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Affinity-based profiling of the adenosine receptors

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Chapter 3

Development of subtype-selective covalent ligands for the adenosine A_{2B} receptor by tuning the reactive group

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Abstract
Signaling through the adenosine receptors (ARs), in particular through the adenosine A_{2B} receptor (A_{2B}AR), has shown to play a role in a variety of pathological conditions, ranging from immune disorders to cancer. Covalent ligands for the A_{2B}AR have the potential to irreversibly block the receptor, as well as inhibit all A_{2B}AR-induced signaling pathways. This will allow a thorough investigation of the pathophysiological role of the receptor. In this study, we synthesized and evaluated a set of potential covalent ligands for the A_{2B}AR. The ligands all contain a core scaffold consisting of a substituted xanthine, varying in type and orientation of electrophilic group (warhead). Here, we find that the right combination of these variables is necessary for a high affinity, irreversible mode of binding and selectivity towards the A_{2B}AR. Altogether, this is the case for sulfonyl fluoride **24** (LUF7982), a covalent ligand that allows for novel ways to interrogate the A_{2B}AR.

RSC Med Chem **2022**, *13*, 850–856.

Introduction

The endogenous molecule adenosine acts as a signaling molecule on the G Protein-Coupled Receptor (GPCR) subfamily of adenosine receptors (ARs): the A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (A_1AR, A_{2A}AR, A_{2B}AR and A_3AR).[1] Elevated concentrations of adenosine have been observed in various pathological conditions, e.g. cancer, inflammation and hypoxia, implying an important role for AR signaling.[2,3] Antagonizing ARs and blocking the adenosine-induced signaling pathways is therefore an interesting strategy to tackle a broad spectrum of pathological conditions.[4]

A_{2B}AR receptor activation has been linked to hallmarks of cancer, i.e. cancer cell proliferation, tumor growth, tumor metastasis and the suppression of surrounding immune cells, among others.[5–7] In fact, multiple clinical trials are currently investigating the inhibition of the A_{2B}AR in cancers, e.g. in combination with an A_{2A}AR antagonist or immune stimulants.[5] Nevertheless, persistent high levels of extracellular adenosine in the tumor microenvironment might hinder the proper inhibition of A_{2B}AR-induced signaling pathways.

Covalent A_{2B}AR ligands on the other hand, cause an 'infinite' blockade of the A_{2B}AR which constitutes a new strategy that may be deployed in targeting cancer progression as well as studying the inhibition of A_{2B}AR signaling in cancerous cell lines and tissues.[8] After binding reversibly, covalent ligands react with an electrophilic substituent ('warhead') to a nearby amino acid residue, allowing the formation of an irreversible bond with the target protein.[9] This in turn leads to an 'infinite' occupancy of the ligand binding pocket, which in case of the A_{2B}AR would prevent even high levels of adenosine from binding to and activating the receptor.

Besides their medicinal potential, covalent ligands have proven especially useful as tools to study GPCR functioning, as they 'lock' the highly dynamic GPCRs into one conformation.[10] This facilitates purification, isolation and crystallization of the receptor and allows for a more thorough pharmacological characterization on a molecular level.[9,11]

Over the past decades, various high affinity xanthine derivatives have been developed as antagonists for the adenosine receptors.[12] In case of the A_{2B}AR these are mostly N^1, N^3-dipropylxanthines, developed by the lab of Jacobson,[13,14] and N^1-propylxanthines, developed by the lab of Müller.[15–17] While both classes exhibit high affinity, the latter type of compounds generally show higher selectivity towards the A_{2B}AR over the other adenosine receptors. This prompted us to design covalent xanthine derivatives, based on the N^1, N^3-dipropyl and N^1-propyl series. Looking at the A_{2A}AR, structurally the most similar to the A_{2B}AR, multiple covalent ligands have been developed.[18–20] A lysine residue on the second extracellular loop (EL2) of the A_{2A}AR is the target of at least one these ligands.[20] We therefore decided to substitute the herein synthesized xanthines with various electrophilic groups known to react with lysine residues. To increase the chances of covalent binding, we also varied the location of the warhead: either *meta*- or *para*-substituted at the C^8-phenyl ring.

Altogether, we have developed a set of twelve potential xanthine-based covalent ligands. Here, we show the synthesis, affinity, selectivity and covalent mode of action of these ligands.

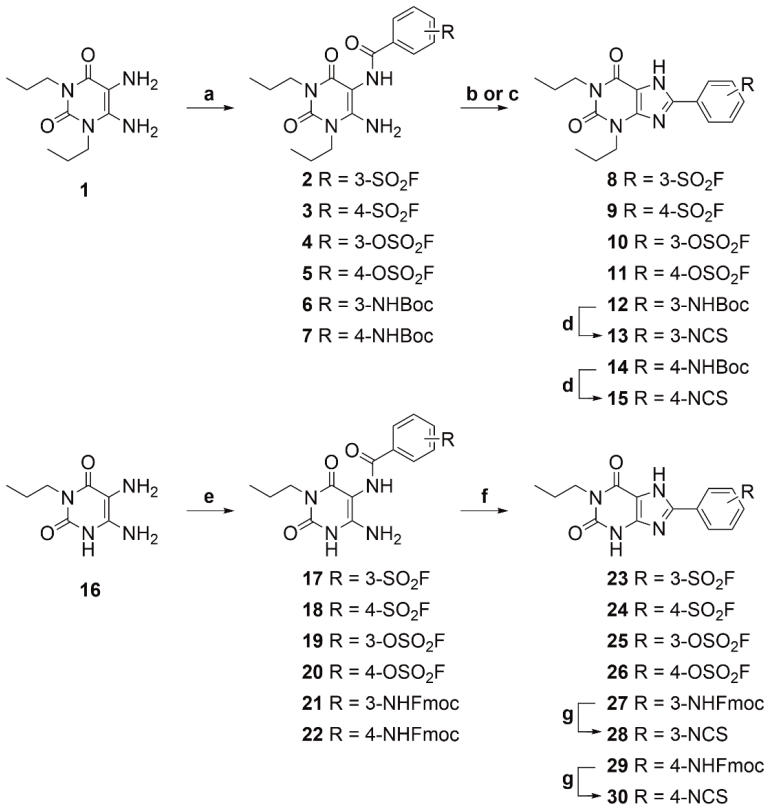
Results and Discussion

Design of covalent A_{2B}AR ligands

Analyzing the binding mode of xanthines into the A_{2A}AR binding pocket,[21,22] as well as the amino acid sequences of the A_{2A}AR and A_{2B}AR, we found three interesting potential anchors for covalent binding: lysines $K265^{EL3}$, $K267^{EL3}$ and $K269^{7.32}$.[23] In this respect, sulfonyl fluoride, fluorosulfonate and isothiocyanate groups were chosen to target either one of these lysine residues. Sulfonyl fluoride groups (-SO_2F) have recently emerged as warheads that have a weak intrinsic reactivity, are often stable under physiological conditions and, under the same conditions, can be directed to react selectively with lysine or tyrosine moieties on drug targets.[24–26] These beneficial properties have helped to coin the term 'SuFEx' (Sulfonyl Fluoride Exchange) as a type of 'click' chemistry.[27] However, even before the use of sulfonyl fluorides in click chemistry applications, they were incorporated in ligands for the A_1, A_{2A} and A_3 adenosine receptors.[20,28–31] Besides sulfonyl fluorides, we also decided to synthesize ligands containing fluorosulfonate groups (-OSO_2F). Fluorosulfonate groups have shown to bear a much lower intrinsic reactivity, as compared to sulfonyl fluoride groups,[25,32] which might reduce off-target binding events. Lastly, we chose the isothiocyanate group (-NCS) as warhead to be incorporated in the series of ligands. Although known for its reactivity towards cysteine residues, the isothiocyanate group has shown to form a more stable product upon reacting with lysine residues.[33,34] Moreover, the isothiocyanate group has been used to develop potent agonists and antagonists that irreversibly bind to the A_1AR.[35–37] In recent work from our lab, the isothiocyanate group was incorporated in a putative covalent ligand for the A_{2B}AR.[38] This inspired us to further investigate this electrophilic substituent as a warhead to target the A_{2B}AR.

Synthesis of covalent A_{2B}AR ligands

Twelve potential covalent ligands were targeted for synthesis, each containing one of the abovementioned electrophilic warheads at the *meta* or *para* position on the C8-substituted phenyl ring of the xanthines. The synthesis started with 1,3-dipropyl 5,6-diamino uracil (**1**) (commercially obtained), or 1-propyl 5,6-diamino uracil (**16**), synthesized according to procedures reported by Müller *et al.*[39–42] These building blocks were subjected to an EDC-mediated peptide coupling, using 3- or 4-fluorosulfonyl benzoic acid (**2**, **3**, **17** and **18**), 3- or 4-fluorosulfonate benzoic acid (**4**, **5**, **19** and **20**), or benzoic acid containing a protected amine group at the 3- or 4-position (**6**, **7**, **21** and **22**) (Scheme 1). Purification of Boc-protected anilines turned out to be cumbersome in case of the N^1-propyl series, therefore an Fmoc-protection was chosen instead. Next, the substituted uracil derivatives were subjected to a ring closure using trimethylsilyl polyphosphate (PPSE).[43,44] Gratifyingly, the electrophilic sulfonyl fluoride and fluorosulfonate groups stayed intact upon heating at 170 °C and in the presence of PSSE for several hours. In case of the Boc-protected anilines, basic conditions (reflux in 2 M NaOH) were chosen to achieve ring closure.[41] The anilines were then deprotected and subsequently subjected to thiophosgene to yield the corresponding isothiocyanates. Altogether this yielded sulfonyl fluoride-containing ligands **8**, **9**, **23** and **24**, fluorosulfonate-containing ligands **10**, **11**, **25** and **26**, and isothiocyanate-containing ligands **13**, **15**, **28** and **30.**



Scheme 1. Synthesis of potential covalent ligands for the A_{2B}AR. Reagents and conditions: (a) EDC·HCl, DIPEA, respective benzoic acid, dry DMF, rt, 2-20 h, 41-68%; (b) PPSE, 170 °C, 1-4 h, 10-53%; (c) 2 M NaOH, dioxane, 120 °C, 2-3 h, 60-84%; (d) (i) TFA, DCM, rt, 1 h; (ii) thiophosgene, 3 M HCl, rt, 2 h, 68-77%; (e) EDC·HCl, respective benzoic acid, dry DMF, rt, 1 h – 2 days, 13-54%; (f) PPSE, 150-170 °C, 2-7 h, 55-88%; (g) (i) piperidine, DMF, rt, 5 min; (ii) thiophosgene, 3 M HCl, rt, 2-4 h, 71-75%.

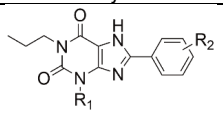
Assessment of time-dependent affinity towards the A_{2B}AR

To investigate the affinity of the twelve ligands and their potential to bind irreversibly to the A_{2B}AR, radioligand displacement assays were carried out using CHO-spap membranes stably overexpressing the A_{2B}AR. Two different conditions were chosen: no pre-incubation of receptor with ligand (pre 0 h) or a 4 h pre-incubation of receptor with ligand (pre 4 h), prior to the addition of radioligand. The pre-incubation step should allow any covalently binding ligand to irreversibly block the available receptor binding sites, thus increasing its apparent affinity for the receptor.[20,30,31] The reference A_{2B}AR antagonist PSB-1115 was taken along as a non-covalent control.

Interestingly, substitution of the chosen warheads onto the xanthines mostly increased the apparent affinity towards the A_{2B}AR (Table 1; pre 0 h), as compared to the affinity of PSB-1115 in our hands.[15] Various patterns were deducted. First of all, the *para*-substituted xanthines all show a higher apparent affinity than their *meta*-substituted counterparts at 0 h of pre-incubation. Secondly, at 0 h of pre-incubation, the N^1-propyl xanthines show a higher apparent affinity than the N^1, N^3-dipropyl xanthines. The best performing compounds are thus N^1-propyl

xanthines containing a *para*-substituted group. This is in line with the compounds presented in literature.[14–17] Looking at 4 h of pre-incubation (Table 1; pre 4 h), the SO_2F-substituted xanthines and NCS-substituted xanthines all show decent shifts in K_i (>3), regardless of the positioning of the warhead (*meta* or *para*) (Examples depicted in Figures 1A and 1C). On the other hand, the shifts observed for the OSO_2F-substituted xanthines are rather small, close to the values found for PSB-1115 (example in Figure 1B). This suggests a reversible binding mode. The SO_2F-containing xanthines have a higher affinity and K_i shift when substituted at the 4-position, while the NCS-containing xanthines show a higher shift when substituted at the 3-position. The biggest shifts are observed for the 3-NCS-substituted xanthines **13** and **28** (K_i shift of 19 and 74). This is probably the result of a low apparent affinity at 0 h of pre-incubation, in combination with the relatively high reactivity of the NCS group. This K_i shift data hints towards a covalent mode of action among the majority of the xanthine-based ligands.

Table 1. Time-dependent characterization of the synthesized adenosine A_{2B} receptor ligands.



