR subtype (not shown). Despite displaying similar degrees of maximal agonist effect, differences in potencies were noted between the compounds. In particular, **15d** showed a significantly greater selectivity for the A_1AR than any of the other subtypes, whereas **15b** and **15c** showed similar potencies across all subtypes (Table 2). Although beyond the scope of the current study, this finding suggests that these compounds can also be used for structural or chemical biology studies at other AR subtypes if an irreversible mode of interaction can be validated at those subtypes. In contrast, the high selectivity of **15d** for the A_1AR identifies this compound as a novel tool that can be used specifically for targeting the A_1AR over other subtypes, perhaps even in native tissues or cells that express a mixture of ARs.

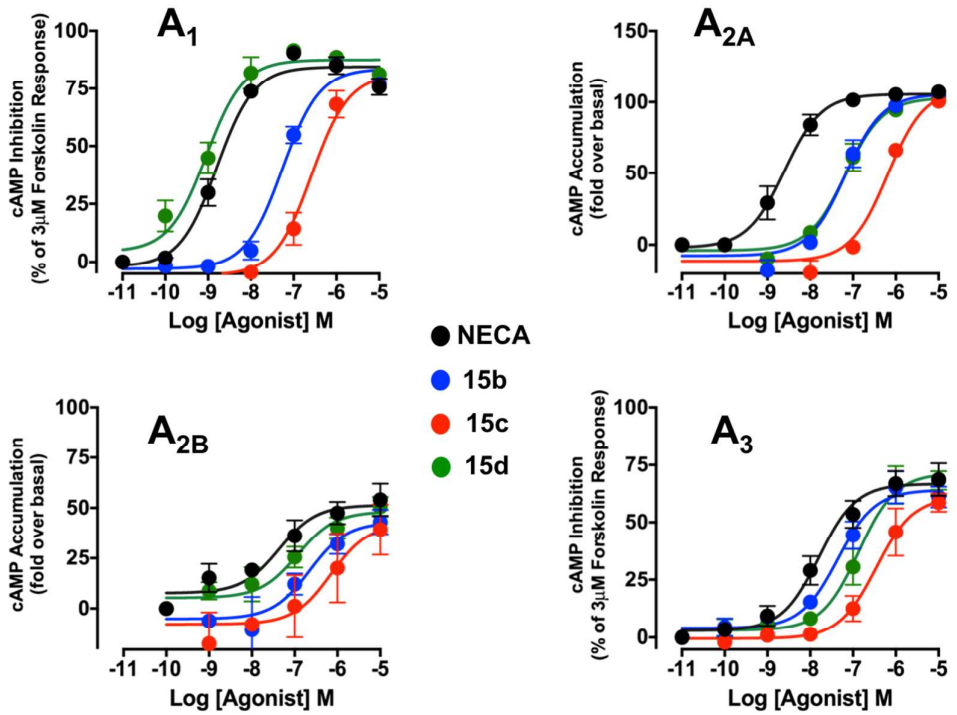


Figure 6. Adenosine receptor subtype selectivity of selected agonists. Functional cAMP concentration-response curves to NECA or test compounds **15b**, **15c** or **15d** at the indicated AR subtype stably expressed in FlpInCHO cells. Data points represent the mean ± SEM of three to four independent experiments performed in duplicate.

Table 2: AR receptor subtype potency (pEC_{50}) values of selected test compounds on cAMP accumulation or inhibition in human AR-expressing FlpIn CHO cells

Compound	AR Subtype			
	A_1	A_{2A}	A_{2B}	A_3
NECA	8.79 ± 0.09	8.60 ± 0.09	7.37 ± 0.32*	7.80 ± 0.15
15b	7.26 ± 0.09	7.17 ± 0.11	6.65 ± 0.32	7.35 ± 0.15
15c	6.57 ± 0.11	6.19 ± 0.13	6.15 ± 0.32	6.46 ± 0.17
15d	9.04 ± 0.12**	7.17 ± 0.09	6.93 ± 0.24	6.87 ± 0.14

Values represent the mean ± SEM from three to four independent experiments performed in duplicates.

* Significantly different ($p < 0.05$) from pEC_{50} value at the A_1 and A_{2A} ARs.

**Significantly different ($p < 0.01$) from pEC_{50} value at A_{2A}, A_{2B} and A_3 ARs. (One-way ANOVA, Tukey's post-hoc test)

■ CONCLUSIONS

We synthesized and evaluated a series of novel adenosine analogs bearing electrophilic substituents, aiming to identify novel agonists capable of irreversible binding and persistent activation the A_1AR. Four compounds, **8b**, **15a**–**15b** and **15d**, showed similar potency and efficacy to our reference (reversible) agonist, NECA, in a pERK1/2 assay. Sensitivity to antagonist addition in a real-time, label-free xCELLigence assay was subsequently used to identify compounds that likely mediated their agonism through an irreversible interaction with the A_1AR. Compounds **8b** and **15b**–**15d** were insensitive to the addition of a high concentration of antagonist, compound **16**, indicating pseudo- or complete irreversibility. Two compounds, **15b** and **15d**, which emerged as most promising from both the pERK1/2 and xCELLigence assays, were more directly validated as irreversible agonists of the A_1AR using membrane-based [^{3}H]DPCPX and [^{35}S]GTPγS binding experiments. Both ligands bound tightly to A_1ARs in FlpInCHO cell membranes and could not be displaced even after extensive washing, as evidenced by the inability of [^{3}H]DPCPX to bind to these membranes, and the persistent maximal binding of [^{35}S]GTPγS to activated G proteins in a manner resistant to both agonist or antagonist addition. To our knowledge, no A_1AR agonists have been examined for the ability to promote receptor thermostability to date, although such a property could prove extremely beneficial for structural biology studies. Thus, we also found that both **15b** and **15d** stabilised purified, detergent-solubilised A_1ARs in a ThermoFluor assay to a significantly higher degree than NECA. Compound **15d** also shows a high degree of selectivity for the A_1AR subtype relative to other ARs, and thus may be prove useful for additional chemical biology applications in native tissues or cells that express mixtures of AR subtypes.

■ EXPERIMENTAL SECTION

Chemistry. Chemicals and solvents were purchased from standard suppliers and used without further purification. Davisil® silica gel (40-63 μm) for flash column chromatography was supplied by Grace Davison Discovery Sciences (Victoria, Australia) and deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (USA, distributed by Novachem PTY. Ltd, Victoria, Australia).

