Non-ribose ligands for the human adenosine A1 receptor

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CHAPTER 6

LUF6037, a non-adenosine agonist with picomolar potency for the adenosine A_1 receptor is unable to internalize the receptor

We and others have recently shown that a novel class of non-adenosine compounds interacts with adenosine A_1 receptors. Here we report on a new derivative, LUF6037, a very potent non-adenosine A_1 receptor agonist. The striking structural differences between traditional adenosine-like agonists and LUF6037 prompted us to investigate its interaction with the receptor and its ability to induce internalization. Radioligand binding and cAMP studies were carried out with CHO cells expressing the human adenosine A_1 receptor. The ability of LUF6037 to internalize the receptor was determined with an adenosine A_1 receptor equipped with a yellow fluorescent protein (YFP) tag. LUF6037 recognized two binding states/sites on the human adenosine A_1 receptor with affinities of 44 ± 30 pM and 8.7 ± 3.7 nM, both much higher than the reference agonist cyclopentyladenosine (CPA) with affinities of 2.2 ± 0.9 nM and 338 ± 24 nM. The interaction of LUF6037 with the receptor did not change in the presence of the allosteric enhancer PD81,723, whereas PD81,723 increased CPA's affinity. LUF6037 behaved as a full agonist in thermodynamic radioligand binding experiments as well as in a second messenger assay, where it completely inhibited forskolin-stimulated cAMP production with an EC_{50} value of 60 ± 5 pM. Finally, whereas CPA dose-dependently induced receptor internalization, LUF6037 had no effect. LUF6037 fully activates the human adenosine A_1 receptor just like traditional agonists such as CPA. However, unlike CPA the activation by LUF6037 is insensitive to the allosteric modulator PD81,723. Moreover, LUF6037 does not induce receptor internalization. Apparently full agonism does not necessarily lead to receptor internalization. This behaviour may be therapeutically advantageous as drug resistance, as a result of receptor internalization, may less readily occur.

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Introduction

During the past decades, several agonists with high affinity for the human adenosine A_1 receptor have been developed. These ligands are structurally related to the endogenous agonist adenosine, consisting of the adenine base coupled to a ribose moiety. To increase the affinity and the selectivity of the agonists for the human adenosine A_1 receptor, adenosine has been substituted at different positions. Especially substitutions at the N^6-position often lead to potent and selective adenosine A_1 receptor agonists[1].

Recently, a new class of adenosine ligands has been described in the patent literature, the 2-amino-4-(3,4 substituted phenyl)-6-(2-hydroxyethylsulfanyl)-pyridin-3,5-dicarbonitriles[2,3]. Although this class of ligands shows very little structural similarity to adenosine, it was observed that several compounds displayed a significant affinity and efficacy towards different adenosine receptor subtypes[2,3,4,5]. A more detailed pharmacological characterization of one of these compounds, LUF5831, revealed that this compound was a partial agonist with an affinity of 18 nM for the adenosine A_1 receptor[6].

In this report we characterized a new non-adenosine agonist, LUF6037 (Figure 6.1), and demonstrated it is a full agonist with very high picomolar affinity, largely surpassing the potency of the reference agonist, N^6-cyclopentyladenosine (CPA). However, unlike CPA, we found that this full agonist is insensitive to the allosteric enhancer PD81,723. LUF6037 was also tested for its ability to induce internalization of the human adenosine A_1 receptor. Again unlike CPA, LUF6037 did not cause any translocation of a YFP-tagged A_1 receptor from the cell membrane to the cytoplasm. This observation is first-time evidence that full activation of this receptor does not necessarily lead to internalization.