Compound	R_1	R_2	*p*Ki (pre 0 h)[a]	*p*Ki (pre 4 h)[b]	Ki shift[c]
8	Propyl	3-SO_2F	6.02 ± 0.10	6.80 ± 0.16*	7.1
9	Propyl	4-SO_2F	7.22 ± 0.28	8.62 ± 0.18*	27.3
10	Propyl	3-OSO_2F	6.25 ± 0.14	6.28 ± 0.09	1.2
11	Propyl	4-OSO_2F	7.31 ± 0.04	7.29 ± 0.04	1.0
13	Propyl	3-NCS	6.21 ± 0.21	7.31 ± 0.15*	19.3
15	Propyl	4-NCS	7.62 ± 0.11	8.49 ± 0.18*	7.7
23	H	3-SO_2F	6.88 ± 0.08	7.88 ± 0.14**	10.2
24 (LUF7982)	H	4-SO_2F	8.10 ± 0.06	9.17 ± 0.12**	12.1
25	H	3-OSO_2F	6.85 ± 0.44	7.15 ± 0.32	1.6
26 (LUF7993)	H	4-OSO_2F	7.93 ± 0.22	8.26 ± 0.20	2.2
28	H	3-NCS	6.96 ± 0.27	8.55 ± 0.08**	74.3
30 (LUF8002)	H	4-NCS	8.67 ± 0.14	9.18 ± 0.01*	3.4
PSB-1115	H	4-SO_2OH	6.71 ± 0.09	6.72 ± 0.18	1.1

[a] Apparent affinity determined from displacement of specific [^{3}H]PSB-603 binding on CHO-spap cell membranes stably expressing hA_{2B}AR at 25 °C after 0.5 h co-incubation; [b] Apparent affinity determined from displacement of specific [^{3}H]PSB-603 binding on CHO-spap cell membranes stably expressing hA_{2B}AR at 25 °C with compounds pre-incubated for 4 h, followed by a 0.5 h co-incubation with [^{3}H]PSB-603. [c] K_i shift determined by ratio K_i(0 h)/K_i(4 h). Data represent the mean ± SEM of three individual experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$ compared to the *p*K_i values obtained from the displacement assay with 0 h pre-incubation of [^{3}H]PSB-603, determined by a two-tailed unpaired Student's t-test.

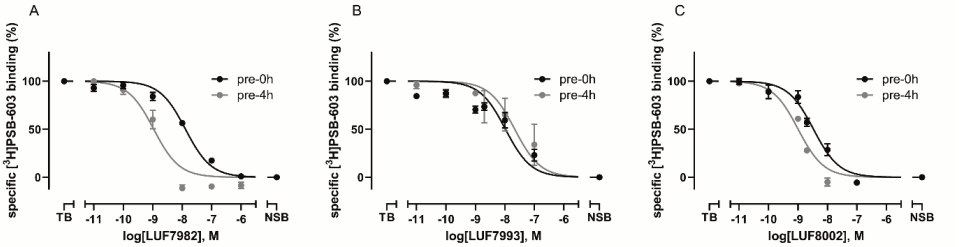


Figure 1. Displacement of [^{3}H]PSB-603 from the A_{2B}AR by (A) SO_2F-substituted LUF7982; (B) OSO_2F-substituted LUF7993 and (C) NCS-substituted LUF8002. Displacement measured after 0 or 4 h of pre-incubation of the respective ligand with CHO-spap membranes stably overexpressing the A_{2B}AR. Data represent the mean ± SEM of three individual experiments performed in duplicate.

Evaluation of binding towards the other adenosine receptors

As mentioned in the introduction, xanthine-based ligands are prone to promiscuous AR binding. To investigate the selectivity of the synthesized ligands towards the A_{2B}AR specifically, radioligand displacement experiments were carried out using 1 µM of ligand on CHO (A_1AR and A_3AR) or HEK (A_{2A}AR) membranes stably overexpressing the respective other adenosine receptor (Table 2). Similar to the experiments for the A_{2B}AR, the compounds were tested either with or without 4 hours of pre-incubation prior to radioligand addition. In our experiments, hardly any ligand showed a strong displacement of radioligand from the structurally similar A_{2A}AR. Only compound **11** seems to bind decently, showing a displacement that exceeds 50%. In case of the A_1AR, all of the N^1,N^3-dipropyl xanthines (**8**-**13** and **15**) show a strong displacement (>50%) of radioligand from the receptor. This is in line with earlier reports on such substituted N^1,N^3-dipropyl xanthines as generally excellent A_1AR antagonists.[28,45,46]

Considering the isothiocyanates (**13**, **15**, **28** and **30**), a moderate to high displacement of radioligand from A_1 and A_3 receptors was observed. Interestingly, the 3-NCS substituted xanthines (**13** and **28**) seem to perform especially well at the A_3AR. A notable loss of displacement at the other adenosine receptors is observed upon removal of the N^3-propyl group. Also the introduction of a sulfonyl group has a beneficial effect on selectivity towards the A_{2B}AR over the other ARs. This group might be stabilized by interactions with K269$^{7.32}$, not present in any of the other ARs.[17] This is especially seen for the OSO_2F-containing **26** (LUF7993), showing the highest selectivity for the A_{2B}AR. Among the compounds with the highest apparent pK_i values, **24** (LUF7982) shows a good selectivity towards the A_{2B}AR and about 50% displacement of radioligand at the A_{2A}AR. The latter suggests **24** (LUF7982) displays a 100-fold selectivity for the A_{2B}AR without pre-incubation (and >1000-fold after 4 h of pre-incubation). Besides, the displacement at the A_{2A}AR is not time-dependent and therefore it is expected that **24** (LUF7982) does not bind covalently to the A_{2A}AR. The high affinity compound **30** (LUF8002) on the other hand, also binds to the A_1AR and A_3AR.

Table 2. Radioligand displacement of the synthesized adenosine A_{2B} receptor ligands on other adenosine receptors.

	(%) displacement at 1 µM					
	hA$_1$AR[a]		hA$_{2A}$AR[b]		hA$_3$AR[c]	
Compound	**0 h**	**4 h**	**0 h**	**4 h**	**0 h**	**4 h**
8	52	61	19	23	30	43
9	61	69	54	55	12	5
10	76	77	13	12	64	58
11	79	85	63	69	47	49
13	73	96	47	34	89	100
15	77	92	40	39	48	96
23	6	36	20	6	14	10
24 (LUF7982)	29	41	52	43	7	9
25	6	1	2	0	14	5
26 (LUF7993)	17	12	35	24	8	8
28	7	16	0	6	58	95
30 (LUF8002)	51	97	25	27	15	66

[a] % displacement at 1 µM concentration of specific [^{3}H]DPCPX binding on CHO cell membranes stably expressing hA$_1$AR pre-incubated with the compounds for 4 or 0 hours at 25 °C, followed by a co-incubation with [^{3}H]DPCPX for 0.5h at 25 °C. [b] % displacement at 1 µM concentration of specific [^{3}H]ZM241385 binding on HEK293 cell membranes stably expressing hA$_{2A}$AR pre-incubated with the compounds for 4 or 0 hours at 25 °C, followed by a co-incubation with [^{3}H]ZM241385 for 0.5h at 25 °C. [c] % displacement at 1 µM concentration of specific [^{3}H]PSB-11 binding on CHO cell membranes stably expressing hA$_3$AR pre-incubated with the compounds for 4 or 0 hours at 25 °C, followed by a co-incubation with [^{3}H]PSB-11 for 0.5h at 25 °C. Data represent the mean of two individual experiments performed in duplicate.

Evaluation of the covalent mode of action of selected compounds

As final validation of the putative covalent mode of binding, wash-out experiments were performed using the compounds highest in affinity and selectivity: **24** (LUF7982), **26** (LUF7993) and **30** (LUF8002). PSB-1115 was taken along as reversible control compound. CHO-spap membranes stably overexpressing the A_{2B}AR were incubated with ligand, followed by either a four-cycle wash treatment or no washing (control), before being exposed to radioligand (Figure 2). Both PSB-1115 and **26** (LUF7993) show an almost full recovery of radioligand binding after washing, indicating that all receptor-bound ligand has been washed away. These results correspond to the previously observed K_i shifts (Table 1), in which no great shifts were observed for PSB-1115 and the OSO_2F-containing xanthines. Of note: it is possible that LUF7993 forms an adduct with the receptor, which is then hydrolyzed to produce a sulfonylated lysine and a reversibly bound phenol.[32] **24** (LUF7982) and **30** (LUF8002) on the other hand, show a persistent mode of binding, with no recovery of radioligand binding after four wash treatments (Figure 2). **24** (LUF7982) and **30** (LUF8002) thus form a stable adduct with the A_{2B}AR, resistant to multiple washing steps and are therefore most likely covalent ligands for the A_{2B}AR. **24** (LUF7982) is the most interesting of these two irreversible compounds due to its high selectivity towards the A_{2B}AR. This compound was therefore further examined in docking experiments.

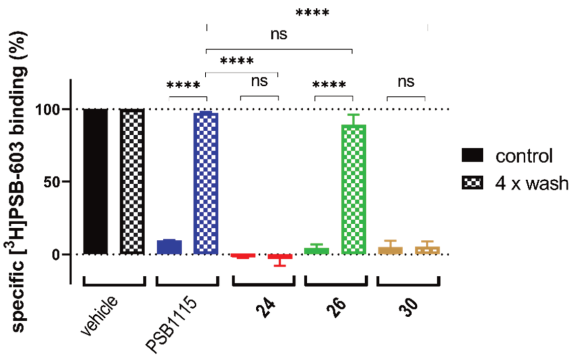


Figure 2. Wash-out assays on the adenosine A_{2B} receptor using the *N*^{1}-propyl xanthines with *para*-substituted warheads. CHO-spap cell membranes stably expressing the adenosine A_{2B} receptor were pre-incubated with buffer (vehicle) or 1 µM of ligand (10 µM in case of PSB-1115), followed by a four-cycle washing treatment (4 x wash) or no washing at all (control) before being exposed to [^{3}H]PSB-603. Data represent the mean ± SEM of three individual experiments performed in duplicate. Statistics were determined using unpaired student's *t* tests. ns: no significant difference; ****P < 0.0001.

Docking of LUF7982 into the A_{2B}AR binding pocket

To predict the binding mode of LUF7982, we generated a model of the A_{2B}AR-LUF7982 binding site based on homology modelling and docking. The first step was to identify the orientation of the xanthine core. The orientation of this chemotype in the AR family binding site is well studied and typically involves hydrogen bonding with N254$^{6.55}$ and π-π stacking with F173^{EL2}.[47] This pattern was also observed for the predicted A_{2B}AR-LUF7982 binding complex (Figure 3A). This leaves the warhead of LUF7982 oriented towards the extracellular vestibule, pointing towards the region of the third extracellular loop (EL3). As mentioned in the introduction, three lysine residues (K265^{EL3}, K267^{EL3} and K269$^{7.32}$) in and near EL3 were identified as potential attachment point for covalent binding of the compounds herein reported. In our model, two out of three lysine residues were in close vicinity to the warhead, namely K267^{EL3} and K269$^{7.32}$ (5.7 and 3.9 Å, respectively) (Figure 3B). K267^{EL3} is predicted to form a salt bridge with E174^{EL2}, similar to the salt bridge observed between a histidine and glutamic acid in the A_{2A}AR.[47]

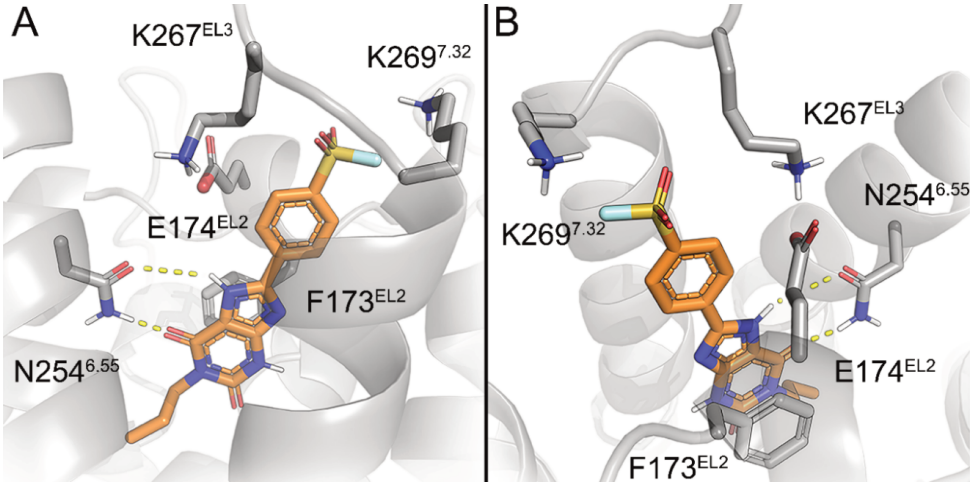


Figure 3. Predicted binding mode of LUF7982. Panel A: overview of the key interactions of LUF7982 in the binding site, which include two hydrogen bonds (yellow dashed lines) with N254$^{6.55}$ and π-π stacking with F173^{EL2}, both are conserved interactions in adenosine receptor ligand recognition. The sulfonyl fluoride warhead points towards the extracellular vestibule. Panel B: top view of the A_{2B}AR-LUF7982 binding pocket, showing potential lysine residues involved in covalent binding (K267^{EL3} and K269$^{7.32}$).

K265^{EL3} on the other hand was too far away from the warhead in our model (10.6 Å) to form a plausible target for covalent attachment. Whilst K267^{EL3} thus is within range, it would be energetically more unfavorable to disrupt the formed salt-bridge, and we therefore expect that K269$^{7.32}$ is the most likely target for covalent attachment. Lysine residues K265^{EL3} and K269$^{7.32}$ are not present on the other three adenosine receptors, while K267^{EL3} is also present on the A_1AR. A covalent mode of binding involving K269$^{7.32}$ might therefore further explain the selectivity of **24** (LUF7982) towards the A_{2B}AR. Further studies using single point mutations in the receptor would need to be carried out to prove this.