Unless otherwise stated, reactions were carried out at ambient temperature. Reactions were monitored by thin layer chromatography on commercially available pre-coated aluminium-backed plates (Merck Kieselgel 60 F_{254}). Visualisation was by examination under UV light (254 and 366 nm). All organic extracts collected after aqueous work-up procedures were dried over anhydrous $MgSO_4$ or Na_2SO_4 before gravity filtering and evaporation to dryness. Organic solvents were evaporated *in vacuo* at ≤ 40°C (water bath temperature). Purification using preparative layer chromatography (PLC) was carried out on Analtech preparative TLC plates (200 mm x 200 mm x 2 mm).

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance Nanobay III 400MHz Ultrashield Plus spectrometer at 400.13 MHz and 100.62 MHz, respectively. Chemical shifts (δ) are recorded in parts per million (ppm) with reference to the chemical shift of the deuterated solvent. Coupling constants (J) and carbon-fluorine coupling constants (J_{CF}) are recorded in Hz and the significant multiplicities described by singlet (s), doublet (d), triplet (t), quadruplet (q), broad (br), multiplet (m), doublet of doublets (dd), doublet of triplets (dt).

Preparative HPLC was performed using an Agilent 1260 infinity coupled with a binary preparative pump and Agilent 1260 FC-PS fraction collector, using Agilent OpenLAB CDS software (Rev C.01.04), and an Altima 5μM C8 22 x 250 mm column. The following buffers were used; buffer A: H_2O; buffer B: MeCN, with sample being run at a gradient of 30% buffer B to 100% buffer B over 10 min, at a flow rate of 20 mL/min.

LCMS were run to verify reaction outcome and purity using an Agilent 6120 Series Single Quad coupled to an Agilent 1260 Series HPLC. Unless stated otherwise, the compounds which underwent pharmacological evaluation were >95% pure. The following buffers were used; buffer A: 0.1% formic acid in H_2O; buffer B: 0.1% formic acid in MeCN. The following gradient was used with a Poroshell 120 EC-C18 50 x 3.0 mm 2.7 micron column, and a flow rate of 0.5 mL/min and total run time of 5 min; 0–1 min 95% buffer A and 5% buffer B, from 1-2.5 min up to 0% buffer A and 100% buffer B, held at this composition until 3.8 min, 3.8–4 min 95% buffer A and 5% buffer B, held until 5 min at this composition. Mass spectra were acquired in positive and negative ion mode with a scan range of 100–1000 *m/z*. UV detection was carried out at 214 and 254 nm. All retention times (t_R) are quoted in min. HRMS analyses were carried out on an Agilent 6224 TOF LC/MS Mass Spectrometer coupled to an Agilent 1290 Infinity (Agilent, Palo Alto, CA). All data were acquired and reference mass corrected via a dual-spray electrospray ionisation (ESI) source. Acquisition was performed using the Agilent Mass Hunter Data Acquisition software version B.05.00 Build 5.0.5042.2 and analysis was performed using Mass Hunter Qualitative Analysis version B.05.00 Build 5.0.519.13. Melting points were measured on a Gallenkamp Melting Point Apparatus.

General procedure A: N^6-Substituted adenosines. 6-Chloropurine riboside (**1**) (1.0 eq), diaminoalkane (5.0 eq) and triethylamine (1.0 eq) were refluxed in ethanol (100 mL/ 2.5 g of the 6-chloropurine riboside) for 3 h under N_2. Upon cooling to rt, a white solid precipitated was formed. The suspension was filtered, washed with cold ethanol and dried under high vacuum to afford the title product as a white powder.

General procedure B: Bicyclo[2.2.2]octa-2,5-diene irreversible ligands. Bicyclo[2.2.2]octa-2,5-diene-2-carboxylic acid (1.50 eq), the respective amine (1.00 eq), BOP reagent (2.00 eq) and triethylamine (4.00 eq) were dissolved in DMF (3 mL/ 100 mg of the amine). The mixture was stirred at rt for 24 h. The mixture was diluted with water (50 mL) and extracted with ethyl acetate

(2 × 50 mL). The combined organic layers were washed with water (2 × 50 mL) and brine (50 mL), dried with Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by column chromatography (CH_2Cl_2: MeOH 100:0 → 90:10) to yield the desired product.

General procedure C: 1-(4-isothiocyanatophenyl)thiourea irreversible ligands. 1,4-Phenylene diisothiocyanate (3.0 eq) dissolved in CH_2Cl_2 (3 mL) was dropwise added to a mixture the respective amine (1.0 eq) in CH_2Cl_2 (5 mL/ 100 mg of the amine). The reaction mixture was stirred at rt under nitrogen for 5-22 h. The reaction mixture was absorbed on Celite® and purified via column chromatography to obtain the title compound.

General procedure D: Isothiocyanate irreversible ligands. *N*,*N*'-Thiocarbonyldiimidazole (TCDI) (1.00 eq) dissolved in CH_2Cl_2 (2 mL) was dropwise added to a mixture of the respective amine (1.00 eq) and CH_2Cl_2 (5 mL/ per 100 mg of the amine). The reaction mixture was stirred for 42 h at rt under nitrogen. The reaction mixture was absorbed on Celite® and purified via column chromatography to obtain the title compound.

General procedure E: 4-Fluorosulfonylphenyl irreversible ligands. 4-(Fluorosulfonyl)benzoic acid (1.50 eq), the respective amine (1.00 eq), BOP reagent (2.00 eq) and triethylamine (4.00 eq) were dissolved in DMF (3 mL/ 100 mg of the amine). The mixture was stirred at rt for 6 h. The mixture was diluted with water (50 mL) and extracted with ethyl acetate (2 × 50 mL). The combined organic layers were washed with water (2 × 50 mL) and brine (50 mL), dried with Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by column chromatography to yield the title product.

*(2*R*,3*R*,4*S*,5*R*)-2-(6-((2-Aminoethyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**5**).* General procedure A. White powder (735 mg, 68%). ^{1}H NMR (d_6-DMSO) δ 8.36 (d, *J* = 5.7 Hz, 1H), 8.21 (s, 1H), 7.82 (br s, 1H), 5.88 (d, *J* = 6.2 Hz, 1H), 5.70–5.00 (m, 2H), 4.69–4.56 (m, 1H), 4.15 (dd, *J* = 4.9, 3.0 Hz, 1H), 3.99–3.95 (m, 1H), 3.68 (dd, *J* = 12.1, 3.6 Hz, 1H),

3.55 (dd, *J* = 12.1, 3.6 Hz, 1H), 3.50–3.43 (m, 2H), 3.32 (br s, 3H), 2.75 (t, *J* = 6.6 Hz, 2H); mp: 90-92 °C; *m/z* MS (TOF ES$^+$) 311.1 [M+H]$^+$; LC-MS t_R: 0.45.

