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

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Signalling through the adenosine receptors (ARs), in particular through the adenosine A_{2B} receptor (A_{2B}AR), has been 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 signalling 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.

Introduction

The endogenous molecule adenosine acts as a signalling 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 signalling pathways is therefore an interesting strategy to tackle a broad spectrum of pathological conditions.[4]

A_{2B}AR activation has been linked to hallmarks of cancer, *i.e.* cancer cell proliferation, tumour growth, tumour 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 tumour microenvironment might hinder the proper inhibition of A_{2B}AR-induced signalling 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 signalling 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

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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

Analysing 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.

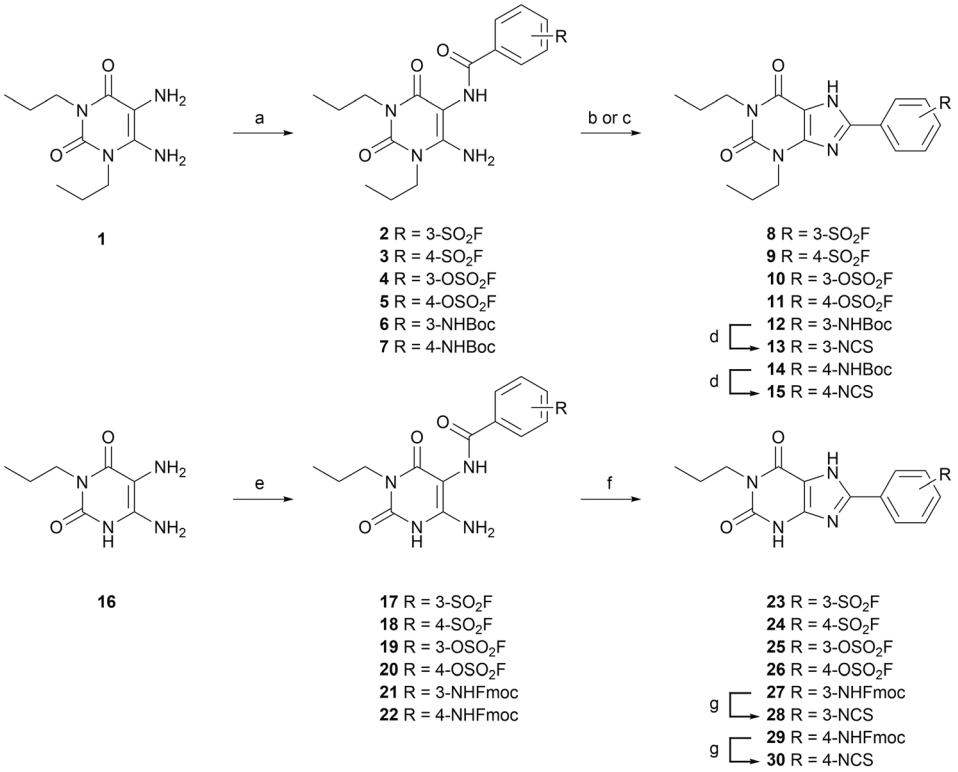
Synthesis of covalent A_{2B}AR ligands

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**.

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

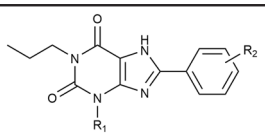
To investigate the affinity of the twelve ligands and their potential to bind irreversibly to the A_{2B}AR, radioligand displacement assays were carried out using CHO-spap membranes stably overexpressing the A_{2B}AR. Two different conditions were chosen: no pre-incubation of receptor with ligand (pre 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 Fig. 1A and C). 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 Fig. 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



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%.

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



Compound	R_1	R_2	pK_i[a] (pre 0 h)	pK_i[b] (pre 4 h)	K_i 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 pK_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.

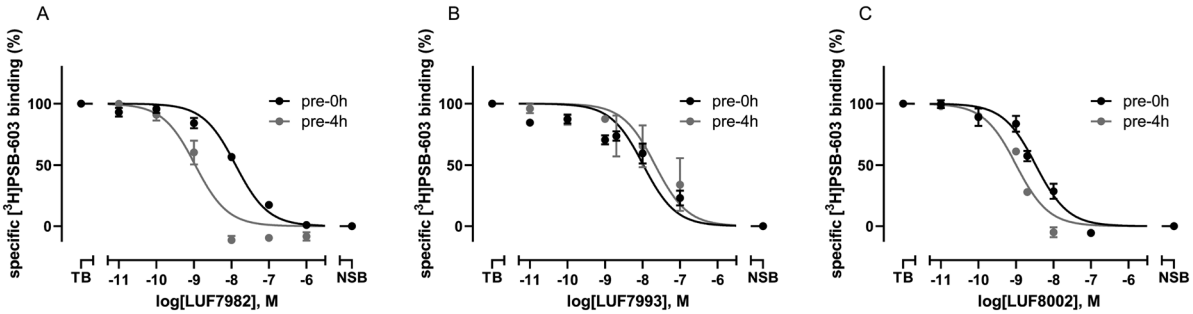


Fig. 1 Displacement of [^{3}H]PSB-603 from the A_{2B}AR by (A) SO_2F-substituted LUF7982; (B) OSO_2F-substituted LUF7993 and (C) NCS-substituted LUF8002. Displacement measured after 0 or 4 h of pre-incubation of the respective ligand with CHO-spap membranes stably overexpressing the A_{2B}AR. Data represent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

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.