Conclusion

Herein we present the development of a set of twelve novel xanthine ligands for the A_{2B}AR, all containing electrophilic groups to covalently target lysine residue(s) on the receptor. The xanthine moiety is a well-known and promiscuous scaffold for all four of the adenosine receptors. Nevertheless, among the synthesized ligands, sulfonyl fluoride- and fluorosulfonate-substituted xanthines appear to be highly selective towards the A_{2B}AR over the other adenosine receptors. This selectivity might be explained by the covalent (SO_2F) and/or non-covalent (OSO_2F) interactions with lysine residue K269$^{7.32}$. Isothiocyanate (NCS)-containing ligands on the other hand, showed to be less selective towards the A_{2B}AR. This is most likely due to a higher intrinsic reactivity of the NCS group. Furthermore, sulfonyl fluoride **24** (LUF7982) showed persistent binding to the A_{2B}AR in radioligand displacement and wash-out assays. This points towards a covalent mode of action of the respective compound.

The A_{2B}AR is an emerging drug target that has been found to play a role in a broad spectrum of pathologies, such as cancers and immune disorders. Antagonizing adenosine signaling through inhibition of the A_{2B}AR is therefore an interesting strategy to tackle a broad spectrum of conditions. Having covalent ligands for the A_{2B}AR will pave the way for studies towards irreversible blockade of the receptor, e.g. in biochemical assays. LUF7982 might thus be used to study the behavior of the A_{2B}AR in pathological conditions, to obtain insight in the structure of the A_{2B}AR and to pharmacologically characterize the receptor.

Experimental

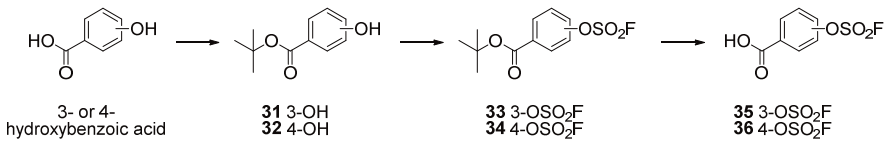
Chemistry

General

All commercially available reagents and solvents were obtained from Sigma Aldrich, Fisher Scientific, VWR chemicals, Biosolve and J&K Scientific. All reactions were carried out under an N_2 atmosphere, unless noted otherwise. Thin layer chromatography was performed on TLC Silica gel 60 F254 (Merck) and visualized using UV irradiation at a wavelength of 254 or 366 nM. ^{1}H NMR, ^{13}C NMR and ^{19}F NMR spectra were recorded on a Bruker AV-400 (400 MHz), Bruker AV-500 spectrometer (500 MHz) or Bruker AV-600 spectrometer (600 MHz). Chemical shift values are reported in ppm (δ) using tetramethylsilane or solvent resonance as the internal standard. Coupling constants (J) are reported in Hz. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), h (hexuplet) or m (multiplet) followed by the number of represented hydrogen atoms. Compound purity was determined by LC-MS, using a LCMS-2020 system (Shimadzu) coupled to a Gemini® 3 µm C18 110Å column (50 x 3 mm). In brief, compounds were dissolved in H2O:MeCN:t-BuOH 1:1:1, injected onto the column and eluted with a linear gradient of H2O:MeCN 90:10 + 0.1% formic acid to H2O:MeCN 10:90 + 0.1% formic acid over the course of 15 minutes.

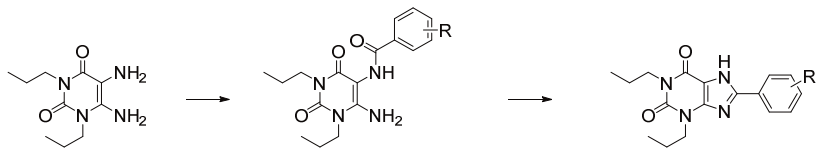
Synthetic Procedures

Dipropyl-substituted xanthines



3- or 4-hydroxybenzoic acid | **31** 3-OH, **32** 4-OH | **33** 3-OSO_2F, **34** 4-OSO_2F | **35** 3-OSO_2F, **36** 4-OSO_2F

Scheme 1 Synthesis of 3- and 4-fluorosulfonate benzoic acid.

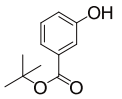


1

2 R = 3-SO_2F
3 R = 4-SO_2F
4 R = 3-OSO_2F
5 R = 4-OSO_2F
6 R = 3-NHBoc
7 R = 4-NHBoc

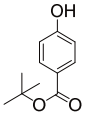
8 R = 3-SO_2F
9 R = 4-SO_2F
10 R = 3-OSO_2F
11 R = 4-OSO_2F
12 R = 3-NHBoc → **13** R = 3-NCS
14 R = 4-NHBoc → **15** R = 4-NCS

Scheme 2 Synthesis of the N^1, N^3-dipropyl-substituted xanthines.



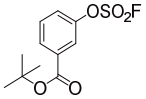
***tert*-Butyl 3-hydroxybenzoate (31)**[48]

3-Hydroxybenzoic acid (849 mg, 6.15 mmol, 1.0 eq) was suspended in dry benzene (100 mL) and refluxed. N,N-Dimethylformamide di-*tert*-butyl acetal (5.0 g, 24.59 mmol, 4.0 eq) was added dropwise over 20 minutes and the mixture was refluxed an additional 30 minutes. The mixture was then cooled, washed with water (50 mL), a saturated $NaHCO_3$ solution (2 x 50 mL) and brine (50 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (Pentane:EtOAc 5:1) to yield **31** as a colorless oil (440 mg, 2.27 mmol, 37%). **TLC** (Pentane:EtOAc 5:1) R_f = 0.43. **^{1}H NMR** (400 MHz, $CDCl_3$) δ [ppm] = 7.64 (dd, *J* = 2.7, 1.6 Hz, 1H), 7.54 (dt, *J* = 7.7, 1.3 Hz, 1H), 7.48 (s, 1H), 7.27 (t, *J* = 7.9 Hz, 1H), 7.08 (ddd, *J* = 8.1, 2.6, 1.0 Hz, 1H), 1.58 (s, 9H). **HPLC** 99%, RT 9.686 min. **LC-MS** [ESI - H]$^+$: 193.00.



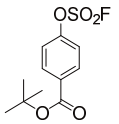
***tert*-Butyl 4-hydroxybenzoate (32)**[49]

tert-Butanol (49.5 mL, 521 mmol, 36.0 eq) was added to a solution of 4-hydroxybenzoic acid (2.0 g, 14.48 mmol, 1.0 eq) and DMAP (88 mg, 0.72 mmol, 0.05 eq) in dry THF (50 mL). A solution of DCC (3.0 g, 14.48 mmol, 1.0 eq) in dry THF (50 mL) was added dropwise over 30 minutes. The mixture was stirred overnight at rt. The formed side-product was removed by filtration and the filtrate was concentrated, dissolved in DCM. A saturated $NaHCO_3$ solution was added and the aqueous layer was extracted with DCM (3 x), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (Pentane:EtOAc 5:1) to yield **32** as a white solid (1.4 g, 7.41 mmol, 51%). **TLC** (Pentane:EtOAc 5:1) R_f = 0.50. **^{1}H NMR** (400 MHz, $CDCl_3$) δ [ppm] = 7.89 (d, *J* = 8.8 Hz, 2H), 6.86 (d, *J* = 8.8 Hz, 2H), 1.59 (s, 9H).



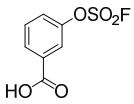
***tert*-Butyl 3-((fluorosulfonyl)oxy)benzoate (33)**[50]

[4-(Acetylamino)phenyl]imidosulfuryl difluoride (AISF) (854 mg, 2.72 mmol, 1.2 eq) was added to a solution of **31** (440 mg, 2.27 mmol, 1.0 eq) in dry THF (10 mL). DBU (751 µL, 4.98 mmol, 2.2 eq) was added and the mixture was stirred at rt for 1 h. EtOAc (50 mL) and 0.5 M HCl (50 mL) were added and the mixture was extracted with EtOAc (3 x 50 mL). The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (Pentane:EtOAc 9:1) to yield **33** as a colorless oil (571 mg, 2.07 mmol, 91%). **TLC** (Pentane:EtOAc 9:1) R_f = 0.90. **^{1}H NMR** (400 MHz, $CDCl_3$) δ [ppm] = 8.05 (dt, *J* = 7.4, 1.5 Hz, 1H), 7.96 – 7.92 (m, 1H), 7.58 – 7.53 (m, 1H), 7.53 – 7.48 (m, 1H), 1.61 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ [ppm] = 163.7, 150.0, 134.9, 130.4, 129.7, 124.9, 122.1, 82.5, 28.2.



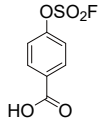
***tert*-Butyl 4-((fluorosulfonyl)oxy)benzoate** (**34**)

AISF (777 mg, 2.47 mmol, 1.2 eq) was added to a solution of **32** (400 mg, 2.06 mmol, 1.0 eq) in dry THF (10 mL). DBU (683 µL, 4.53 mmol, 2.2 eq) was added and the mixture was stirred at rt for 1 h. EtOAc (50 mL) and 0.5 M HCl (50 mL) were added and the aqueous layer was extracted with EtOAc (3 x 50 mL). The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (Pentane:EtOAc 9:1) to yield **34** as a colorless oil (494 mg, 1.79 mmol, 87%). **TLC** (DCM:MeOH 98:2) R_f = 0.83. **^{1}H NMR** (400 MHz, $CDCl_3$) δ [ppm] = 8.12 (d, *J* = 8.9 Hz, 2H), 7.39 (dd, *J* = 9.0, 0.9 Hz, 2H), 1.60 (s, 9H).



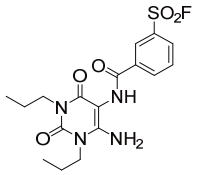
3-((Fluorosulfonyl)oxy)benzoic acid (**35**)

TFA (8.27 mL, 107 mmol, 60.0 eq) was added to a solution of **33** (571 mg, 2.07 mmol, 1.0 eq) in DCM (8 mL). The mixture was stirred for 3 h at rt, after which the reaction showed full completion. The solvents were evaporated under reduced pressure to yield **35** as a white solid (455 mg, 2.07 mmol, quant). **^{1}H NMR** (400 MHz, $CDCl_3$) δ [ppm] = 11.83 (s, 1H), 8.23 – 8.17 (m, 1H), 8.10 (s, 1H), 7.68 – 7.60 (m, 2H).



4-((Fluorosulfonyl)oxy)benzoic acid (**36**)

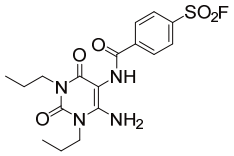
TFA (8.27 mL, 107 mmol, 60.0 eq) was added to a solution of **34** (494 mg, 1.79 mmol, 1.0 eq) in DCM (8 mL). The mixture was stirred for 3 h at rt, after which the reaction showed full completion. The solvents were evaporated under reduced pressure to yield **36** as a white solid (394 mg, 1.79 mmol, quant). **^{1}H NMR** (400 MHz, $CDCl_3$) δ [ppm] = 8.26 (d, *J* = 9.0 Hz, 2H), 7.48 (d, *J* = 8.5 Hz, 2H).



3-((6-Amino-2,4-dioxo-1,3-dipropyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)benzenesulfonyl fluoride (**2**)

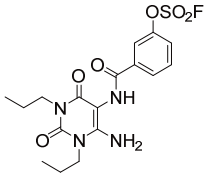
EDC·HCl (933 mg, 4.86 mmol, 1.1 eq) and DIPEA (770 µL, 4.42 mmol, 1.0 eq) were added to a solution of 5,6-diamino-1,3-dipropyluracil (**1**) hydrochloride (1511 mg, 5.75 mmol, 1.3 eq) and 3-(fluorosulfonyl)benzoic acid (903 mg, 4.42 mmol, 1.0 eq) in dry DMF (21 mL). The mixture was stirred overnight at rt. EtOAc (15 mL) was added and the organic layer was washed with water (400 mL) and brine (100 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 99:1 →

97:3) to yield **2** as yellow solid (1085 mg, 2.63 mmol, 60%). **TLC** (DCM:MeOH 98:2) R_f = 0.27. **^{1}H NMR** (400 MHz, CD_3OD) δ [ppm] = 8,75 (s, *J* = 1.8 Hz, 1H), 8.51 (d, *J* = 7.9, 1.4 Hz, 1H), 8.28 (d, *J* = 8.1, 1.3Hz, 1H), 7,89 (t, *J* = 7.9 Hz, 1H), 3.98 – 3.92 (t, 2H), 3.92 – 3.86 (t, 2H), 1.79 – 1.70 (d, 2H), 1.70 – 1.61 (d, 2H), 1.02 (t, *J* = 7.4 Hz, 3H), 0.96 (t, *J* = 7.5 Hz, 3H).