*(2*R*,3*R*,4*S*,5*R*)-2-(6-((6-Aminohexyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**6**).* General procedure A. White powder (6.04 g, 90%). ^{1}H NMR (d_6-DMSO) δ 8.34 (s, 1H), 8.21 (s, 1H), 7.90 (br s, 1H), 5.88 (d, *J* = 6.2 Hz, 1H), 5.46 (br s, 1H), 5.23 (br s, 1H), 4.62 (dd, *J* = 5.5, 5.5 Hz, 1H), 4.15 (dd, *J* = 4.8, 3.0 Hz, 1H), 4.00–3.94 (m, 1H), 3.68 (dd, *J* = 12.1, 3.6 Hz, 1H), 3.55 (dd, *J* = 12.1, 3.5 Hz, 1H), 3.50 – 3.42 (m, 2H), 3.33 (br s, 3H), 2.50–2.46 (m, 2H), 1.65–1.53 (m, 2H), 1.38–1.24 (m, 6H); mp: 163-166 °C; *m/z* MS (TOF ES$^+$) 367.3 [M+H]$^+$; LC-MS t_R: 2.71.

*(2*R*,3*R*,4*S*,5*R*)-2-(6-((10-Aminodecyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (7).* General procedure A. White powder (1.26 g, 86%). ^{1}H NMR (d_6-DMSO) δ 8.34 (s, 1H), 8.20 (s, 1H), 7.89 (br s, 1H), 5.88 (d, *J* = 6.2 Hz, 1H), 5.70–4.80 (m, 2H), 4.61 (dd, *J* = 5.5, 5.5 Hz, 1H), 4.15 (dd, *J* = 4.7, 3.1 Hz, 1H), 3.99–3.95 (m, 1H), 3.68 (dd, *J* = 12.1, 3.5 Hz, 1H), 3.55 (dd, *J* = 12.1, 3.5 Hz, 1H), 3.51–3.40 (m, 2H), 3.30 (br s, 3H), 2.50–2.45 (m, 2H), 1.66–1.50 (m, 2H), 1.26 (d, *J* = 22.1 Hz, 14H); mp: 153-156 °C; *m/z* MS (TOF ES$^+$) 423.3 [M+H]$^+$; LC-MS t_R: 2.75.

N-*(2-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)ethyl)bicyclo[2.2.2]octa-2,5-diene-2-carboxamide (**8a**).* General procedure B. A second silica column chromatography (EtOAc: MeOH 100:0 → 80:20) was performed to yield the desired product as a white resin (15 mg, 11%). ^{1}H NMR (d_6-DMSO) δ 8.37 (s, 1H), 8.22 (s, 1H), 7.99–7.90 (m, 2H), 6.96–6.92 (m, 1H), 6.37–6.25 (m, 2H), 5.89 (d, *J* = 6.1 Hz, 1H), 5.45 (d, *J* = 6.2 Hz, 1H), 5.41 (dd, *J* = 7.2, 4.5 Hz, 1H), 5.20 (d, *J* = 4.7 Hz, 1H), 4.61 (dd, *J* = 11.2, 6.1 Hz, 1H), 4.17–4.13 (m, 1H), 4.12–4.08 (m, 1H), 3.97 (dd, *J* = 6.6, 3.4 Hz, 1H), 3.79–3.64 (m, 2H), 3.62–3.52 (m, 3H), 3.44–3.38 (m, 2H), 1.27–1.10 (m, 4H); *m/z* MS (TOF ES$^+$) 443.1 [M+H]$^+$; LC-MS t_R: 2.85; HRMS - $C_{21}H_{27}N_6O_5$ [M+H]$^+$ calcd 443.2043; found 443.2046.

*1-(2-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)ethyl)-3-(4-isothiocyanatophenyl)thiourea (**8b**).* General procedure C. The purification was performed via column chromatography (CH_2Cl_2: MeOH 100:0 → 80:20). The title compound was obtained as a white solid (38 mg, 24%). ^{1}H NMR (d_6-DMSO) δ 9.77 (br s, 1H), 8.38 (s, 1H), 8.17 (s, 1H), 8.13 – 7.92 (m, 2H), 7.57 – 7.47 (m, 2H), 7.40 – 7.31 (m, 2H), 5.90 (d, *J* = 6.1 Hz, 1H), 5.44 (d, *J* = 6.3 Hz, 1H), 5.39 (dd, *J* = 7.0, 4.6 Hz, 1H), 5.20 (d, *J* = 4.7 Hz, 1H), 4.61 (dd, *J* = 11.3, 6.0 Hz, 1H), 4.18–4.12 (m, 1H), 3.97 (dd, *J* = 6.5, 3.3 Hz, 1H), 3.84–3.63 (m, 5H), 3.61–3.51 (m, 1H); mp: 165-170 °C; *m/z* MS (TOF ES$^+$) 503.1 $[M+H]^+$; LC-MS t_R: 3.03; HRMS - $C_{20}H_{23}N_8O_4S_2$ $[M+H]^+$ calcd 503.1284; found 503.1287.

*(2*R*,3*S*,4*R*,5*R*)-2-(Hydroxymethyl)-5-(6-((2-isothiocyanatoethyl)amino)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol (**8c**).* General procedure D. The purification was performed twice via column chromatography (CH_2Cl_2: MeOH 100:0 → 8:2). The title compound was obtained as a white solid (17 mg, 15%). ^{1}H NMR (CD_3OD) δ 8.75 (s, 1H), 8.69 (s, 1H), 6.16 (d, *J* = 5.6 Hz, 1H), 4.77 (t, *J* = 5.3 Hz, 1H), 4.59–4.51 (m, 2H), 4.39 (dd, *J* = 5.0, 3.7 Hz, 1H), 4.19 (m, *J* = 6.4, 3.1 Hz, 1H), 3.92 (dd, *J* = 12.4, 2.9 Hz, 1H), 3.85–3.76 (m, 3H); *m/z* MS (TOF ES$^+$) 353.1 $[M+H]^+$; LC-MS t_R: 0.79; HRMS - $C_{13}H_{17}N_6O_4S$ $[M+H]^+$ calcd 353.1032; found 353.1030.