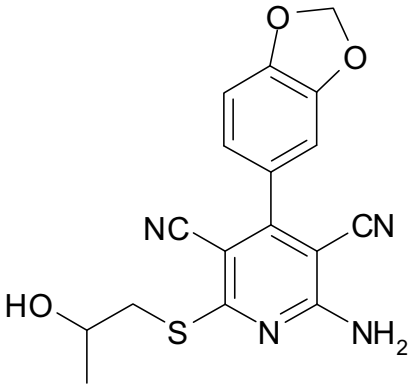

Figure 6.1. Structure of LUF6037

Methods

Materials

N^6-cyclopentyladenosine (CPA) was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), bovine serum albumin (BSA), forskolin, DEAE dextran and chloroquine were from Sigma (St. Louis MO, U.S.A.). Adenosine deaminase (ADA) was purchased from Roche Biochemicals (Mannheim, Germany) and Bicinchonic acid (BCA) protein assay reagent was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). [^{3}H] 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX -specific activity 124 Ci/mmol) was purchased from

NEN (Du Pont Nemours, 's Hertogenbosch, The Netherlands). [^{3}H] cyclic adenosine monophosphate ([^{3}H] cAMP – specific activity 32.1 Ci/mmol) was obtained from Perkin Elmer Life Sciences Inc. (Boston, MA, U.S.A.). G418 (neomycin) was obtained from Stratagene (Cedar Creek, U.S.A.). (2-Amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]-methanone (PD81,723) was synthesized in our laboratory as described by Van der Klein et al.[7]. Protein kinase A (PKA) was isolated from bovine adrenal glands according to Leurs et al.[8]. LUF6037 was synthesized in our laboratory, as described for the 2-amino-4-(3,4 substituted phenyl)-6-(2-hydroxyethylsulfanyl)-pyridin-3,5-dicarbonitrile compounds[5]. Chinese hamster ovary (CHO) cells stably expressing the human adenosine A_1 receptor were obtained from A. Townsend – Nicholson[9]. CHO cells stably expressing the human adenosine A_1YFP receptor were made in our laboratory[10]. All other chemicals were of analytical grade and obtained from standard commercial sources.

Cell culture

CHO cells stably expressing the human adenosine A_1 receptor or the human adenosine A_1YFP receptor were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% newborn calf serum, streptomycin (50 µg/mL), penicillin (50 IU/mL) and 0.2 or 0.8 mg/mL neomycin (G418), respectively. The cells were maintained in a humidified atmosphere at 37 ºC and 5% CO_2, and subcultured twice weekly.

Preparation of cell membranes

Membranes of CHO cells stably expressing the human adenosine A_1 receptor were prepared as previously described[10]. The protein concentration in the membrane preparation was determined using the BCA method[11] with BSA as a standard.

Radioligand displacement assay

Radioligand binding studies were carried out as previously described[12]. In short, membrane aliquots containing 10 µg of protein were incubated in 400 µL of 50 mM Tris-HCl buffer, pH 7.4 at 25 ºC for 60 min or at 0 ºC for 120 min in the presence of ADA (1 U/mL) and approximately 1.6 nM [^{3}H]DPCPX. Increasing concentrations of cold ligand were added in the presence or absence of 10 µM PD81,723. This concentration of PD81,723 was chosen based on its EC_{50} value of 9.8 µM[13]. Non-specific binding was measured in the presence of 10 µM CPA. Incubations were stopped by rapid dilution with 1 mL ice-cold 50 mM Tris-HCl, pH 7.4 and bound radioligand was separated from free radioligand by rapid filtration through Whatman GF/B filters using a Brandel harvester. Filters were subsequently washed three times with 2 mL ice-cold buffer. Filter-bound radioactivity was measured by liquid

scintillation counting (Tri-Carb 2900TR, Perkin Elmer) after the addition of 3.5 mL of Packard Emulsifier Safe. Experiments were performed at least in triplicate.