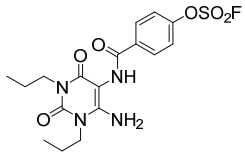
4-((6-Amino-2,4-dioxo-1,3-dipropyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)benzenesulfonyl fluoride (**3**)

EDC·HCl (932 mg, 4.86 mmol, 1.1 eq) and DIPEA (752 µL, 4.32 mmol, 1.0 eq) were added to a solution of 5,6-diamino-1,3-dipropyluracil (**1**) hydrochloride (1161 mg, 4.42 mmol, 1.0 eq) and 4-(fluorosulfonyl)benzoic acid (902 mg, 4.42 mmol, 1.0 eq) in dry DMF (20 mL). The mixture was stirred for 2.5 h at rt. EtOAc (150 mL) was then added and the organic layer was washed with water (150 mL). The aqueous layer was extracted with EtOAc (2 x 50 mL). The organic layers were combined, washed with water (3 x 100 mL), brine (100 mL), combined, dried over $MgSO_4$ and concentrated under reduced pressure to yield **3** as an off-white solid (749 mg, 1.82 mmol, 41%). **TLC** (Pentane:EtOAc 1:1) R_f = 0.49. **^{1}H NMR** (400 MHz, $(CD_3)_2CO$) δ [ppm] = 8.34 (d, *J* = 8.3 Hz, 2H), 8.19 (d, *J* = 8.5 Hz, 2H), 6.46 (s, 1H), 3.97 (t, *J* = 7.8 Hz, 2H), 3.82 (t, *J* = 7.3 Hz, 2H), 1.72 (h, *J* = 7.4 Hz, 2H), 1.58 (h, *J* = 7.5 Hz, 2H), 0.94 (t, *J* = 7.4 Hz, 3H), 0.87 (t, *J* = 7.5 Hz, 3H).



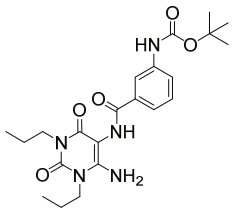
3-((6-Amino-2,4-dioxo-1,3-dipropyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)phenyl sulfurofluoridate (**4**)

EDC·HCl (420 mg, 2.19 mmol, 1.1 eq) was added to a solution of **35** (439 mg, 1.99 mmol, 1.0 eq) in dry DMF (10 mL). 5,6-Diamino 1,3-dipropyl uracil (**1**) hydrochloride (524 mg, 1.99 mmol, 1.0 eq) was added and the mixture became a pink solution. DIPEA (693 µL, 3.98 mmol, 2.0 eq) was added and to form a clear orange solution. The mixture was then stirred for 4 h, upon which no starting material was detected anymore by LCMS. EtOAc (50 mL) was added and the organic mixture was washed with brine (3 x 50 mL). The aqueous layers were combined and back-extracted with EtOAc (3 x 50 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica column chromatography to yield **4** as yellow substance (329 mg, 0.77 mmol, 39%). **TLC** (DCM:MeOH 96:4): Rf = 0.41. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 9.17 (s, 1H), 8.16 – 8.10 (m, 2H), 7.80 (dd, *J* = 8.3, 1.4 Hz, 1H), 7.72 (t, *J* = 8.0 Hz, 1H), 6.84 (s, 2H), 3.85 (t, *J* = 8.1, 7.2 Hz, 2H), 3.73 (t, *J* = 7.2 Hz, 2H), 1.60 – 1.54 (m, 2H), 1.53 – 1.47 (m, 2H), 0.89 (t, *J* = 7.4 Hz, 3H), 0.83 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 164.6, 159.0, 151.7, 150.4, 149.4, 137.3, 130.7, 128.7, 123.8, 120.5, 86.8, 43.7, 41.9, 20.9, 20.8, 11.2, 10.7. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ 39.0.



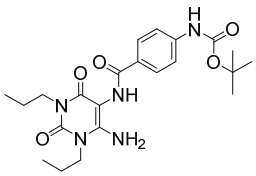
4-((6-Amino-2,4-dioxo-1,3-dipropyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)phenyl sulfurofluoridate (**5**)

EDC·HCl (312 mg, 1.63 mmol, 0.9 eq) was added to a solution of **36** (376 mg, 1.71 mmol, 0.9 eq) in dry DMF (7.5 mL). The mixture was stirred for 30 min and then 5,6-diamino 1,3-dipropyl uracil (**5**) hydrochloride (476 mg, 1.81 mmol, 1.0 eq) and DIPEA (257 µL, 1.48 mmol, 1.0 eq) were added. The mixture was stirred for 5 h, upon which DIPEA (257 µL, 1.48 mmol, 1.0 eq) was added. The mixture was then stirred overnight. EtOAc (25 mL) was added and the organic layer was washed with water (2 x 25 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (PE:EtOAc 7:3 → 0:1) to yield **5** as an orange solid (387 mg, 0.90 mmol, 50%). **TLC** (DCM:MeOH 98:2) R_f = 0.53. **^{1}H NMR** (500 MHz, $(CD_3)_2CO$) δ [ppm] = 9.13 (s, 1H), 8.18 (d, *J* = 8.9 Hz, 2H), 7.44 (d, *J* = 8.7 Hz, 2H), 6.63 (s, 2H), 3.88 (t, *J* = 7.8 Hz, 2H), 3.77 (t, *J* = 7.2 Hz, 2H), 1.65 (h, *J* = 7.2 Hz, 2H), 1.52 (h, *J* = 7.5 Hz, 2H), 0.91 (t, *J* = 7.4 Hz, 3H), 0.82 (t, *J* = 7.5 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2CO$) δ [ppm] = 165.1, 160.5, 152.1, 151.6, 150.2, 134.6, 130.4, 120.5, 88.0, 44.2, 42.3, 21.0, 21.0, 10.6, 10.2. **^{19}F NMR** (471 MHz, $(CD_3)_2CO$) δ [ppm] = 38.2.

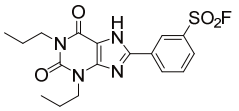


***tert*-Butyl (3-((6-amino-2,4-dioxo-1,3-dipropyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)phenyl)carbamate** (**6**)

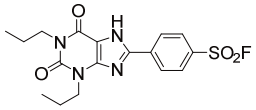
3-(Boc-amino)benzoic acid (474 mg, 2.00 mmol, 1.0 eq), EDC·HCl (422 mg, 2.20 mmol, 1.1 eq) and DIPEA (350 µL, 2.00 mmol, 1.0 eq) were added to a solution of 5,6-diamino 1,3-dipropyl uracil (**1**) hydrochloride (525 mg, 2.00 mmol, 1.0 eq) in dry DMF (10 mL). The mixture was stirred for 3 h, after which another 2.0 equivalents of DIPEA were added (350 µL, 2.00 mmol, 1.0 eq). The mixture was stirred for another hour and then diluted with EtOAc (80 mL). The organic layer was washed with water (3 x 80 mL), brine (80 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 90:10 → 95:5) to yield **6** as an off-white solid (446 mg, 1.00 mmol, 50%). **TLC** (DCM:MeOH 97:3) R_f = 0.42. **^{1}H NMR** (400 MHz, $(CD_3)_2CO$) δ [ppm] = 8.05 (t, *J* = 2.0 Hz, 1H), 7.70 (dd, *J* = 8.0, 1.5 Hz, 1H), 7.62 (ddd, *J* = 7.8, 1.8, 1.1 Hz, 1H), 7.36 (t, *J* = 7.9 Hz, 1H), 3.94 (t, *J* = 7.8 Hz, 2H), 3.80 (t, *J* = 7.3 Hz, 2H), 1.72 (h, *J* = 7.1 Hz, 2H), 1.65 – 1.51 (m, 2H), 1.49 (s, 9H), 0.94 (t, *J* = 7.4 Hz, 3H), 0.87 (t, *J* = 7.5 Hz, 3H).



***tert*-Butyl (4-((6-amino-2,4-dioxo-1,3-dipropyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)phenyl)carbamate** (**7**)
4-(Boc-amino)benzoic acid (237 mg, 1.00 mmol, 1.0 eq), EDC·HCl (211 mg, 1.10 mmol, 1.1 eq) and DIPEA (174 µL, 1.00 mmol, 1.0 eq) were added to a solution of 5,6-diamino 1,3-dipropyl uracil (**1**) hydrochloride (263 mg, 1.00 mmol, 1.0 eq) in dry DMF (5 mL). The mixture was stirred for 4 h, after which LCMS indicated full consumption of starting material. EtOAc (50 mL) was added and the organic layer was washed with water (2 x 50 mL) and brine (50 mL). The aqueous layers were combined and back-extracted with EtOAc (2 x 50 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 99:1 → 90:10) to yield **7** as an off-white solid (302 mg, 0.68 mmol, 68%). **TLC** (DCM:MeOH 95:5) R_f = 0.27. **^{1}H NMR** (400 MHz, CD_3OD) δ [ppm] = 7.93 (d, *J* = 8.8 Hz, 2H), 7.49 (d, *J* = 8.8 Hz, 2H), 3.88 – 3.81 (m, 2H), 3.81 – 3.75 (m, 2H), 1.68 – 1.56 (m, 4H), 1.54 (s, 9H), 0.96 (t, *J* = 7.4 Hz, 3H), 0.90 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (101 MHz, MeOD) δ [ppm] = 170.3, 162.1, 154.7, 154.2, 152.2, 144.4, 130.0, 128.4, 118.4, 89.0, 81.2, 45.7, 44.0, 28.7, 22.2, 22.0, 11.6, 11.2.

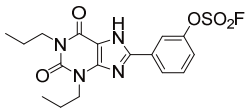


3-(2,6-Dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)benzenesulfonyl fluoride (**8**)
PPSE (approximately 5 mL) was added to **2** (1085 mg, 2.63 mmol, 1.0 eq). The mixture was refluxed for 4 h at 170 °C and afterwards cooled down to rt overnight. MeOH (50 mL) was added and the formed residue was collected by filtration and subsequently purified by silica column chromatography (DCM:MeOH 99.5:0.5 → 85:15) to yield **8** as an off-white solid (281 mg, 0.71 mmol, 27%). **TLC** (DCM:MeOH 99.5:0.5) R_f = 0.60. **^{1}H NMR** (400 MHz, $(CD_3)_2SO$) δ [ppm] = 14.32 (s, 1H), 8.83 (t, *J* = 1.7 Hz, 1H), 8.61 (d, *J* = 8.0 Hz, 1H), 8.22 (d, *J* = 8.9 Hz, 1H), 7.93 (t, *J* = 8.0 Hz, 1H), 4.02 (t, *J* = 7.4 Hz, 3H), 3.86 (t, *J* = 7.4 Hz, 2H), 1.74 (hept, *J* = 7.4 Hz, 2H), 1.58 (hept, *J* = 7.5 Hz, 2H), 0.91 (d, *J* = 7.4 Hz, 3H), 0.87 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (101 MHz, $(CD_3)_2SO$) δ [ppm] = 155.1, 151.5, 149.0, 147.9, 134.8, 133.6 (d, *J* = 24.1 Hz), 132.4, 131.7. 130.3, 126.4, 109.6, 45.5, 43.2, 21.8, 21.8, 12.1, 12.0. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 66.5. **HPLC** 100%, RT 11.292 min. **LC-MS** $[ESI + H]^+$: 395.05.

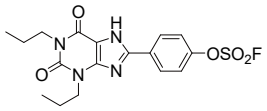


4-(2,6-Dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)benzenesulfonyl fluoride (**9**)
PPSE (approximately 2 mL) was added to **3** (500 mg, 1.21 mmol, 1.0 eq), refluxed for 1 h at 170 °C and afterwards cooled down to rt. MeOH was added and the product was allowed to crystallize overnight. The residue was collected and purified by silica column chromatography (DCM:MeOH 99.5:0.5 → 98.5:1.5) to yield **9** as an off-white solid (46 mg, 0.12 mmol, 10%). **TLC** (DCM:MeOH 98:2) R_f = 0.40. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 14.41 (s, 1H), 8.47 (d, *J* = 8.6 Hz, 2H), 8.29 (d, *J* = 8.7 Hz, 2H), 4.03 (t, *J* = 7.2 Hz, 2H), 3.88 (t, *J* = 7.3 Hz, 2H), 1.75 (h, *J* = 7.4 Hz, 2H), 1.59 (h, *J* = 7.5 Hz, 2H), 0.91 (t, *J* = 7.5 Hz, 3H), 0.88 (t, *J* = 7.4 Hz,

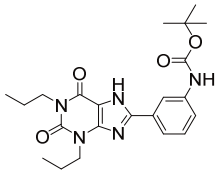
3H). **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 66.8. **HPLC:** 97%, RT 11.395 min. LC-MS [ESI + H]+: 395.05.



3-(2,6-Dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)phenyl sulfurofluoridate (**10**)
PPSE (2 mL) was added to **4** (329 mg, 0.77 mmol, 1.0 eq) and refluxed at 170 °C. The mixture was stirred for 4 h and afterwards cooled down to rt. Water (50 mL) was added and the aqueous mixture was extracted with DCM (3 x 50 mL). The organic layers were combined, washed with brine (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 99.5:0.5 → 98:2) to yield **10** as a white solid (186 mg, 0.45 mmol, 59%). **TLC** (DCM:MeOH 99.5:0.5): Rf = 0.44. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ 14.11 (s, 1H), 8.28 – 8.20 (m, 2H), 7.77 – 7.68 (m, 2H), 4.01 (t, *J* = 7.2 Hz, 2H), 3.86 (t, *J* = 7.4 Hz, 2H), 1.74 (h, *J* = 7.4 Hz, 2H), 1.58 (h, *J* = 7.4 Hz, 2H), 0.89 (dt, *J* = 12.1, 7.4 Hz, 6H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ 154.2, 150.6, 150.0, 148.1, 147.5, 131.7, 131.4, 127.0, 122.5, 118.5, 108.4, 44.5, 42.2, 20.8, 11.2, 11.0. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ 39.6.