N-*(6-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)hexyl)bicyclo[2.2.2]octa-2,5-diene-2-carboxamide (**9a**).* General procedure B. White solid (53 mg, 39%). ^{1}H NMR (d_6-DMSO) δ 8.34 (s, 1H), 8.20 (br s, 1H), 7.87 (br s, 1H), 7.75 (br t, *J* = 5.5 Hz, 1H), 6.97–6.87 (m, 1H), 6.36–6.26 (m, 2H), 5.88 (d, *J* = 6.2 Hz, 1H), 5.50–5.40 (m, 2H), 5.18 (d, *J* = 4.6 Hz, 1H), 4.62 (dd, *J* = 11.2, 5.8 Hz, 1H), 4.18–4.13 (m, 1H), 4.12–4.08 (m, 1H), 4.00–3.95 (m, 1H), 3.73–3.64 (m, 2H), 3.60–3.53 (m, 1H), 3.51–3.39 (m, 2H), 3.15–3.02 (m, 2H), 1.66–1.54 (m, 2H), 1.51–1.38 (m, 2H), 1.36–1.09 (m, 8H); mp: 108-111 °C; *m/z* MS (TOF ES$^+$) 499.3 $[M+H]^+$; LC-MS t_R: 2.98; HRMS - $C_{25}H_{35}N_6O_5$ $[M+H]^+$ calcd 499.2669; found 499.2673.

*1-(6-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)hexyl)-3-(4-isothiocyanatophenyl)thiourea (**9b**).* General procedure C. The purification was performed via column chromatography (CH_2Cl_2: MeOH 100:0 → 90:10). The title compound was obtained as a white solid (80 mg, 35%). ^{1}H NMR (d_6-DMSO) δ 9.62 (br s, 1H), 8.34 (s, 1H), 8.21 (br s, 1H), 8.00–7.83 (m, 2H), 7.60–7.50 (m, 2H), 7.44–7.29 (m, 2H), 5.88 (d, *J* = 6.2 Hz, 1H), 5.48–5.40 (m, 2H), 5.20 (d, *J* = 4.6 Hz, 1H), 4.62 (dd, *J* = 11.3, 6.1 Hz, 1H), 4.18–4.11 (m, 1H), 3.97 (dd, *J* = 6.5, 3.3 Hz, 1H), 3.74–3.65 (m, 1H), 3.63–3.53 (m, 1H), 3.53–3.38 (m, 4H), 1.69–1.46 (m, 4H), 1.42–1.27 (m, 4H); mp: 131-135 °C; *m/z* MS (TOF ES$^+$) 559.2 $[M+H]^+$; LC-MS t_R: 3.14; HRMS - $C_{24}H_{31}N_8O_4S_2$ $[M+H]^+$ calcd 559.1910; found 559.1913.

*(2*R*,3*S*,4*R*,5*R*)-2-(Hydroxymethyl)-5-(6-((6-isothiocyanatohexyl)amino)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol (**9c**).* General procedure D. The purification was performed via column chromatography (CH_2Cl_2: MeOH 100:0 → 80:20). The title compound was obtained as a white solid (10 mg, 9%). ^{1}H NMR (CD_3OD) δ 8.15 (s, 1H), 8.12 (br s, 1H), 5.85 (d, *J* = 6.5 Hz, 1H), 4.64 (dd, *J* = 6.2, 5.3 Hz, 1H), 4.22 (dd, *J* = 5.1, 2.4 Hz, 1H), 4.12–4.03 (m, 1H), 3.79 (dd, *J* = 12.6, 2.4 Hz, 1H), 3.64 (dd, *J* = 12.6, 2.5 Hz, 1H), 3.59–3.40 (m, 4H), 1.68–1.53 (m, 4H), 1.46–1.32 (m, 4H); *m/z* MS (TOF ES$^+$) 409.1 $[M+H]^+$; LC-MS t_R: 3.04; HRMS - $C_{17}H_{25}N_6O_4S$ $[M+H]^+$ calcd 409.1658; found 409.1663.

*4-((6-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)hexyl)carbamoyl)benzenesulfonyl fluoride (**9d**).* General procedure E. The purification was performed via column chromatography (CH_2Cl_2: methanol 100:0 → 8:2) and (EtOAc: MeOH 100:0 → 80:20). The title compound was obtained as a white solid (50 mg, 10%). ^{1}H NMR (d_6-DMSO) δ 8.85 (br t, *J* = 5.5 Hz, 1H), 8.34 (s, 1H), 8.28–8.23 (m, 2H), 8.22–8.13 (m, 3H), 7.95–7.88 (m, 1H), 5.88 (d, *J* = 6.2 Hz, 1H), 5.47–5.41 (m, 2H), 5.19 (d, *J* = 4.6 Hz, 1H), 4.61 (dd, *J* = 11.3, 6.1 Hz, 1H), 4.17–4.12 (m, 1H), 3.97 (dd, *J* = 6.5, 3.4 Hz, 1H), 3.71–3.63 (m, 1H), 3.59–3.51 (m, 1H), 3.60–3.51 (m 2H), 3.28 (dd, *J* = 12.9, 6.7 Hz, 2H), 1.67–1.48 (m, 4H), 1.42–1.30 (m, 4H);

m/z MS (TOF ES$^+$) 553.2 [M+H]$^+$; LC-MS t_R: 3.33; HRMS - $C_{23}H_{30}FN_6O_7S$ [M+H]$^+$ calcd 553.1881; found 553.1888.

N-*(10-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)decyl)bicyclo[2.2.2]octa-2,5-diene-2-carboxamide (**10a**).* General procedure B. White solid (53 mg, 34%). ^{1}H NMR (d_6-DMSO) δ 8.34 (s, 1H), 8.20 (br s, 1H), 7.94–7.84 (m, 1H), 7.75 (br t, *J* = 5.5 Hz, 1H), 6.91 (dd, *J* = 6.3, 1.7 Hz, 1H), 6.38–6.25 (m, 2H), 5.88 (d, *J* = 6.2 Hz, 1H), 5.49–5.39 (m, 2H), 5.19 (d, *J* = 4.6 Hz, 1H), 4.61 (dd, *J* = 11.3, 6.0 Hz, 1H), 4.16–4.12 (m, 1H), 4.12–4.07 (m, 1H), 4.01–3.92 (m, 1H), 3.74–3.63 (m, 2H), 3.60–3.51 (m, 1H), 3.50–3.39 (m, 2H), 3.14–2.99 (m, 2H), 1.64–1.52 (m, 2H), 1.46–1.36 (m, 2H), 1.35–1.10 (m, 16H); mp: 88-90 °C; *m/z* MS (TOF ES$^+$) 555.3 [M+H]$^+$; LC-MS t_R: 3.15; HRMS - $C_{29}H_{43}N_6O_5$ [M+H]$^+$ calcd 555.3296; found 555.3301.