Thermodynamic data determination

The values of the thermodynamic parameters were obtained by measuring K_i values at 0 ºC and at 25 ºC, followed by van 't Hoff analysis[14]. The standard free energy, ΔG^0, was calculated according to $\Delta G^0 = -RT \ln K_A$ with $K_A = 1/K_i$. The van 't Hoff equation yields a graph of ln K_A versus $1/T$. The standard enthalpy, ΔH^0, can be calculated from the slope ($\Delta H^0/R$), whereas the standard entropy, ΔS^0, can be calculated either from the intercept ($\Delta S^0/R$) or from $\Delta S^0 = (\Delta H^0 - \Delta G^0)/T$, with R = 8.314 $JK^{-1}mol^{-1}$ and T = 298.15 K

cAMP assay

cAMP assays were performed according to Kourounakis et al.[15]. In short, cells stably expressing the human adenosine A_1 receptor were harvested using a trypsin solution (0.25% in PBS/EDTA), resuspended in medium and plated in 24-well plates at 500 µl or 2×10^5 cells/well. After 24 hr, the cells were washed two times with 500 µl DMEM containing 50 mM HEPES, pH 7.4. Subsequently, cells were incubated at 37 ºC in 250 µL DMEM + HEPES supplemented with ADA (2 IU/mL), rolipram (50 µM) and cilostamide (50 µM). After 30 min of preincubation, ligands were added, to obtain the concentrations as indicated. After 10 min of incubation (37 ºC), 100 µL forskolin was added (final concentration 10 µM) and cells were incubated for an additional 15 min at 37 ºC. The assay was terminated by quick aspiration of the medium and cells were lysed by adding 200 µL of 0.1 M ice-cold HCl. Plates were stored at -20 ºC until further use. To determine the amount of cAMP produced, 100 µL PKA in buffer (150 mM K_2HPO_4; 10 mM EDTA; 0.2% BSA, pH 7.5), 50 µL [^{3}H]cAMP (20,000 dpm) in K_2HPO_4/EDTA buffer (150 mM K_2HPO_4; 10 mM EDTA, pH 7.7) and 50 µL of the cell lysate or cAMP solution for generation of a standard curve (0-16 pmol/200 µL 0.1 M HCl) were incubated on ice for 2.5 hr. Separation of bound and free radioligand was performed by rapid filtration through Whatman GF/C filters, that were subsequently washed once with ice-cold buffer. Scintillation fluid (Emulsifier Safe, Packard Bioscience), 3.5 mL, was added and after 2 hr extraction, radioactivity was counted using a Tri-Carb 2900TR, Perkin Elmer scintillation counter. Experiments were performed at least in triplicate.

Internalization experiments and quantification

CHO cells stably expressing the human adenosine A_1YFP receptor were exposed to the reference agonist CPA, to LUF6037 and to the inverse agonist DPCPX at a concentration of 4 µM to observe if internalization occurred. Internalization experiments were performed as described before[10]. Briefly, 3×10^4 cells were plated

on coverslips in a 24 wells plate (500 µL), and allowed to attach for 24 hr. Subsequently, cells were exposed for 16 hr to the ligands, washed, fixed with 4.0% formaldehyde (pH 7.0 - 7.2) and mounted on object glasses using Aqua Polymount® (Polysciences). Images of representative transsections of the cells were obtained using confocal microscopy (Nikon Eclipse TE 2000-U) with excitation at 520 nm and emission at 532 nm under 60 × oil enlargement. In representative transsections of cells, total cell fluorescence and fluorescence in the cytosol were measured using the computer program Image Pro Plus (MediaCybernetics, Germany). Transsections of at least three different cells were analyzed. The percentage plasma membrane staining relative to total was subsequently calculated according to the method described in Klaasse et al.[10].