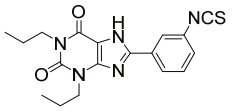


4-(2,6-Dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)phenyl sulfurofluoridate (**11**)
PPSE (2 mL) was added to **5** (387 mg, 0.90 mmol, 1.0 eq) and refluxed at 170 °C. The mixture was stirred for 4 h and afterwards cooled down to rt. Water (50 mL) was added and the aqueous layer was extracted with DCM (3 x 25 mL). The organic layers were combined, washed with brine (50 mL) and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 99.75:0.25 → 99.5:0.5) to yield **11** as a white solid (198 mg, 0.48 mmol, 53%). **TLC** (DCM:MeOH 98:2) R_f = 0.68. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 14.06 (s, 1H), 8.32 – 8.26 (m, 2H), 7.76 (d, *J* = 8.9 Hz, 2H), 4.01 (t, *J* = 6.9 Hz, 2H), 3.90 – 3.83 (m, 2H), 1.74 (h, *J* = 7.4 Hz, 2H), 1.58 (h, *J* = 7.5 Hz, 2H), 0.90 (t, *J* = 7.4 Hz, 3H), 0.87 (t, *J* = 7.5 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 154.2, 150.6, 150.3, 148.2, 148.0, 129.6, 128.8, 121.9, 108.4, 44.5, 42.2, 20.8, 20.8, 11.2, 11.0. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 39.0. **HPLC** 98%, RT 11.524 min. **LC-MS** $[ESI + H]^+$: 411.10.

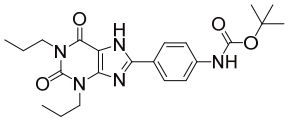


***tert*-Butyl (3-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)phenyl)carbamate** (**12**)
2 M NaOH (5 mL) was added to a solution of **6** (446 mg, 1.00 mmol, 1.0 eq) in dioxane (5 mL). The mixture was refluxed for 3 h at 120 °C. The reaction was then cooled down to rt and water (65 mL) was added. The aqueous layer was washed with EtOAc (4 x 80 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 99:1 → 97:3) to yield **12** (170 mg, 0.40 mmol, 40%) and Boc-deprotected **12** (65 mg, 0.20 mmol, 20%), both as an off-

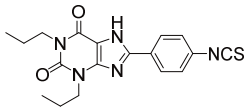
white powder. The products were combined and used directly in the next reaction (0.60 mmol, 60%). **TLC** (DCM:MeOH 98:2) R_f = 0.42. **^{1}H NMR** (400 MHz, $(CD_3)_2SO$) δ [ppm] = 13.88 (s, 1H), 9.57 (s, 1H), 8.27 (s, 1H), 7.75 (d, *J* = 7.7 Hz, 1H), 7.55 (d, *J* = 8.0 Hz, 1H), 7.41 (t, *J* = 7.9 Hz, 1H), 4.06 (t, *J* = 6.9 Hz, 2H), 3.90 (t, *J* = 7.5 Hz, 2H), 1.85 – 1.72 (m, 2H), 1.71 – 1.55 (m, 2H), 1.53 (s, 9H), 0.95 (t, *J* = 6.2 Hz, 3H), 0.91 (t, *J* = 6.4 Hz, 3H).



8-(3-Isothiocyanatophenyl)-1,3-dipropyl-3,7-dihydro-1H-purine-2,6-dione (**13**)
TFA (3 mL) was added to a suspension of **12** (0.60 mmol, 1.0 eq) in DCM (7 mL) and the mixture was stirred for 1 h at rt. TLC indicated full consumption of starting material and therefore 2 M NaOH (65 mL) was added to neutralize the reaction. The aqueous layer was extracted with EtOAc (4 x 60 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated to yield the respective amine. 3 M HCl (32 mL) was added to the crude amine to form a suspension. Thiophosgene (450 µL, 5.87 mmol, 9.8 eq) was added and the mixture was stirred for 2 h at rt. The mixture was then diluted with water (75 mL) and the aqueous layer was extracted with EtOAc (4 x 100 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 98:2 → 90:10) to yield **13** as an off-white powder (173 mg, 0.46 mmol, 77% over two steps). **TLC** (DCM:MeOH 9:1) R_f = 0.26. **^{1}H NMR** (400 MHz, $(CD_3)_2SO$) δ [ppm] = 13.99 (s, 1H), 8.13 (s, 1H), 8.09 (d, *J* = 7.9 Hz, 1H), 7.58 (t, *J* = 7.9 Hz, 1H), 7.51 (d, *J* = 9.0 Hz, 1H), 4.01 (t, *J* = 6.8 Hz, 2H), 3.86 (t, *J* = 7.3 Hz, 2H), 1.74 (h, *J* = 7.4 Hz, 2H), 1.58 (h, *J* = 7.5 Hz, 2H), 0.91 (t, *J* = 7.4 Hz, 3H), 0.87 (t, *J* = 3.6 Hz, 3H). **^{13}C NMR** (101 MHz, $(CD_3)_2SO$): δ [ppm] = 154.1 , 150.6 , 148.1 , 148.0, 134.9, 131.0, 130.7, 130.4, 127.1, 125.6, 123.7, 108.2, 44.5, 42.2, 20.9, 20.9, 11.2, 11.1. **HPLC** 97%, RT 12.229 min. **LC-MS** $[ESI + H]^+$: 370.10.

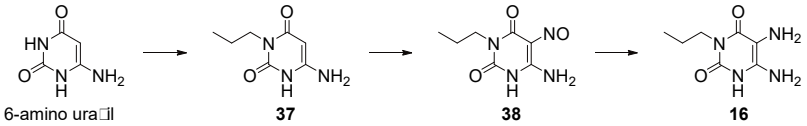


***tert*-Butyl (4-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)phenyl)carbamate** (**14**)
2 M NaOH (10 mL) was added to a solution of **7** (302 mg, 0,68 mmol, 1.0 eq) in dioxane (10 ml). The mixture was refluxed at 120 °C for 2 h and afterwards allowed to cool down to rt. Water (50 mL) was then added and the aqueous layer was extracted with EtOAc (3 x 50 mL). The organic layers were combined, dried with $MgSO_4$, filtered and concentrated under reduced pressure to yield **14** as an off-white solid (242 mg, 0,566 mmol, 84 % yield). **TLC** (DCM:MeOH 98:2): Rf = 0.42. **^{1}H NMR** (400 MHz, $CDCl_3$) δ [ppm] = 13.11 (s, 1H), 8.22 (d, *J* = 8.3 Hz, 2H), 7.51 (d, *J* = 8.3 Hz, 2H), 4.16 (t, *J* = 7.4 Hz, 2H), 4.06 (t, *J* = 7.6 Hz, 2H), 1.93 – 1.81 (m, 2H), 1.78 – 1.69 (m, 2H), 1.54 (s, 9H), 1.02 – 0.93 (m, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ [ppm] = 155.8, 152.7, 151.8, 151.2, 149.9, 140.8, 128.1, 123.5, 118.3, 107.9, 67.2, 45.4, 43.4, 28.5, 21.5, 21.5, 11.6, 11.3.

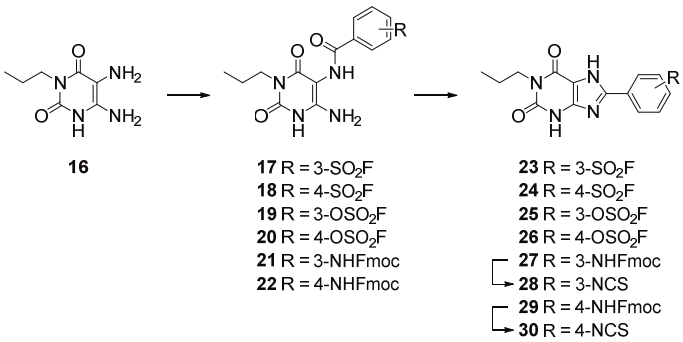


8-(4-Isothiocyanatophenyl)-1,3-dipropyl-3,7-dihydro-1H-purine-2,6-dione (**15**)
TFA (6 mL) was added to a suspension of **14** (242 mg, 0.57 mmol, 1.0 eq) in DCM (12 mL). The mixture immediately became a clear solution. The mixture was stirred for 1 h, upon which TLC showed full consumption of starting material. 2 M NaOH (50 mL) was added and the aqueous phase was extracted with EtOAc (3 x 50 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated. 3 M HCl (30 mL) was added to the crude amine to form a suspension. Thiophosgene (437 µL, 5.70 mmol, 10.0 eq) was added and the mixture was stirred for 2 h at rt. Water (70 mL) was then added and the aqueous layer was extracted with EtOAc (4 x 100 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 99.75:0.25 → 99:1) to yield **15** as a white powder (144 mg, 0.39 mmol, 68% over two steps). **TLC** (DCM:MeOH 9:1) R_f = 0.30. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 13.95 (s, 1H), 8.16 (d, *J* = 8.7 Hz, 2H), 7.56 (d, *J* = 8.7 Hz, 2H), 4.01 (t, *J* = 7.5, 7.0 Hz, 2H), 3.86 (t, *J* = 7.3 Hz, 2H), 1.74 (h, *J* = 7.3 Hz, 2H), 1.58 (h, *J* = 7.4 Hz, 2H), 0.93 – 0.85 (m, 6H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 154.1, 150.6, 148.5, 148.3, 134.7, 131.3, 127.9, 127.7, 126.6, 108.2, 44.4, 42.2, 20.8 (2C), 11.2, 11.0. **HPLC** 98%, RT 12.194 min. **LC-MS** $[ESI + H]^+$: 370.10.

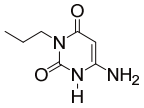
Monopropyl-substituted xanthines



Scheme 3 Synthesis of 1-propyl 5-6-diamino uracil.

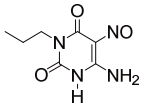


Scheme 4 Synthesis of the *N*^{1}-propyl-substituted xanthines.



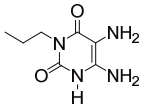
6-Amino-3-propylpyrimidine-2,4(1H,3H)-dione (**37**)[42]

6-aminouracil (20.00 g, 157 mmol, 1.0 eq) and ammonium sulfate (500 mg, 3.78 mmol) were added to a three-neck flask. HMDS (99 ml, 472 mmol, 3.0 eq) was added and the suspension was refluxed at 200 °C. After 2 h the suspension became a clear solution. The solution was cooled down to 80 °C and the HMDS was distilled off by boiling at 200 °C for 8 h. The solution was then cooled to 70 °C and iodine (150 mg, 0.591 mmol, cat) and 1-bromopropane (29 mL, 314 mmol, 2.0 eq) were added. The mixture was refluxed at 70 °C overnight. Extra 1-bromopropane (14.5 mL, 157 mmol, 1.0 eq) was added and the mixture was refluxed at 120 °C for 8 h, following by stirring at 70 °C overnight. Full conversion of starting material was observed and the mixture was put on ice. A saturated bicarb solution (400 mL) was gradually added. The dirty pink suspension was filtered over a glass filter and subsequently washed with water (100 mL), toluene (100 mL) and diethyl ether (100 mL). This yielded pure **37** as an orange/brown solid (21.439 g, 127 mmol, 81 % yield). **TLC** (DCM:MeOH 9:1): R_f = 0.46. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 10.31 (s, 1H), 6.16 (s, 2H), 4.53 (d, *J* = 1.9 Hz, 1H), 3.71 – 3.50 (m, 2H), 1.46 (h, *J* = 7.5 Hz, 2H), 0.81 (t, *J* = 7.5 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 162.9, 153.5, 151.0, 74.1, 40.2, 21.0, 11.2.



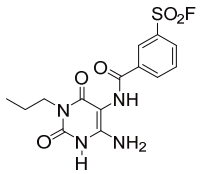
6-Amino-5-nitroso-3-propylpyrimidine-2,4(1H,3H)-dione (**38**)[42]

37 (21.439 g, 127 mmol, 1.0 eq) was dissolved in 300 mL H_2O:AcOH 1:1 at 65 °C. Sodium nitrite (10.97 g, 159 mmol, 1.3 eq) was added in parts to the stirring solution. An immediate color change was observed from brown to purple and back to brown. After approximately 30 minutes brown vapors started to form (NO_x). At this point the reaction was cooled on ice, filtered over a glass filter and washed thoroughly with water. This yielded **38** as a brown solid (18.429 g, 93 mmol, 73 % yield). **TLC** (DCM:MeOH 9:1): R_f = 0.29. **^{1}H NMR** (400 MHz, $(CH_3)_2SO$) δ [ppm] = 11.40 (s, 1H), 8.03 (s, 1H), 3.79 (t, *J* = 7.4 Hz, 2H), 1.59 (h, *J* = 7.5 Hz, 2H), 0.89 (t, *J* = 7.5 Hz, 3H). **^{13}C NMR** (101 MHz, $(CH_3)_2SO$) δ [ppm] = 161.2, 149.2, 144.5, 139.7, 41.4, 20.8, 11.2.



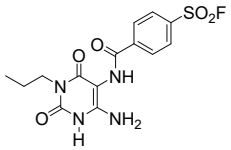
5,6-Diamino-3-propylpyrimidine-2,4(1H,3H)-dione (**16**)[51]

38 (1000 mg, 5.05 mmol, 1.0 eq) was dissolved in MeOH (40 ml) and brought under an N_2 atmosphere. platinum(IV) oxide (20 mg, cat) was added and the mixture was flushed two times with H_2 (g). The mixture was stirred for 1 h under H_2(g), after which a white/grey precipitate had formed. DCM (180 mL) was added and the mixture was filtered over Celite. The Celite was washed with 10% MeOH in DCM (100 mL) and the filtrate was concentrated under reduced pressure. This yielded **16** as an orange/brown solid (806 mg, 4.38 mmol, 87 % yield). This was used in the next steps without further purification. **^{1}H NMR** (400 MHz, $(CD_3)_2SO$) δ [ppm] = 5.56 (s, 2H), 3.70 – 3.59 (m, 2H), 1.48 (m, 2H), 0.81 (t, *J* = 7.5 Hz, 3H).