*1-(10-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)decyl)-3-(4-isothiocyanatophenyl)thiourea (**10b**).* General procedure C. The purification was performed twice via column chromatography (CH_2Cl_2: MeOH 100:0 → 80:20). The title compound was obtained as a white solid (26 mg, 18%). ^{1}H NMR (d_6-DMSO) δ 9.64 (br s, 1H), 8.34 (s, 1H), 8.20 (br s, 1H), 7.99–7.80 (m, 2H), 7.58–7.51 (m, 2H), 7.41–7.33 (m, 2H), 5.88 (d, *J* = 6.2 Hz, 1H), 5.47–5.40 (m, 2H), 5.19 (d, *J* = 4.6 Hz, 1H), 4.61 (dd, *J* = 11.3, 6.0 Hz, 1H), 4.17–4.12 (m, 1H), 3.97 (dd, *J* = 6.5, 3.4 Hz, 1H), 3.72–3.63 (m, 1H), 3.63–3.52 (m, 1H), 3.51–3.39 (m, 4H), 1.66–1.46 (m, 4H), 1.39–1.22 (m, 12H); mp: 88-93 °C; *m/z* MS (TOF ES$^+$) 615.2 [M+H]$^+$; LC-MS t_R: 3.33; HRMS - $C_{28}H_{39}N_8O_4S_2$ [M+H]$^+$ calcd 615.2536; found 615.2566.

*(2*R*,3*S*,4*R*,5*R*)-2-(Hydroxymethyl)-5-(6-((10-isothiocyanatodecyl)amino)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol (**10c**).* General procedure D. The purification was performed twice via column chromatography (CH_2Cl_2: MeOH 100:0 → 80:20). The title compound was obtained as a white solid (53 mg, 35%). ^{1}H NMR (CD_3OD) δ 8.27 (s, 1H), 8.23 (br s, 1H), 5.97 (d, *J* = 6.5 Hz, 1H), 4.76 (dd, *J* = 6.5, 5.1 Hz, 1H), 4.62 (br s, 1H), 4.34 (dd, *J* = 5.1, 2.4 Hz, 1H), 4.19 (dd, *J* = 5.0,

2.5 Hz, 1H), 3.91 (dd, *J* = 12.6, 2.4 Hz, 1H), 3.76 (dd, *J* = 12.6, 2.6 Hz, 1H), 3.64–3.57 (m, 1H), 3.56 (t, *J* = 6.5 Hz, 2H), 1.77–1.64 (m, 4H), 1.51–1.30 (m, 12H); mp: 111-113 °C; *m/z* MS (TOF ES^+) 456.2 $[M+H]^+$; LC-MS t_R: 3.29; HRMS - $C_{21}H_{33}N_6O_4S$ $[M+H]^+$ calcd 456.2284; found 456.2287.

*4-((10-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)decyl)carbamoyl)benzenesulfonyl fluoride (**10d**).* General procedure E. The purification was performed via column chromatography (CH_2Cl_2: MeOH 100:0 → 80:20) and (EtOAc: MeOH 100:0 → 80:20). The title compound was obtained as a white solid (11 mg, 8%). ^{1}H NMR (d_6-DMSO) δ 8.84 (br t, *J* = 5.5 Hz, 1H), 8.34 (s, 1H), 8.28–8.23 (m, 2H), 8.23–8.13 (m, 3H), 7.93–7.85 (m, 1H), 5.88 (d, *J* = 6.2 Hz, 1H), 5.49–5.39 (m, 2H), 5.19 (d, *J* = 4.6 Hz, 1H), 4.61 (dd, *J* = 11.3, 6.1 Hz, 1H), 4.16–4.12 (m, 1H), 3.97 (dd, *J* = 6.5, 3.4 Hz, 1H), 3.75–3.62 (m, 1H), 3.62–3.52 (m, 1H), 3.50–3.40 (m, 2H), 3.31–3.22 (m, 2H), 1.67–1.49 (m, 4H), 1.34–1.22 (m, 12H); *m/z* MS (TOF ES^+) 609.2 $[M+H]^+$; LC-MS t_R: 3.19; HRMS - $C_{27}H_{38}FN_6O_7S$ $[M+H]^+$ calcd 609.2507; found 609.2517.

*tert-Butyl (4-aminobenzyl)carbamate (**12**).* A solution of di-*tert*-butyl dicarbonate (3.75 g, 17.2 mmol, 1.05 eq) in THF (30 mL) was added to a solution 4-aminobenzylamine (**11**) (2.00 g, 16.4 mmol, 1.00 eq) in THF (50 mL). The reaction was stirred at rt for 5 h. Ethyl acetate (400 mL) was added and the organic layer was washed with 1 M HCl (100 mL), 1 M NaOH (100 mL), sat. $NaHCO_3$ (100 mL) and brine (100 mL). The water layer was extracted with ethyl acetate (3 x 100 mL). The combined organic layers were dried with Na_2SO_4, filtered and the solvent was removed under reduced pressure. The crude material was purified by column chromatography (PET → PET: EtOAc 1:1) to give the desired product was a yellow solid (3.42 g, 94%). ^{1}H NMR ($CDCl_3$) δ 7.15–7.03 (m, 2H), 6.71–6.60 (m, 2H), 4.77 (br s, 1H), 4.20 (d, *J* = 5.4 Hz, 2H), 3.74 (br s, 2H), 1.48 (s, 9H); mp: 70-73 °C; *m/z* MS (TOF ES^+) 223.1 $[M+H]^+$; LC-MS t_R: 2.73.

tert-*Butyl (4-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)benzyl)carbamate **(13)**.* Triethylamine (714 µL, 5.23 mmol, 1.00 eq) was added to a mixture of 6-chloropurine riboside (**1**) (1.50 g, 5.23 mmol, 1.00 eq) and *tert*-butyl (4-aminobenzyl)carbamate (**12**) (3.49 g, 15.7 mmol, 3.00 eq) in ethanol (50 mL). The reaction mixture was stirred at reflux for 26 h, before it was cooled to rt. Upon cooling, a white solid precipitated was formed. The suspension was filtered, washed with cold ethanol and dried under high vacuum to afford the title product as a white powder (2.17 g, 88%). ^{1}H NMR (d_6-DMSO) δ 9.93 (s, 1H), 8.54 (s, 1H), 8.39 (s, 1H), 7.90–7.77 (m, 2H), 7.36 (t, J = 6.1 Hz, 1H), 7.27–7.13 (m, 2H), 5.96 (d, J = 6.0 Hz, 1H), 5.50 (d, J = 6.2 Hz, 1H), 5.31 (dd, J = 6.8, 4.8 Hz, 1H), 5.23 (d, J = 4.7 Hz, 1H), 4.64 (dd, J = 11.2, 6.0 Hz, 1H), 4.18 (dd, J = 8.1, 4.8 Hz, 1H), 4.09 (d, J = 6.1 Hz, 2H), 3.99 (dd, J = 6.5, 3.6 Hz, 1H), 3.77–3.64 (m, 1H), 3.62–3.50 (m, 1H), 1.40 (s, 9H); mp: 178-181 °C; *m/z* MS (TOF ES$^+$) 473.2 $[M+H]^+$; LC-MS t_R: 3.03.