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]

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

	(%) displacement at 1 μM					
	hA_1AR^a		$hA_{2A}AR^b$		hA_3AR^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_1AR pre-incubated with the compounds for 4 or 0 hours at 25 °C, followed by a co-incubation with [^{3}H]DPCPX for 0.5 h 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 0.5 h at 25 °C. [c] % displacement at 1 μM concentration of specific [^{3}H]PSB-11 binding on CHO cell membranes stably expressing hA_3AR pre-incubated with the compounds for 4 or 0 hours at 25 °C, followed by a co-incubation with [^{3}H]PSB-11 for 0.5 h at 25 °C. Data represent the mean of two individual experiments performed in duplicate.

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.

Investigation 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

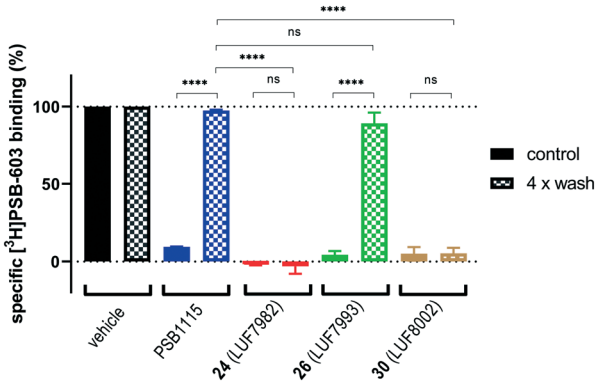


Fig. 2 Wash-out assays on the adenosine A_{2B} receptor using the N^1-propyl xanthines with *para*-substituted warheads. CHO-spap cell membranes stably expressing the adenosine A_{2B} receptor were pre-incubated with buffer (vehicle) or 1 μM of ligand (10 μM in case of **PSB-1115**), followed by a four-cycle washing treatment (4× 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; ****$P < 0.0001$.

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 (Fig. 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 hydrolysed 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 (Fig. 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.

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 (Fig. 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) (Fig. 3B).

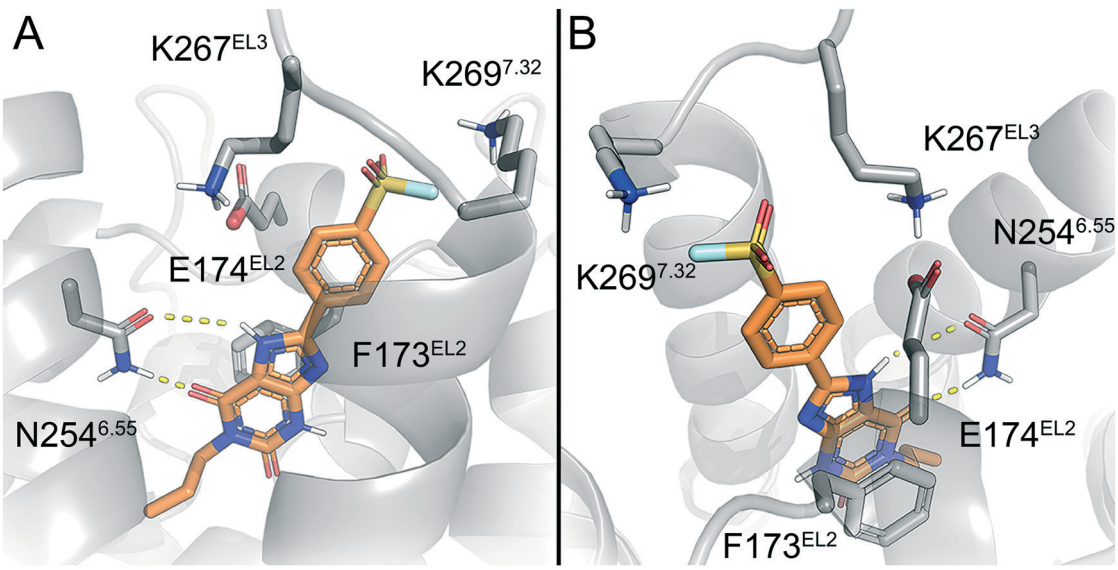


Fig. 3 Predicted binding mode of LUF7982. Panel A: Overview of the key interactions of LUF7982 in the binding site, which include two hydrogen bonds (yellow dashed lines) with $N254^{6.55}$ and π–π stacking with $F173^{EL2}$, both are conserved interactions in adenosine receptor ligand recognition. The 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 $K269^{7.32}$).

$K267^{EL3}$ 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] $K265^{EL3}$ 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 unfavourable 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 signalling 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 behaviour 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.

Author contributions

Conceptualization: BLHB, APIJ and DE. Investigation and validation: BLHB, XW, MA, LNA, JV, WJ and RL. Data curation, formal analysis and methodology: BLHB, XW, WJ, LHH, APIJ and DE. Funding acquisition, project administration, resources and supervision: LHH, APIJ and DE. Writing – original draft: BLHB and WJ. Writing – review & editing: BLHB, XW, WJ, LHH, APIJ and DE.

Conflicts of interest

The authors declare no conflicts of interest.

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