Data analysis

Data of radioligand binding and cAMP experiments were analyzed using the non-linear regression curve fitting program Prism v. 4.0.2 (GraphPad, San Diego, CA, USA). Radioligand displacement curves were fitted to one and two state/site binding curves. For LUF6037 and CPA the data were best fitted to a two state/site binding model. K_i values were derived from the IC_{50} values according to the Cheng & Prusoff equation $K_i = IC_{50}/(1+[L^*]/K_d)$, where $[L^*]$ is the radioligand concentration, and K_d its dissociation constant[16]. The K_d values of [^{3}H]DPCPX were 0.69 nM at 0 ºC, and 1.6 nM and 8.8 nM at 25 ºC in the absence and presence of PD81,723, respectively[6].

Results

Affinity of LUF6037 for the human adenosine A_1 receptor

To determine the affinity of LUF6037, radioligand binding studies were performed on membranes of CHO cells stably expressing the human adenosine A_1 receptor with [^{3}H]DPCPX as the radioligand (see Figure 6.2). LUF6037 recognized two binding states/sites with affinities of 44 ± 30 pM and 8.7 ± 3.7 nM for the high and low affinity sites, respectively (see Table 6.1). Both affinities are substantially higher than the previously determined affinities of the reference agonist CPA, which were 2.2 ± 0.9 nM and 338 ± 24 nM, for the high and low affinity sites, respectively[6].

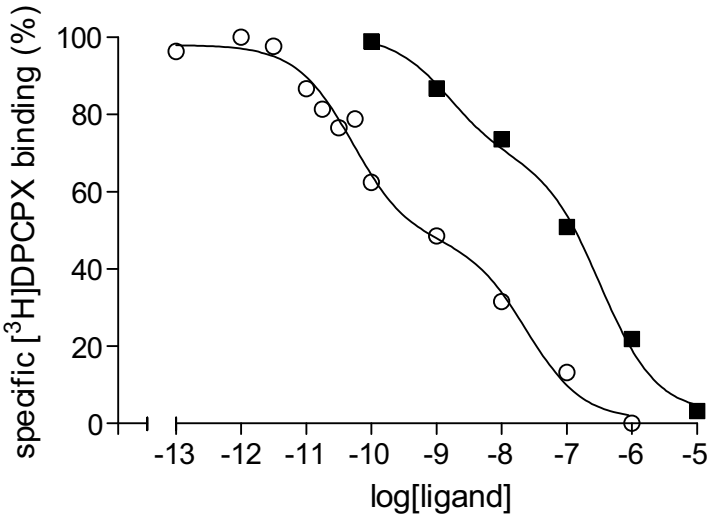

Figure 6.2. Displacement of [^{3}H]DPCPX from the human adenosine A_1 receptor expressed on CHO membranes by LUF6037 (○) or CPA (■). A representative curve from one experiment performed in duplicate is shown.

Effect of the allosteric modulator PD81,723 on the affinity of LUF6037

Radioligand binding experiments were performed in the presence and absence of 10 μM of the allosteric modulator PD81,723 (see Figure 6.3 and Table 6.1). In contrast to CPA (Heitman et al.[6], Table 6.1), LUF6037 was virtually insensitive to this compound. The $K_{i,H}$ and $K_{i,L}$ values were 73 ± 32 pM and 9.9 ± 4.3 nM, respectively, almost identical to the values reported in the absence of PD81,723 (Table 6.1).