*(2*R*,3*R*,4*S*,5*R*)-2-(6-((4-(Aminomethyl)phenyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol **(14)**.* *tert*-Butyl (4-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)benzyl)carbamate (1.00 g, 2.12 mmol, 1.00 eq) (**13**) was dissolved in CH_2Cl_2 (20 mL)and trifluoroacetic acid (2 mL) was added and the reaction was stirred at rt. After 6 h DCM (50 mL) was added, followed by the addition of 1 M NaOH until the reaction mixture basic (pH= 12). The layers were separated and the water layer reduced under *vacuo*. The residue was purified via reversed phase chromatography (C18 column) (H_2O: MeOH 95:5 → 0:100) to obtain the titled compound as a white solid (450 mg, 70%). ^{1}H NMR (d_6-DMSO) δ 9.88 (s, 1H), 8.53 (s, 1H), 8.38 (s, 1H), 7.87 – 7.81 (m, 2H), 7.31 – 7.25 (m, 2H), 5.96 (d, J = 6.0 Hz, 1H), 5.66 (br s, 1H), 5.40 (br s, 1H), 5.35 – 5.28 (m, 1H), 4.63 (t, J = 5.4 Hz, 1H), 4.24 – 4.14 (m, 1H), 3.99 (q, J = 3.6 Hz, 1H), 3.75 – 3.66 (m, J = 13.7 Hz, 3H), 3.62 – 3.53 (m, 1H), 1.72 (br s, 2H); *m/z* MS (TOF ES$^+$) 373.0 $[M+H]^+$; LC-MS t_R: 1.38. Remark: Amine **14** was successfully used as the TFA salt for further reactions, however, in case of the synthesis of compounds **15b** and **15d** better results were obtained when using the free amine.

N-*(4-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)benzyl)bicyclo[2.2.2]octa-2,5-diene-2-carboxamide (**15a**).* General procedure B. White solid (113 mg, 42%). ^{1}H NMR (d_6-DMSO) δ 9.93 (s, 1H), 8.54 (s, 1H), 8.38 (s, 1H), 8.35 (br t, *J* = 6.1 Hz, 1H), 7.87–7.81 (m, 2H), 7.25–7.17 (m, 2H), 7.02 (dd, *J* = 6.3, 1.9 Hz, 1H), 6.40–6.28 (m, 2H), 5.96 (d, *J* = 6.0 Hz, 1H), 5.50 (d, *J* = 6.1 Hz, 1H), 5.30 (dd, *J* = 6.7, 4.8 Hz, 1H), 5.23 (d, *J* = 4.7 Hz, 1H), 4.64 (dd, *J* = 11.1, 5.8 Hz, 1H), 4.28 (d, *J* = 5.9 Hz, 2H), 4.20–4.14 (m, 2H), 3.99 (dd, *J* = 7.0, 3.6 Hz, 1H), 3.77–3.66 (m, 2H), 3.61–3.52 (m, 1H), 1.30–1.14 (m, 4H); mp: 195-200 °C; *m/z* MS (TOF ES$^+$) 505.2 $[M+H]^+$; LC-MS t_R: 3.04; HRMS - $C_{26}H_{29}N_6O_5$ $[M+H]^+$ calcd 505.2199; found 505.2203.

*1-(4-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)benzyl)-3-(4-isothiocyanatophenyl)thiourea (**15b**).* General procedure C. 1,4-Phenylene diisothiocyanate (23 mg, 118 μmol, 1.10 eq) was added to a solution of (2R,3R,4S,5R)-2-(6-((4-(aminomethyl)phenyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**14**) (40 mg, 107 μmol, 1.00 eq) in DMF (4 mL). The reaction mixture was stirred at rt for 2 h, then water was added until a white precipitate formed. The solid was allowed to settle to the ground and the liquid phase was decanted. The residue was taken up in DMF (2 mL) and purified via preparative RP-HPLC (30-100% ACN) to obtain the titled compound as a white solid (10 mg, 17%). ^{1}H NMR (d_6-DMSO) δ 10.02 (s, 1H), 9.83 (br s, 1H), 8.60 (s, 1H), 8.45 (s, 1H), 8.41–8.30 (m, 1H), 7.99–7.92 (m, 2H), 7.68–7.61 (m, 2H), 7.49–7.43 (m, 2H), 7.41–7.34 (m, 2H), 6.02 (d, *J* = 6.0 Hz, 1H), 5.54 (d, *J* = 6.2 Hz, 1H), 5.35 (dd, *J* = 6.7, 4.8 Hz, 1H), 5.28 (d, *J* = 4.7 Hz, 1H), 4.75 (d, *J* = 5.0 Hz, 2H), 4.72–4.67 (m, 1H), 4.27–4.21 (m, 1H), 4.04 (dd, *J* = 3.6 Hz, 1H), 3.80–3.72 (m, 1H), 3.69–3.60 (m, 1H); ^{1}H NMR (d_6-DMSO) δ 180.9, 152.6, 152.4, 149.8, 141.2, 139.7, 138.9, 133.3, 133.1, 128.2, 126.7, 125.3, 123.9, 121.5, 120.8, 88.3, 86.3, 74.1, 71.0, 62.0, 47.4; mp: 172-176 °C; *m/z* MS (TOF ES$^+$) 565.1 $[M+H]^+$; LC-MS t_R: 3.22; HRMS – $C_{25}H_{25}N_8O_4S_2$ $[M+H]^+$ calcd 565.1440; found 565.1447.

(2R,3S,4R,5R)-2-(Hydroxymethyl)-5-(6-((4-(isothiocyanatomethyl)phenyl)amino)-9H-purin-9-yl)tetrahydrofuran-3,4-diol ***(15c)***. General procedure D. The purification was performed via column chromatography (CH_2Cl_2: MeOH 100:0 → 8:2) and preparative TLC (CH_2Cl_2: MeOH 95:5). The title compound was obtained as a white solid (5 mg, 3%). ^{1}H NMR (d_6-DMSO) δ 10.08 (s, 1H), 8.57 (s, 1H), 8.43 (s, 1H), 8.04–7.97 (m, 2H), 7.40–7.33 (m, 2H), 5.97 (d, J = 6.0 Hz, 1H), 5.51 (d, J = 6.2 Hz, 1H), 5.28 (dd, J = 6.6, 4.9 Hz, 1H), 5.23 (d, J = 4.8 Hz, 1H), 4.89 (s, 2H), 4.67–4.61 (m, 1H), 4.21–4.15 (m, 1H), 4.01–3.97 (m, 1H), 3.73–3.68 (m, 1H), 3.61–3.54 (m, 1H); *m/z* MS (TOF ES$^+$) 415.1 $[M+H]^+$; LC-MS t_R: 3.15; HRMS – $C_{18}H_{19}N_6O_4S$ $[M+H]^+$ calcd 415.1188; found 415.1184.