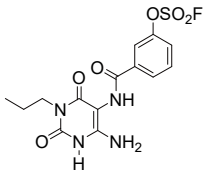
3-((6-Amino-2,4-dioxo-3-propyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)benzenesulfonyl fluoride (**17**)

EDC·HCl (688 mg, 3.59 mmol, 1.20 eq) was added to a solution of crude **16** (551 mg, 2.99 mmol, 1.0 eq) and 3-(fluorosulfonyl)benzoic acid (641 mg, 3.14 mmol, 1.05 eq) in dry DMF (10 mL). The mixture was stirred overnight at rt. Water (80 mL) was then added and the aqueous layer was extracted with EtOAc (13x). The organic layers were combined, washed with brine (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 96:4 → 90:10) to yield **17** as a white solid (598 mg, 2.99 mmol, 54%). **TLC** (DCM:MeOH 9:1): R_f = 0.48. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 10.55 (s, 1H), 9.37 (s, 1H), 8.66 (t, *J* = 1.8 Hz, 1H), 8.45 (dt, *J* = 7.9, 1.4 Hz, 1H), 8.31 (ddd, *J* = 8.0, 2.0, 1.1 Hz, 1H), 7.92 (t, *J* = 7.9 Hz, 1H), 6.28 (s, 2H), 3.66 (t, *J* = 7.2 Hz, 2H), 1.51 (h, *J* = 7.5 Hz, 2H), 0.84 (t, *J* = 7.5 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 164.3, 160.6, 150.6, 149.9, 136.6, 135.8, 131.6 (d, *J* = 23.6 Hz), 130.7, 130.6, 127.6, 86.3, 40.9, 21.0, 11.2. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 66.1. **HPLC** RT 7.393 min. **LC-MS** $[ESI + H]^+$: 371.00.



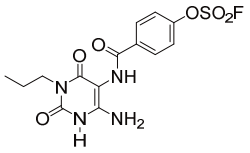
4-((6-Amino-2,4-dioxo-3-propyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)benzenesulfonyl fluoride (**18**)

EDC·HCl (513 mg, 2.67 mmol, 1.3 eq) was added to a solution of crude **16** (379 mg, 2.06 mmol, 1.0 eq) and 4-(fluorosulfonyl)benzoic acid (462 mg, 2.26 mmol, 1.1 eq) in dry DMF (8 mL). The mixture was stirred at rt overnight. Water (80 mL) was then added and the aqueous layers was extracted with EtOAc (7x). The organic layers were combined, washed with brine (10 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 95:5 → 90:10) to yield **18** as a yellow solid (100 mg, 0.27 mmol, 13%). **TLC** (DCM:MeOH 9:1): R_f = 0.48. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 10.55 (s, 1H), 9.30 (s, 1H), 8.27 (s, 4H), 6.26 (s, 2H), 3.66 (t, *J* = 7.4 Hz, 2H), 1.55 – 1.46 (m, 2H), 0.84 (t, *J* = 7.5 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 164.8, 160.5, 150.5, 149.9, 141.8, 133.4 (d, *J* = 23.7 Hz), 129.7, 128.3, 86.3, 40.9, 21.0, 11.2. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 66.0. **HPLC** RT 7.370 min. **LC-MS** $[ESI + H]^+$: 371.00.



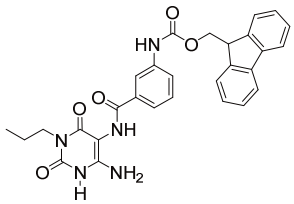
3-((6-amino-2,4-dioxo-3-propyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)phenyl sulfurofluoridate (**19**)

EDC·HCl (428 mg, 2.24 mmol, 1.2 eq) was added to a solution of crude **16** (343 mg, 1.86 mmol, 1.0 eq) and **35** (430 mg, 1.96 mmol, 1.05 eq) in dry DMF (10 mL). The mixture was stirred overnight at rt. TLC and LCMS showed full conversion of starting material and therefore water (80 mL) was added. The formed precipitate was collected and the filtrate was extracted with 5% MeOH in hot $CHCl_3$. The organic layer was concentrated and both residues were purified by column chromatography ($CHCl_3$:MeOH 98:2 → 92.5:7.5), combined and re-crystallized in MeOH to yield **19** as a white solid (237 mg, 0.61 mmol, 33%). **TLC** ($CHCl_3$:MeOH 95:5) R_f = 0.32. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 10.54 (s, 1H), 9.17 (s, 1H), 8.11 (d, *J* = 7.6 Hz, 2H), 7.86 – 7.76 (m, 1H), 7.72 (t, *J* = 8.0 Hz, 1H), 6.24 (s, 2H), 3.66 (t, *J* = 7.3 Hz, 2H), 1.50 (h, *J* = 7.5 Hz, 2H), 0.83 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 164.5, 160.6, 150.6, 150.0, 149.4, 137.3, 130.8, 128.6, 123.8, 120.5, 86.4, 41.0, 21.0, 11.2. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 38.9. **HPLC** 100%, RT 7.817 min. **LC-MS** [ESI + H]$^+$: 386.95.



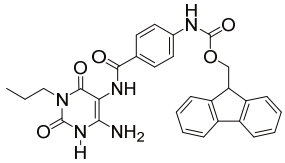
4-((6-Amino-2,4-dioxo-3-propyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)phenyl sulfurofluoridate (**20**)

EDC·HCl (446 mg, 2.33 mmol, 1.3 eq) was added to a solution of crude **16** (330 mg, 1.79 mmol, 1.0 eq) and **36** (394 mg, 1.79 mmol, 1.0 eq) in dry DMF (10 mL). The mixture was stirred overnight at rt. TLC and LCMS showed full conversion of starting material and therefore water (80 mL) was added. The aqueous layer was extracted with EtOAc (4 x). The organic layers were combined, washed with brine (10 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (DCM:MeOH 94:6 → 90:10) and re-crystallized in MeOH to yield **20** as a white solid (104 mg, 0.27 mmol, 15%). **TLC** ($CHCl_3$:MeOH 94:6) R_f = 0.32. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 10.51 (s, 1H), 9.09 (s, 1H), 8.14 (d, *J* = 8.5 Hz, 2H), 7.72 (d, *J* = 8.4 Hz, 2H), 6.19 (s, 2H), 3.66 (t, *J* = 7.2 Hz, 2H), 1.50 (h, *J* = 7.4 Hz, 2H), 0.84 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 164.0, 159.6, 150.2, 149.6, 149.0, 134.4, 129.6, 119.9, 85.5, 39.9, 20.0, 10.2. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 38.9. **HPLC** 100%, RT 7.875 min. **LC-MS** [ESI + H]$^+$: 386.95.



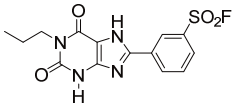
(9H-Fluoren-9-yl)methyl (3-((6-amino-2,4-dioxo-3-propyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)phenyl)carbamate (**21**)

3-(Fmoc-amino)benzoic acid (1.34 g, 3.74 mmol, 1.05 eq) and EDC·HCl (819 mg, 4.27 mmol, 1.2 eq) were added to a solution of crude **16** (656 mg, 3.56 mmol, 1.0 eq) in dry DMF (20 mL). The mixture was stirred for 2 days, after which LCMS and TLC showed full conversion of starting material. The DMF was removed by heating under reduced pressure and water (100 mL) was added. The mixture was extracted with EtOAc (4 x 100 mL), washed with brine (100 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was collected, washed several times with MeOH and acetone and further purified by column chromatography ($CHCl_3$:MeOH 95:5) to yield **21** as an off-white solid (397 mg, 0.76 mmol, 21%). **TLC** ($CHCl_3$:MeOH 95:5) R_f = 0.20. **^{1}H NMR** (400 MHz, $(CD_3)_2SO$) δ [ppm] = 10.47 (s, 1H), 9.89 (s, 1H), 8.84 (s, 1H), 7.99 (s, 1H), 7.91 (d, *J* = 7.5 Hz, 2H), 7.77 (d, *J* = 7.4 Hz, 2H), 7.65 (d, *J* = 8.1 Hz, 2H), 7.43 (t, *J* = 7.4 Hz, 2H), 7.35 (td, *J* = 7.4, 1.3 Hz, 3H), 6.07 (s, 2H), 4.47 (d, *J* = 6.8 Hz, 2H), 4.32 (t, *J* = 6.8 Hz, 1H), 3.66 (t, *J* = 7.6, 7.1 Hz, 2H), 1.50 (h, *J* = 7.5 Hz, 2H), 0.84 (t, *J* = 7.5 Hz, 3H).

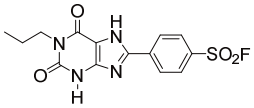


(9H-fluoren-9-yl)methyl (4-((6-amino-2,4-dioxo-3-propyl-1,2,3,4-tetrahydropyrimidin-5-yl)carbamoyl)phenyl)carbamate (**22**)

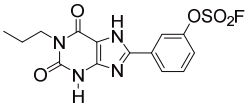
4-(Fmoc-amino)benzoic acid (1.89 g, 5.25 mmol, 1.2 eq) and EDC·HCl (1.09 g, 5.70 mmol, 1.3 eq) were added to a solution of **16** (806 mg, 4.38 mmol, 1.0 eq) in dry DMF (16 mL). The mixture was stirred for 1 h, after which LCMS showed completion of the reaction. EtOAc (250 mL) was added and the organic layer was washed with brine (3 x 100 mL). Upon addition of brine, a precipitate formed in the organic layer. The precipitate was collected and recrystallized in MeOH to yield **22** as yellow/green solid (1.17 g, 2.22 mmol, 51%). **TLC** (DCM:MeOH 98:2): Rf = 0.44. **^{1}H NMR** (400 MHz, $(CD_3)_2SO$) δ [ppm] = 10.62 (s, 1H), 10.01 (s, 1H), 8.76 (s, 1H), 7.92 (dd, *J* = 13.8, 7.9 Hz, 4H), 7.78 (d, *J* = 7.5 Hz, 2H), 7.54 (s, 2H), 7.45 (t, *J* = 7.4 Hz, 2H), 7.37 (t, *J* = 6.8 Hz, 2H), 6.20 (s, 2H), 4.53 (d, *J* = 6.7 Hz, 2H), 4.34 (t, *J* = 6.6 Hz, 1H), 3.66 (t, *J* = 7.5 Hz, 2H), 1.51 (h, *J* = 7.1 Hz, 2H), 0.84 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (101 MHz, $(CD_3)_2SO$) δ [ppm] = 165.8, 160.7, 153.3, 150.6, 149.9, 143.7, 141.7, 140.8, 128.8, 128.3, 127.7, 127.2, 125.2, 120.2, 117.1, 87.0, 65.8, 46.6, 40.9, 21.0, 11.2.



3-(2,6-Dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)benzenesulfonyl fluoride (**23**)
Trimethylsilyl polyphosphate (PPSE) (2.50 g, 13.72 mmol, 9.0 eq) was added to **17** (568 mg, 1.53 mmol, 1.0 eq). The mixture was refluxed at 150 °C for 3.5 h under an N_2 atmosphere. LCMS measurements indicated full conversion of the reaction. The mixture was cooled on ice and MeOH (25 mL) was added. The mixture was stirred for 15 minutes and filtrated. The filtrate was stirred in MeOH (15 mL) and filtrated again. The residues were combined, washed with cold MeOH and dried *in vacuo* to yield **23** as a white powder (467 mg, 1.33 mmol, 86%). **TLC** (DCM:MeOH 98:2) R_f = 0.44. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 14.11 (s, 1H), 11.95 (s, 1H), 8.78 (s, 1H), 8.55 (d, *J* = 8.0 Hz, 1H), 8.17 (d, *J* = 8.0 Hz, 1H), 7.89 (t, *J* = 7.9 Hz, 1H), 3.80 (t, *J* = 7.4 Hz, 2H), 1.56 (h, *J* = 7.5 Hz, 2H), 0.87 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 154.9, 150.9, 147.4, 147.0, 133.5, 132.6 (d, *J* = 24.0 Hz), 131.4, 130.8, 129.1, 125.5, 108.5, 41.5, 20.9, 11.2. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 66.0. **HPLC:** 100%, RT 9.095 min **LC-MS** $[ESI + H]^+$: 353.00.

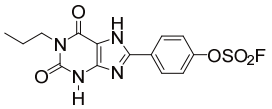


4-(2,6-Dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)benzenesulfonyl fluoride (**24**) **(LUF7982)**
PPSE (430 mg, 2.36 mmol, 9.0 eq) was added to **18** (97 mg, 0.26 mmol, 1.0 eq) and refluxed at 150 °C for 7 h under an N_2 atmosphere. More PPSE (430 mg, 2.36 mmol, 9.0 eq) was added and the mixture was refluxed for another 4 h, upon which LCMS measurements indicated full conversion of starting material. The mixture was cooled on ice, MeOH (25 mL) was added and the formed crystals were filtrated. The filtrate was stirred in MeOH (15 mL) for 15 minutes and filtrated again. The residues were combined, washed with cold MeOH and dried *in vacuo* to yield **24** (LUF7982) as a white powder (68 mg, 0.19 mmol, 74%). **TLC** (DCM:MeOH 98:2) R_f = 0.44. **^{1}H NMR** (300 MHz, $(CD_3)_2SO$) δ [ppm] = 14.24 (s, 1H), 12.05 (s, 1H), 8.43 (d, *J* = 8.3 Hz, 2H), 8.28 (d, *J* = 8.3 Hz, 2H), 3.83 (t, *J* = 7.5 Hz, 2H), 1.69 – 1.47 (m, 2H), 0.88 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 154.98, 150.92, 147.68, 146.98, 135.92, 131.67 (d, *J* = 23.9 Hz), 129.23, 128.02, 109.08, 41.54, 20.85, 11.19. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 66.8. **HPLC** 99%, RT 9.124 min. **LC-MS** $[ESI + H]^+$: 353.00.