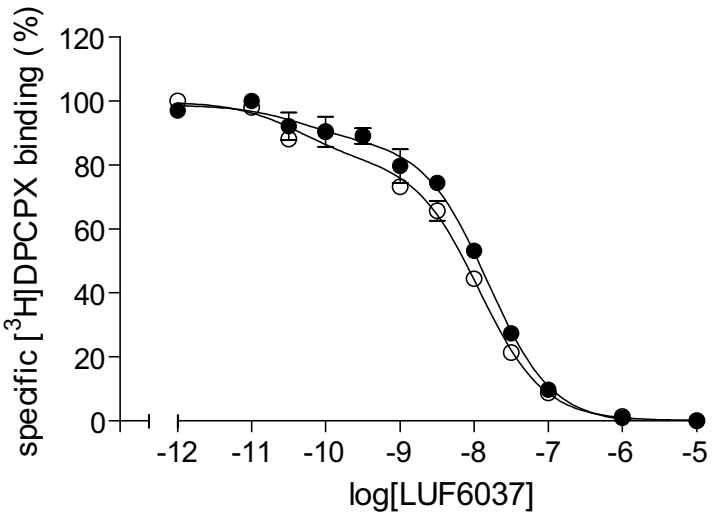

Figure 6.3. Displacement of [^{3}H]DPCPX from the human adenosine A_1 receptor expressed on CHO membranes by LUF6037 in the presence (closed symbols) or absence (open symbols) of 10 μM PD81,723.

Table 6.1. Effect of PD81,723 (10 μM) and temperature (25 ºC versus 0 ºC) on the high ($K_{i,H}$) and low ($K_{i,L}$) affinity binding of LUF6037 to the human adenosine A_1 receptor. Data for CPA are shown for comparison.

Condition	LUF6037		CPA[a]	
	$K_{i,H}$ in pM	$K_{i,L}$ in nM	$K_{i,H}$ in nM	$K_{i,L}$ in nM
25 ºC	44 ± 30	8.7 ± 3.7	2.2 ± 0.9	338 ± 24
25 ºC + PD81,723	73 ± 32	9.9 ± 4.3	2.3 ± 0.4	93 ± 22
0 ºC	55 ± 30	3.9 ± 0.3	7.0 ± 3.0	128 ± 64

[a]: data are from Heitman et al. 2006[6].

Effect of temperature on the affinity of LUF6037

To study the effect of temperature on the displacement of [^{3}H]DPCPX with LUF6037, radioligand binding studies were performed at 25 ºC and 0 ºC (see Table 6.1). Neither the affinity for the high affinity site nor the affinity for the low affinity site was strongly affected.

From these affinities the equilibrium binding association constants, K_A (=1/K_i), were calculated. The thermodynamic parameters ΔG^0 (Gibbs energy), ΔH^0 (standard enthalpy) and ΔS^0 (standard entropy) were determined with a van 't Hoff plot (Figure 6.4 and Table 6.2). The interaction of both CPA[6] and LUF6037 with the high affinity site is entropy driven and endothermic. The interaction with the low affinity state/site was also similar for the two compounds, being exothermic and partially entropy and partially enthalpy driven.

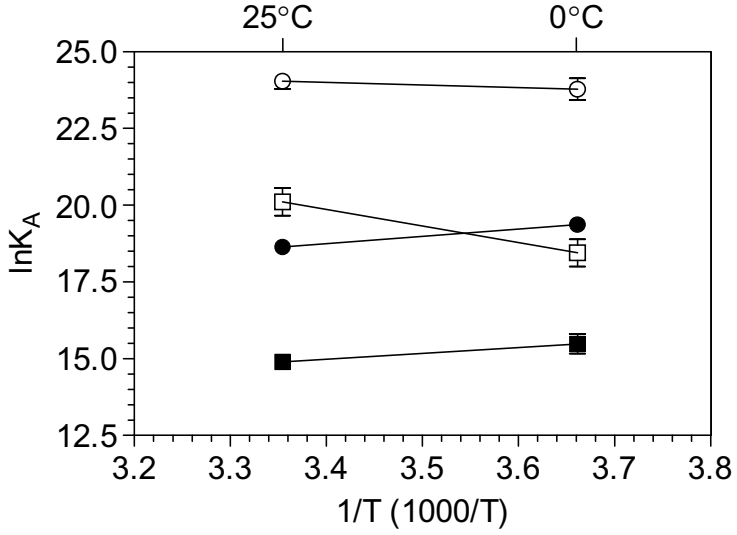

Figure 6.4. Van 't Hoff plots showing the effect of temperature on the equilibrium binding constants, K_A, for the high and low affinity binding sites of LUF6037 (open and closed circles, respectively) and CPA (open and closed squares, respectively). The thermodynamic parameters are presented in Table 6.2.