*4-((4-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)benzyl)carbamoyl)benzenesulfonyl fluoride* ***(15d)***. (Fluorosulfonyl)benzoic acid (24 mg, 118 µmol, 1.1 eq), (2R,3R,4S,5R)-2-(6-((4-(aminomethyl)phenyl)amino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (**14**) (40 mg, 107 µmol, 1.00 eq), BOP reagent (95 mg, 224 µmol, 2.00 eq) and 2,6-lutidine (62 µL, 537 µmol, 5.00 eq) were dissolved in DMF (8 mL). The mixture was stirred at rt for 1 h. The mixture was diluted with water (50 mL) and extracted with ethyl acetate (2 × 50 mL). The combined organic layers were washed with water (2 × 50 mL) and brine (50 mL), dried with Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was suspended in a mixture of MeOH and DCM (10 mL). The solid was allowed to settle on the ground and the liquid layer was decanted. The solid was dried under a nitrogen flow to give the titled compound as a white powder (41 mg, 68%). ^{1}H NMR (d_6-DMSO) δ 9.96 (s, 1H), 9.42 (br t, J = 5.6 Hz, 1H), 8.54 (s, 1H), 8.38 (s, 1H), 8.30–8.20 (m, 4H), 7.91–7.85 (m, 2H), 7.36–7.27 (m, 2H), 5.96 (d, J = 6.0 Hz, 1H), 5.51–5.42 (m, 1H), 5.32–5.15 (m, 2H), 4.64 (t, J = 5.6 Hz, 1H), 4.49 (d, J = 5.7 Hz, 2H), 4.20–4.14 (m, 1H), 4.00–3.96 (m, 1H), 3.70 (dd, J = 11.8, 3.6 Hz, 1H), 3.58 (dd, J = 12.5, 4.2 Hz, 1H), strong concentration dependent effects of nonexchangeable proton resonances[50, 51] were observed for the multiplicity of the signal at 8.30–8.20 ppm.; ^{1}H NMR (d_6-DMSO) δ 164.8, 152.6, 152.4, 149.8, 141.9, 141.1, 138.8, 133.9, 133.8, 129.7, 129.1, 128.1, 121.5,

120.7, 88.3, 86.3, 74.1, 71.0. 62.0, 43.1; mp: >350 °C (degraded/ turned black); *m/z* MS (TOF ES^+) 559.1 $[M+H]^+$; LC-MS t_R: 3.08; HRMS - $C_{24}H_{24}FN_6O_7S$ $[M+H]^+$ calcd 559.1411; found 559.1387.

Pharmacology. *ERK 1/2 phosphorylation assays.* FlpIn™ Chinese hamster ovary (FlpInCHO) cells stably expressing the human A_1AR (A_1AR-FlpInCHO) cells were seeded into 96-well culture plates at a density of 4×10^4 cells/well in DMEM containing 10% FBS and maintained at 37°C in a humidified incubator containing 5% CO_2. After 6 h, cells were washed with serum free DMEM and maintained in serum free DMEM for approximately 18 h at 37°C in 5% CO_2 before assaying. Agonist concentration-response curves were generated at the peak-response time point (5 min exposure). In all cases, stimulation with 10% FBS for 5 min was used as a positive control. Stimulation was terminated by the rapid removal of media and addition of 100 μL *SureFire*™ lysis buffer to each well. Plates were then agitated for 5 min and detection performed as previously described.[39] Data were baseline-corrected and then expressed as a percentage the pERK1/2 response in the presence of 1 μM NECA (defined as 100%) and fitted to "log (agonist) vs. response" equation in Prism 6.0 (GraphPad Software).

xCELLigence real-time cell analysis assays. Cellular impedance was measured over time using the label-free technology, xCELLigence Real-Time Cell Analyzer (RTCA) single-plate (SP) instrument (Roche Diagnostics GmbH and ACEA Biosciences). A_1AR-FlpInCHO cells were seeded into 96-well E-plates™ plates at a density of 4×10^4 cells/well in DMEM containing 10% FBS and maintained at 37°C in a humidified incubator containing 5% CO_2. After 6 h, cells were washed with serum free DMEM and maintained in serum free DMEM for approximately 18 h at 37°C in 5% CO_2 before assaying. The E-plate was then plated into the RTCA reader and allowed to equilibrate for at least 1 h at 37°C in 5% CO_2, during which time the cellular impedance was measured at 10 min intervals. Following the equilibration period, cells were exposed to 1 μM agonist or vehicle and changes in cellular impedance measured at 15 s intervals. After 1.7 h, the

A_1AR antagonist **16** (1 μM) or vehicle was added to the wells and changes in cellular impedance measured at 15 s intervals for at least an additional 2 h. Data was baseline corrected to the cellular impedance at the time-point prior to the first ligand addition. The influence of A_1AR agonists and antagonists was then established by normalising each time-point to the NECA treated well (as described in the text).

Radioligand equilibrium binding assays. A_1AR-FlpInCHO were grown in DMEM supplemented with 10% FBS and hygromycin-B (500 μg/mL) and maintained at 37°C in a 5% CO_2 humidified incubator. A_1AR-FlpInCHO cell membranes were prepared as described previously.[52] In brief, A_1AR-FlpInCHO cells were grown to confluence before being harvested and resuspended in HEPES homogenization buffer (10 mM HEPES and 10 mM EDTA, pH 7.4) and homogenized using a hand-held homogenizer (Polytron; Kinematica, Littau-Lucerne, Switzerland). The homogenate was twice centrifuged (40,000*g*, 30 min, 4°C), and the cell pellet was then resuspended in HEPES assay buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4). The protein content was determined using a Pierce® BCA protein assay kit according to manufacturer's instructions.