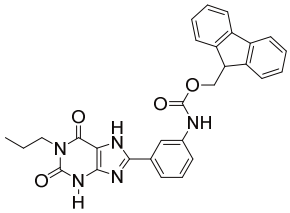
3-(2,6-Dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)phenyl sulfurofluoridate (**25**)
PPSE (2.2 g, 12.07 mmol, 22.2 eq) was added to **19** (210 mg, 0.54 mmol, 1.0 eq) and refluxed at 150 °C. LCMS showed full conversion of starting material after 3h. Therefore the mixture was cooled on ice and MeOH (50 mL) was added. The formed residue was collected and washed with cold MeOH. Recrystallization in $CHCl_3$/MeOH yielded **25** as a white solid (161 mg, 0.44 mmol, 80%). **TLC** (DCM:MeOH 95:5) R_f = 0.63. **^{1}H NMR** (400 MHz, $(CD_3)_2SO$) δ [ppm] = 14.00 (s, 1H), 11.98 (s, 1H), 8.22 (dd, *J* = 6.3, 2.0 Hz, 2H), 7.79 – 7.67 (m, 2H), 3.82 (t, *J* = 7.5 Hz, 2H), 1.57 (h, *J* = 7.5 Hz, 2H), 0.88 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 154.9, 150.9, 150.0, 147.6, 147.5, 131.7, 131.5, 126.7, 122.9, 118.4,

108.3, 41.5, 20.9, 11.2. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 38.9. **HPLC** 97%, RT 9.332 min. **LC-MS** $[ESI + H]^+$: 368.95.



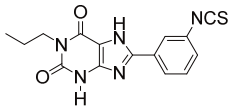
4-(2,6-Dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)phenyl sulfurofluoridate (**26**) **(LUF7993)**

PPSE (1.2 g, 6.59 mmol, 25.7 eq) was added to **20** and refluxed at 150 °C. The mixture was refluxed for 2 h, after which LCMS indicated full conversion of starting material. The mixture was cooled on ice and MeOH (25 mL) was added. The formed precipitate was collected, washed with cold MeOH and recrystallized in MeOH (15 mL). This yielded **26** (LUF7993) as a white powder (83 mg, 0.23 mmol, 88%). **TLC** (DCM:MeOH 95:5) R_f = 0.55. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 13.90 (s, 1H), 11.94 (s, 1H), 8.26 (d, *J* = 8.9 Hz, 2H), 7.74 (d, *J* = 8.4 Hz, 2H), 3.81 (t, *J* = 7.4 Hz, 2H), 1.57 (h, *J* = 7.4 Hz, 2H), 0.87 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 154.9, 151.0, 150.3, 148.1, 147.6, 129.8, 128.7, 121.9, 108.2, 41.5, 20.9, 11.2. **^{19}F NMR** (471 MHz, $(CD_3)_2SO$) δ [ppm] = 38.94. **HPLC** 99%, RT 9.327 min. **LC-MS** $[ESI + H]^+$: 369.00.

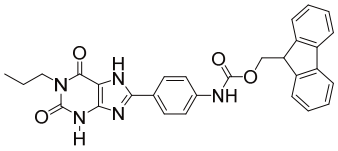


(9H-Fluoren-9-yl)methyl (3-(2,6-dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)phenyl)carbamate (**27**)

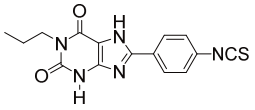
PPSE (3.44 g, 18.88 mmol, 25.0 eq) was added to **21** (397 mg, 0.76 mmol, 1.0 eq) and the mixture was refluxed at 150 °C. After 3 h of stirring, TLC showed full conversion of the reaction. The mixture was cooled down to rt and water (40 mL) and brine (10 mL) were added. The aqueous layer was extracted by EtOAc (6 x 80 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated under reduced pressure. This yielded **27** (269 mg, 0.53 mmol, 70%) as an off-white solid. **TLC** ($CHCl_3$:MeOH 95:5) R_f = 0.55. **^{1}H NMR** (400 MHz, $(CD_3)_2SO$) δ [ppm] = 13.71 (s, 1H), 11.92 (s, 1H), 9.94 (s, 1H), 8.33 (s, 1H), 7.92 (d, *J* = 7.5 Hz, 2H), 7.76 (t, *J* = 7.1 Hz, 3H), 7.43 (t, *J* = 7.5 Hz, 3H), 7.40 – 7.32 (m, 3H), 4.49 (d, *J* = 6.8 Hz, 2H), 4.33 (t, *J* = 6.8 Hz, 1H), 3.82 (t, *J* = 7.5 Hz, 2H), 1.57 (h, *J* = 7.1 Hz, 2H), 0.88 (t, *J* = 7.4 Hz, 3H). **HPLC** 95%, RT 11.075 min. **LC-MS** $[ESI + H]^+$: 508.10.



8-(3-Isothiocyanatophenyl)-1-propyl-3,7-dihydro-1H-purine-2,6-dione (**28**)
20% Piperidine in DMF (6 mL) was added to remove the Fmoc group of **27** (265 mg, 0.52 mmol, 1.0 eq). The deprotection was complete after 5 min of stirring at rt. The solvents were then removed under reduced pressure and the residue was washed with MeOH to remove the remaining N-Fmoc-piperidine. 3 M HCl (25 mL) was added to the crude amine to form a suspension. Thiophosgene (333 µL, 4.35 mmol, 8.4 eq) was added and the mixture was stirred for 4 h at rt. The aqueous suspension was filtered and the precipitate was washed thoroughly with EtOAc to yield **28** as a white solid (129 mg, 0.39 mmol, 75% over two steps). **TLC** (DCM:MeOH 95:5) R_f = 0.62. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 14.01 – 13.74 (s, 1H), 11.94 (s, 1H), 8.12 (t, *J* = 1.9 Hz, 1H), 8.06 (dt, *J* = 7.9, 1.3 Hz, 1H), 7.57 (t, *J* = 7.9 Hz, 1H), 7.48 (dd, *J* = 8.0, 1.0 Hz, 1H), 3.80 (t, *J* = 7.3 Hz, 2H), 1.56 (h, *J* = 7.3 Hz, 2H), 0.87 (t, *J* = 7.4 Hz, 3H). **^{13}C NMR** (126 MHz, $(CD_3)_2SO$) δ [ppm] = 154.8, 150.9, 148.1, 147.4, 135.3, 131.0, 130.7, 130.5, 126.8, 125.5, 123.6, 108.1, 41.4, 20.8, 11.2. **HPLC** 99%, RT 9.825 min. **LC-MS** [ESI - H]$^-$: 326.95.



(9H-Fluoren-9-yl)methyl (4-(2,6-dioxo-1-propyl-2,3,6,7-tetrahydro-1H-purin-8-yl)phenyl)carbamate (**29**)
PPSE (22 mL) was added to **22** (1.17 g, 2.22 mmol, 1.0 eq) and refluxed at 170 °C. After 5 h of stirring, the mixture was cooled down to rt and water (250 mL) was added. The formed precipitate was collected and purified by column chromatography (DCM:MeOH 99.5:0.5 → 95:5) to yield **29** as an off-white solid (618 mg, 1.22 mmol, 55%). **TLC** (DCM:MeOH 98:2) R_f = 0.38. **^{1}H NMR** (400 MHz, $(CD_3)_2SO$) δ [ppm] 13.50 (s, 1H), 11.87 (s, 1H), 9.98 (s, 1H), 8.01 (dd, *J* = 16.6, 8.5 Hz, 2H), 7.92 (d, *J* = 7.4 Hz, 2H), 7.76 (d, *J* = 7.4 Hz, 2H), 7.63 – 7.51 (m, 2H), 7.43 (t, *J* = 7.4 Hz, 2H), 7.36 (t, *J* = 7.3 Hz, 2H), 4.54 (d, *J* = 6.6 Hz, 2H), 4.33 (t, *J* = 6.5 Hz, 1H), 3.81 (t, *J* = 7.5 Hz, 2H), 1.57 (m, 2H), 0.87 (t, *J* = 7.4 Hz, 3H).



8-(4-Isothiocyanatophenyl)-1-propyl-3,7-dihydro-1H-purine-2,6-dione (**30**) **(LUF8002)**
20% Piperidine in DMF (6 mL) was added to remove the Fmoc group of **29** (85 mg, 0.17 mmol, 1.0 eq). The deprotection was complete after 5 min of stirring at rt. The solvents were then removed under reduced pressure and the residue was washed with MeOH to remove the remaining N-Fmoc-piperidine. 3 M HCl (3 mL) was then added to the crude amine to form a suspension. Thiophosgene (150 µL, 1.96 mmol, 11.9 eq) was added and the mixture was stirred for 2 h at rt. LCMS indicated full conversion of starting material, therefore water (50 mL) was added. The formed precipitate was collected and washed with DCM (50 mL) and EtOAc (50 mL). The residue was dried under reduced pressure to yield **30** (LUF8002) as an off-white powder (40 mg, 0.12 mmol, 71% over two steps). **TLC** (DCM:MeOH 95:5) R_f = 0.55. **^{1}H NMR** (500 MHz, $(CD_3)_2SO$) δ [ppm] = 13.81 (s, 1H), 11.94 (s, 1H), 8.13 (d, *J* = 8.7 Hz, 2H), 7.56 (d, *J* = 8.7 Hz, 2H), 3.82 (t, *J* = 7.5 Hz, 2H), 1.57 (h, *J* = 7.3 Hz, 2H), 0.88 (t, *J* = 7.4 Hz, 3H). **^{13}C**

NMR (151 MHz, $(CD_3)_2SO$) δ [ppm] = 154.9, 151.0, 148.6, 147.6, 134.7, 131.3, 128.1, 127.7, 126.7, 108.1, 41.5, 20.9, 11.2. **HPLC** 95%, RT 9.834 min. **LC-MS** $[ESI + H]^+$: 327.95.

Biology

Cell lines

CHO-spap cells stably expressing the human A_{2B} receptor (CHO-spap-hA_{2B}AR) were kindly provided by S.J. Dowell (Glaxo Smith Kline, UK). Chinese hamster ovary (CHO) cells stably expressing the human adenosine A_1 receptor (CHOhA_1AR) were kindly provided by Prof. S.J. Hill (University of Nottingham, UK). Human embryonic kidney 293 cells stably expressing the human adenosine A_{2A} receptor (HEK293hA_{2A}AR) were kindly provided by Dr. J. Wang (Biogen/IDEC, Cambridge, MA). CHO cells stably expressing the human adenosine A_3 receptor (CHOhA_3AR) were a kindly provided by Dr. K.N. Klotz (University of Würzburg, Germany).

Radioligands

$[^3H]$8-(4-(4-(4-Chlorophenyl)piperazide-1-sulfonyl)phenyl)-1-propylxanthine ($[^3H]$PSB-603, specific activity 79 Ci/mmol) was purchased from Quotient Bioresearch. $[^3H]$1,3-dipropyl-8-cyclopentyl-xanthine ($[^3H]$DPCPX, specific activity 137 Ci/mmol) was purchased from ARC, Inc. $[^3H]$4-(-2-[7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazin-5-ylamino)ethyl) phenol ($[^3H]$-ZM241385, specific activity 50 Ci/mmol) was purchased from ARC, Inc. $[^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) was a gift from Prof. C.E. Müller (University of Bonn, Germany).

Chemicals

5'-N-ethylcarboxamidoadenosine (NECA), N^6-Cyclopentyladenosine (CPA) and adenosine deaminase (ADA) were purchased from Sigma Aldrich. ZM241385 was kindly donated by Dr. S.M. Poucher (Astra Zeneca, Manchester, UK). CGS21680 was purchased from Ascent Scientific. PSB 1115 potassium salt was purchased from Tocris Bioscience. All other chemicals were of analytical grade and obtained from standard commercial sources.

Cell culture and membrane preparation

CHO-spap-hA_{2B}R cells, CHOhA_1AR cells, HEK293hA_{2A}AR cells and CHOhA_3AR were cultured and membranes were prepared as previously reported.[52]

Radioligand displacement assays

Single point radioligand displacement assays on CHOhA_1AR cells, HEK293hA_{2A}AR cells and CHOhA_3AR cells were performed as previously reported.[52] Full curve radioligand displacement assays were performed using ChO-spap-hA_{2B}AR membranes and a concentration rage of competing ligand. 30 µg of protein in a total volume of 100 µL assay buffer (0.1% CHAPS in 50 mM Tris-HCl pH 7.4) was taken and pre-incubated for either 0 or 4 h with the competing ligand. ~1.5 nM $[^3H]$PSB-603 was then added and the membranes were co-incubated for 30 min at 25 °C. Nonspecific binding was determined in the presence of 10 µM ZM241385. Incubations were terminated by vacuum filtration to separate the bound and free radioligand through prewetted 96-well GF/C filter plates using a Filtermate-harvester (PerkinElmer). Filters were subsequently washed 5 times with ice-cold wash buffer (0.1% BSA in 50 mM Tris-HCl pH 7.4). The plates were dried at 55 °C after which 25 µL of MicroscintTM-20 cocktail (PerkinElmer) was added to each well. After 3 h the filter-bound radioactivity was determined by scintillation spectrometry using a 2450 MicroBeta^2 Microplate Counter (PerkinElmer).