Table 6.2. Thermodynamic parameters, ΔG^0, ΔH^0 and ΔS^0, for the high and low affinity binding of LUF6037 to the human adenosine A_1 receptors. Data for CPA are shown for comparison.

Compound	ΔG^0 kJ mol^{-1}	ΔH^0 kJ mol^{-1}	ΔS^0 J mol^{-1} K^{-1}
LUF6037 (high)	-59 ± 2	7.0 ± 12	223 ± 40
LUF6037 (low)	-46 ± 9	-19.7 ± 5.7	89 ± 20
CPA (high) [a]	-50 ± 1	45 ± 17	318 ± 60
CPA (low) [a]	-37 ± 1	-16 ± 9	71 ± 31

[a]: data are from Heitman et al. 2006[6].

Efficacy of LUF6037

LUF6037 was further tested in cAMP experiments on CHO cells expressing the human adenosine A_1 receptor. For comparison the reference agonist CPA and the reference inverse agonist DPCPX were also tested. The compounds were tested at the concentrations indicated (see Figure 6.5). The basal cAMP production in the cells was set at 0%, whereas the amount of cAMP produced in the presence of 10 μM forskolin was set at 100%. The reference agonist CPA (30 nM) inhibited the cAMP production to 27 ± 7%, whereas the reference inverse agonist DPCPX (100 nM) increased the cAMP production to 143 ± 15%. LUF6037 (30 nM) was equipotent to CPA resulting in an inhibition of the cAMP production to 34 ± 5%. DPCPX at a concentration of 100 nM was able to antagonize the effect of both CPA and LUF6037 to a similar extent resulting in values of 102 ± 9% and 109 ± 9%, respectively.

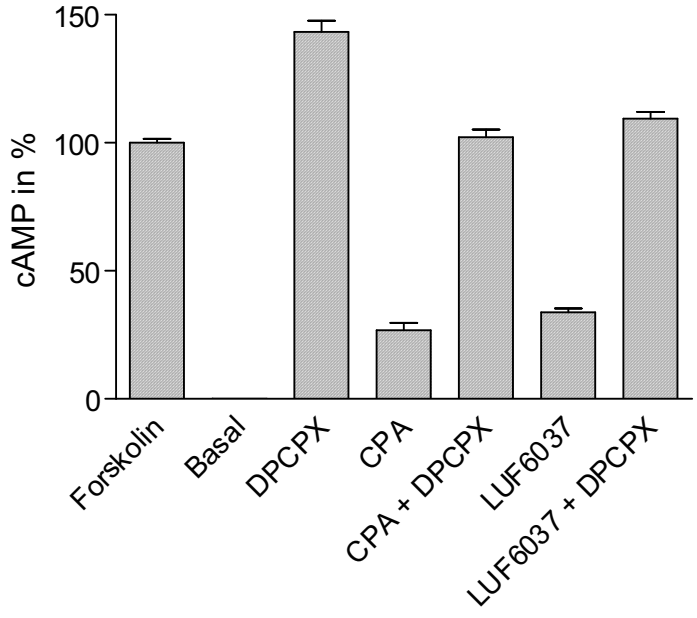

Figure 6.5. Modulation of forskolin-induced cAMP production in CHO cells stably expressing the human adenosine A_1 receptor, after exposure to reference ligands (CPA, DPCPX) or LUF6037. DPCPX was tested at a concentration of 100 nM, whereas CPA and LUF6037 were tested at a concentration of 30 nM.