Membranes were incubated with 10 μM ligand in 1 mL of phosphate buffer saline (PBS, 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; 137 mM NaCl; 2.7 mM KCl) with 1 unit of adenosine deaminase (ADA) for 1 h at 37°C, following by 3 x 1.5 mL washes with PBS buffer (1 h at 37°C incubation between each wash) followed by 30 min centrifugation at 20000g. After the final spin, membranes were resuspended in HME buffer (50 mM HEPES pH7.5, 5 mM $MgCl_2$, 1 mM EDTA), sonicated and their concentration measured using Bradford reagent (Bio-Rad). Radioligand binding was performed in HME buffer supplemented with 1 unit/mL of ADA. [^{3}H]DPCPX concentration ranged from 0.1 - 12 nM, determined by counting stock dilutions for each experiment in TriCarb 2910 TR liquid scintillation analyzer (PerkinElmer) for each experiment. Washed membranes were incubated with [^{3}H]DPCPX for 1 h at 37°C with shaking, harvested on UniFilter GF/C (Whatman) plates using Filtermate 196 harvester (Packard), dried and dissolved in 4 mL of Ultima Gold

scintillation liquid (PerkinElmer) followed by β-counting. Nonspecific binding was measured in the presence of 1 μM **16**. According to an F-test, specific binding, determined by subtracting nonspecific binding from total, could be adequately described by "One site-specific binding" equation in Prism 6.0 (GraphPad Software), using a shared K_d value. ($F_{4, 95}$=0.027; p<0.01 for shared vs. independent K_d values).

[^{35}S]GTPγS binding. Measurement of [^{35}S]GTPγS incorporation was performed on membranes prepared in the same manner as those used for radioligand equilibrium binding experiments. After drug treatments and extensive washing, 2.5 μg of membranes were pre-incubated with 3μM GDP for 30 min at 20°C in the assay buffer (20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 0.1% BSA, 30 μg/mL saponin and 1 U/mL ADA). Where indicated, 10 μM of NECA or **16** were added during this incubation. Reactions were started by the addition of [^{35}S]GTPγS to a final concentration of 300 pM. After 40 min incubation at 20°C, the reaction was terminated by harvesting the membranes on Whatman UniFilter GF/C plates using Filtermate 196 harverster (Packard). Plates were washed 3 times with ice-cold 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 100 mM NaCl, dissolved in 40 μL of MicroScint-O scintillation cocktail (Packard) and counted using MicroBeta LumiJET counter (PerkinElmer). Data from each experiment were normalised to the response engendered by NECA of the control membranes pre-incubated without drugs, but subjected to the same wash procedures.

cAMP accumulation. A_1AR-, A_{2A}AR-, A_{2B}AR-, A_3AR- and non-transfected (NT)-FlpInCHO cells were seeded into 96-well culture plates at a density of 2×10^4 cells/well in DMEM containing 10% FBS, 600 μg/mL hygromycin B and incubated in a humidified incubator at 37^{o}C in 5% CO_2 overnight. For NT-FlpInCHO and FlpInCHO cells expressing $G_{i/o}$-coupled A_1ARs or A_3ARs, media was replaced with stimulation buffer (140 mM NaCl, 5.4 mM KCl, 0.8 μM $MgSO_4$, 0.2 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 5.6 mM D-glucose, 5 mM HEPES, 0.1% bovine serum albumin (BSA), and 10 μM rolipram, pH 7.45) and incubated at 37°C for 30 min.

Concentration response assays were performed by subsequent incubation of agonists (10 pM - 10 μM) and 3 μM forskolin for an additional 30 min at 37°C. The reactions were terminated by the addition of 50 μL ice cold 100% ethanol. The lysis buffer (0.1% BSA, 0.3% tween-20, 5 mM HEPES, pH 7.45) was added to the cells after the compete evaporation of ethanol. Detection of cAMP was performed using LANCE^{TM} cAMP 384 kits (PerkinElmer) following the manufacturer's protocol, and fluorescence was measured with an EnVision® plate reader (PerkinElmer). Agonist concentration-response curves were normalized to the ability to inhibit the response mediated by 3 μM forskolin (0%) or buffer (100%) alone. For FlpInCHO cells expressing G_s-coupled A_{2A}ARs or A_{2B}ARs, media was removed and replaced with stimulation buffer. Cell were then incubated at 37°C for 30 min (A_{2A}AR-FlpInCHO only). Agonists were then added and cells were incubated for 10 min (A_{2B}AR-FlpInCHO) or 30 min (A_{2B}AR-FlpInCHO) followed by termination of the reaction and detection of cAMP as described above. Agonist concentration-response curves were normalized to the response mediated by 3 μM forskolin (100%) or buffer (0%) alone. All experiments were performed in triplicate.

ThermoFluor. The A_1AR was expressed, purified from Sf9 insect cells (Expression Systems) and exchanged into 0.1% Lauryl Maltose Neopentyl Glycol (LMNG)/ 0.01% Cholesteryl Hemisuccinate (CHS). Receptor (0.2 μg) was incubated with 10 μM compounds for 20 h at 4°C in the assay buffer (30 mM HEPES pH 7.5; 100 mM NaCl; 0.01% LMNG; 0.001% CHS; 1 U/mL ADA) under 8 μL of silicone oil (Sigma) in black PCR plates (Bio-Rad). N-[4-(7-diethylamino-4methyl-3-coumarinyl)phenyl]maleimide (CPM; Sapphire Bioscience) was added to a final concentration of 10 μM and incubated at rt for 30 min prior to heating. The plate was gradually heated from 4 to 80 °C in 3° intervals using a PCR machine. Each temperature was held for 90 s followed by a rapid (30 s) fluorescence reading using an EnVision plate reader (Perkin Elmer) with 380/470nm excitation/emission filters. Increases in fluorescence indicated receptor unfolding. Receptor stabilization was monitored by the rightward shift of the meting curve, and increases in

the melting temperature (T_m). Results were fitted to Boltzmann sigmoidal equation in Prism 6.0 (GraphPad Software) and T_m was defined as the halfway of the transition.

■ AUTHOR INFORMATION

Corresponding Authors

*For P.J.S.: phone: +613 9903 9542; E-mail: peter.scammells@monash.edu.

*For A.C.: phone: +613 9903 9067; E-mail: arthur.christopoulos@monash.edu.

Author Contributions

The manuscript was written through contributions of all authors, and all authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; cat, catalytic; DMF, dimethylformamide; eq, equivalent; EtOAc, ethyl

acetate; FCC, flash column chromatography; PE, petroleum spirits 40-60; rt, room temperature; THF, tetrahydrofuran.

■ ASSOCIATED CONTENT

Supporting Information Available: Details of the expression and purification of A_1ARs, reconstitution into high-density lipoproteins particles and pharmacology of the purified A_1AR reconstituted in nanodiscs.

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