Wash-out assays

100 µL of assay buffer (0.1% CHAPS in 50 mM Tris-HCl pH 7.4) containing 1 µM of competing ligand (10 µM in case of PSB 1115) and 200 µL of assay buffer were added to 100 µL of ChO-spap-hA_{2B}AR membrane suspension (80 µg of protein) in a 2 mL Eppendorf tube. The tubes were incubated 2 h at 25 °C while shaking. The 'washed' group of samples was centrifuged (5 min, 13 200 rpm, 4 °C), the supernatant was removed, the pellet was resuspended in 1 mL of assay buffer and incubated for 10 min at 25 °C while shaking at 900 rpm. The washing steps were repeated three times. After the last washing step, the membrane pellets were resuspended in 300 µL of assay buffer to determine radioligand displacement. Both washed and unwashed samples were transferred to test tubes and incubated together with 100 µL of 1.5 nM [^{3}H]PSB-603 for 2 h at 25 °C. Nonspecific binding was determined in the presence of 10 µM ZM241385. The incubation was terminated by vacuum filtration through prewetted 96-well GF/C filter plates using a Brandol M24 Scintillation harvester. Filters were subsequently washed 3 times with ice-cold wash buffer (0.1% BSA in 50 mM Tris-HCl pH 7.4). The filter-bound radioactivity was determined using a Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer).

Data Analysis

All data from radioligand displacement and wash-out assays were analyzed using GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA). IC_{50} values were converted to K_i values using the Cheng-Prusoff equation.[53] The K_D values of [^{3}H]PSB603 at CHO-spap-hA_{2B}AR membranes (1.7 nM) was taken from in-house determinations.

Computational Procedures

Docking of LUF7982 in the adenosine A_{2B} receptor

The A_{2B}AR homology model was retrieved from the GPCRdb.[54] Several orientations of the extracellular loop 3 (EL3) loop region (between residue numbers 258 and 270) were generated using MODELLER,[55] to obtain viable orientations of the Lysine residues in the binding site. 3D coordinates of LUF7982 were generated using rdkit.[56] Thereafter, LUF7982 was manually docked in the receptor model using PyMOL,[57] and subsequently minimized using the all-atom minimization tool in ICM Pro.[58] Residue numbers are presented with their Ballosteros-Weinstein numbering scheme.[23] Distances given in Å are the distances calculated between the N-atom of the lysine residue and the S-atom of the sulfonyl fluoride warhead.

Author Contributions

B.L.H.B., M.A. and J.P.D.V. synthesized compounds. X.W., L.N.A. and R.L. performed radioligand displacement experiments. B.L.H.B., M.A. and W.J. performed molecular docking experiments. L.H.H., A.P.IJ. and D.v.d.E. supervised the project.

References

[1] B. B. Fredholm, A. P. IJzerman, K. A. Jacobson, K.-N. Klotz, J. Linden, *Pharmacol Rev* **2001**, *53*, 527–552.

[2] B. B. Fredholm, A. P. IJzerman, K. A. Jacobson, J. Linden, C. E. Müller, *Pharmacol Rev* **2011**, *63*, 1–34.

[3] B. Allard, D. Allard, L. Buisseret, J. Stagg, *Nat Rev Clin Oncol* **2020**, *17*, 611–629.

[4] J.-F. Chen, H. K. Eltzschig, B. B. Fredholm, *Nat Rev Drug Discov* **2013**, *12*, 265–286.

[5] Z. Gao, K. A. Jacobson, *Int J Mol Sci* **2019**, *20*, 5139.

[6] C. Cekic, J. Linden, *Nat Rev Immunol* **2016**, *16*, 177–192.

[7] P. A. Borea, S. Gessi, S. Merighi, F. Vincenzi, K. Varani, *Physiol Rev* **2018**, *98*, 1591–1625.

[8] T. Zhang, J. M. Hatcher, M. Teng, N. S. Gray, M. Kostic, *Cell Chem Biol* **2019**, *26*, 1486–1500.

[9] D. Weichert, P. Gmeiner, *ACS Chem Biol* **2015**, *10*, 1376–1386.

[10] A. Glukhova, D. M. Thal, A. T. Nguyen, E. A. Vecchio, M. Jörg, P. J. Scammells, L. T. May, P. M. Sexton, A. Christopoulos, *Cell* **2017**, *168*, 867–877.

[11] X. Yang, L. H. Heitman, A. P. IJzerman, D. van der Es, *Purinergic Signal* **2021**, *17*, 85–108.

[12] C. E. Müller, K. A. Jacobson, *Biochim Biophys Acta* **2011**, *1808*, 1290–1308.

[13] K. A. Jacobson, A. P. IJzerman, J. Linden, *Drug Dev Res* **1999**, *47*, 45–53.

[14] Y.-C. Kim, X. Ji, N. Melman, J. Linden, K. A. Jacobson, *J Med Chem* **2000**, *43*, 1165–1172.

[15] A. M. Hayallah, J. Sandoval-Ramírez, U. Reith, U. Schobert, B. Preiss, B. Schumacher, J. W. Daly, C. E. Müller, *J Med Chem* **2002**, *45*, 1500–1510.

[16] T. Borrmann, S. Hinz, D. C. G. Bertarelli, W. Li, N. C. Florin, A. B. Scheiff, C. E. Müller, *J Med Chem* **2009**, *52*, 3994–4006.

[17] J. Jiang, C. J. Seel, A. Temirak, V. Namasivayam, A. Arridu, J. Schabikowski, Y. Baqi, S. Hinz, J. Hockemeyer, C. E. Müller, *J Med Chem* **2019**, *62*, 4032–4055.

[18] X.-D. Ji, C. Gallo-Rodriguez, K. A. Jacobson, *Drug Dev Res* **1993**, *29*, 292–298.

[19] J. C. Shryock, S. Snowdy, P. G. Baraldi, B. Cacciari, G. Spalluto, A. Monopoli, E. Ongini, S. P. Baker, L. Belardinelli, *Circulation* **1998**, *98*, 711–718.

[20] X. Yang, G. Dong, T. J. M. Michiels, E. B. Lenselink, L. H. Heitman, J. Louvel, A. P. IJzerman, *Purinergic Signal* **2017**, *13*, 191–201.

[21] A. S. Doré, N. Robertson, J. C. Errey, I. Ng, K. Hollenstein, B. Tehan, E. Hurrell, K. Bennett, M. Congreve, F. Magnani, C. G. Tate, M. Weir, F. H. Marshall, *Structure* **2011**, *19*, 1283–1293.

[22] R. K. Y. Cheng, E. Segala, N. Robertson, F. Deflorian, A. S. Doré, J. C. Errey, C. Fiez-Vandal, F. H. Marshall, R. M. Cooke, *Structure* **2017**, *25*, 1275–1285.

[23] J. A. Ballesteros, H. Weinstein, in *Methods in Neurosciences* (Ed.: S.C. Sealfon), Academic Press, **1995**, pp. 366–428.

[24] A. Narayanan, L. H. Jones, *Chem Sci* **2015**, *6*, 2650–2659.

[25] H. Mukherjee, J. Debreczeni, J. Breed, S. Tentarelli, B. Aquila, J. E. Dowling, A. Whitty, N. P. Grimster, *Org Biomol Chem* **2017**, *15*, 9685–9695.

[26] D. E. Mortenson, G. J. Brighty, L. Plate, G. Bare, W. Chen, S. Li, H. Wang, B. F. Cravatt, S. Forli, E. T. Powers, K. B. Sharpless, I. A. Wilson, J. W. Kelly, *J Am Chem Soc* **2018**, *140*, 200–210.

[27] J. Dong, L. Krasnova, M. G. Finn, K. B. Sharpless, *Angewandte Chemie - International Edition* **2014**, *53*, 9430–9448.

[28] A. R. Beauglehole, S. P. Baker, P. J. Scammells, *J Med Chem* **2000**, *43*, 4973–4980.

[29] M. Jörg, A. Glukhova, A. Abdul-Ridha, E. A. Vecchio, A. T. N. Nguyen, P. M. Sexton, P. J. White, L. T. May, A. Christopoulos, P. J. Scammells, *J Med Chem* **2016**, *59*, 11182–11194.

[30] X. Yang, J. P. D. van Veldhoven, J. Offringa, B. J. Kuiper, E. B. Lenselink, L. H. Heitman, D. van Der Es, A. P. IJzerman, *J Med Chem* **2019**, *62*, 3539–3552.

[31] X. Yang, M. A. Dilweg, D. Osemwengie, L. Burggraaff, D. van der Es, L. H. Heitman, A. P. IJzerman, *Biochem Pharmacol* **2020**, *180*, DOI 10.1016/j.bcp.2020.114144.

[32] A. Baranczak, Y. Liu, S. Connelly, W.-G. H. Du, E. R. Greiner, J. C. Genereux, R. L. Wiseman, Y. S. Eisele, N. C. Bradbury, J. Dong, L. Noodleman, K. B. Sharpless, I. A. Wilson, S. E. Encalada, J. W. Kelly, *J Am Chem Soc* **2015**, *137*, 7404–7414.

[33] M. Gehringer, S. A. Laufer, *J Med Chem* **2019**, *62*, 5673–5724.

[34] T. Nakamura, Y. Kawai, N. Kitamoto, T. Osawa, Y. Kato, *Chem Res Toxicol* **2009**, *22*, 536–542.

[35] G. L. Stiles, K. A. Jacobson, *Mol Pharmacol* **1988**, *34*, 724–728.

[36] K. A. Jacobson, S. Barone, U. Kammula, G. L. Stiles, *J Med Chem* **1989**, *32*, 1043–1051.

[37] J. Zhang, L. Belardinelli, K. A. Jacobson, D. H. Otero, S. P. Baker, *Mol Pharmacol* **1997**, *52*, 491–498.

[38] A. Vlachodimou, H. de Vries, M. Pasoli, M. Goudswaard, S.-A. Kim, Y.-C. Kim, M. Scortichini, M. Marshall, J. Linden, L. H. Heitman, K. A. Jacobson, A. P. IJzerman, *Biochem Pharmacol* **2022**, *200*, 115027.

[39] C. E. Müller, *Tetrahedron Lett* **1991**, *32*, 6539–6540.

[40] C. E. Müller, *Synthesis (Stuttg)* **1993**, *1*, 125–128.

[41] C. E. Müller, J. Sandoval-Ramirez, *Synthesis (Stuttg)* **1995**, *10*, 1295–1299.

[42] J. Hockemeyer, J. C. Burbiel, C. E. Müller, *Journal of Organic Chemistry* **2004**, *69*, 3308–3318.

[43] Y. Imai, A. Mochizuki, M. Kakimoto, *Synthesis (Stuttg)* **1983**, 851.

[44] C. E. Müller, D. Shi, M. Manning, J. W. Daly, *J Med Chem* **1993**, *36*, 3341–3349.

[45] J. R. Pfister, L. Belardinelli, G. Lee, R. T. Lum, P. Milner, W. C. Stanley, J. Linden, S. P. Baker, G. Schreiner, *J Med Chem* **1997**, 1773–1778.

[46] S. Weyler, F. Fülle, M. Diekmann, B. Schumacher, S. Hinz, K.-N. Klotz, C. E. Müller, *ChemMedChem* **2006**, *1*, 891–902.

[47] W. Jespers, A. C. Schiedel, L. H. Heitman, R. M. Cooke, L. Kleene, G. J. P. van Westen, D. E. Gloriam, C. E. Müller, E. Sotelo, H. Gutiérrez-de-Terán, *Trends Pharmacol Sci* **2018**, *39*, 75–89.

[48] M. G. Moloney, D. R. Paul, R. M. Thompson, E. Wright, *Tetrahedron Asymmetry* **1996**, *7*, 2551–2562.

[49] M. Söftje, S. Acker, R. Plarre, J. C. Namyslo, D. E. Kaufmann, *RSC Adv* **2020**, *10*, 15726–15733.

[50] H. Zhou, P. Mukherjee, R. Liu, E. Evrard, D. Wang, J. M. Humphrey, T. W. Butler, L. R. Hoth, J. B. Sperry, S. K. Sakata, C. J. Helal, C. W. Am Ende, *Org Lett* **2018**, *20*, 812–815.

[51] C. E. Müller, J. Sandoval-Ramirez, *Synthesis (Stuttg)* **1995**, *19995*, 1295–1299.

[52] T. Amelia, J. P. D. Van Veldhoven, M. Falsini, R. Liu, L. H. Heitman, G. J. P. Van Westen, E. Segala, G. Verdon, R. K. Y. Cheng, R. M. Cooke, D. Van Der Es, A. P. IJzerman, *J Med Chem* **2021**, *64*, 3827–3842.

[53] Y.-C. Cheng, W. H. Prusoff, *Biochem Pharmacol* **1973**, *22*, 3099–3108.

[54] A. J. Kooistra, S. Mordalski, G. Pándy-Szekeres, M. Esguerra, A. Mamyrbekov, C. Munk, G. M. Keserű, D. E. Gloriam, *Nucleic Acids Res* **2021**, *49*, D335–D343.

[55] B. Webb, A. Sali, *Curr Protoc Bioinformatics* **2016**, *54*, 5.6.1-5.6.37.

[56] "RDKit: Open-source cheminformatics," can be found under http://www.rdkit.org

[57] Schrödinger LLC, *The PyMOL Molecular Graphics System, Version 2.6.0a0*

[58] M. Totrov & R. Abagyan, in *Drug-Receptor Thermodynamics: Introduction and Applications*, Wiley & Sons, **2001**, pp. 603–624.