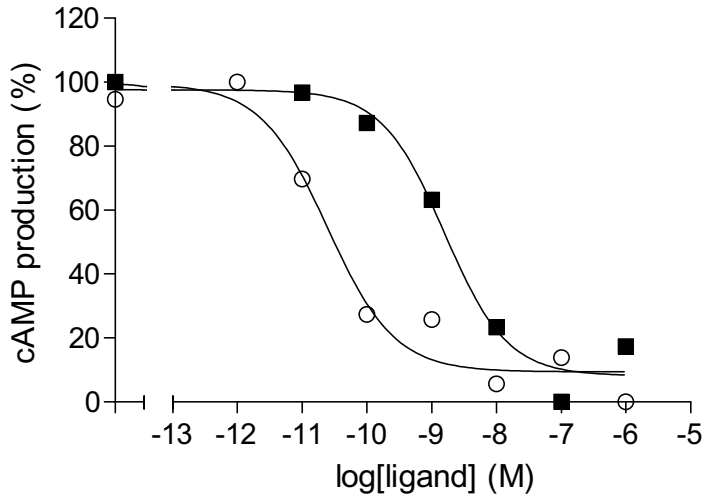

Figure 6.6. Representative curves of the inhibition of cAMP production by CPA (■) and LUF6037 (○) in CHO cells stably expressing the human adenosine A_1 receptor (n = 5).

In addition, full dose response curves for both CPA and LUF6037 were made (Figure 6.6). Analysis of these data revealed EC_{50} values for CPA and LUF6037 of 2.1 ± 0.7 nM and 60 ± 50 pM, respectively. These EC_{50} values are similar to the affinities of CPA and LUF6037 for the high affinity state/site of the receptor, as obtained in the radioligand binding studies.

Internalization of the human adenosine A_1 receptor by LUF6037

CHO cells stably expressing the human adenosine A_1YFP receptor were incubated for 16 h with CPA and LUF6037 at a concentration of 4 μM. Transsections of the cells were studied with confocal microscopy, and the amount of plasma membrane fluorescence was assessed. In Figure 6.7 the percentage fluorescence retained in the plasma membrane is shown for the ligand-free situation as well as upon incubation with either CPA or LUF6037. Whereas CPA significantly reduced the number of plasma membrane bound receptors, LUF6037 was without effect, very similar to a control experiment with the antagonist/inverse agonist DPCPX (4 μM). The respective percentages of fluorescence retained on the plasma membrane were 38 ± 5%, 91 ± 4%, 80 ± 3% and 85 ± 5% for CPA, DPCX, LUF6037 and the control situation, respectively. Representative transsections of confocal images are presented in Figure 6.8.

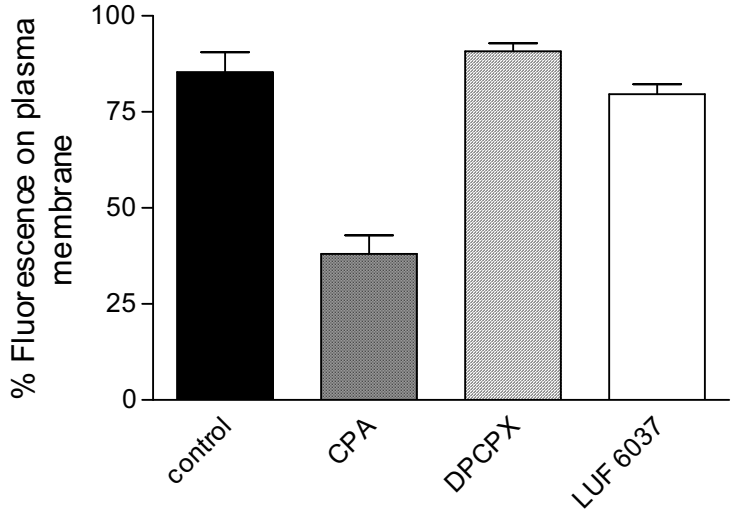

Figure 6.7. Percentage membrane fluorescence on plasma membranes of CHO cells stably expressing human adenosine A_1YFP receptors, after 16 h exposure to CPA, DPCPX or LUF6037 at a concentration of 4 μM, respectively.

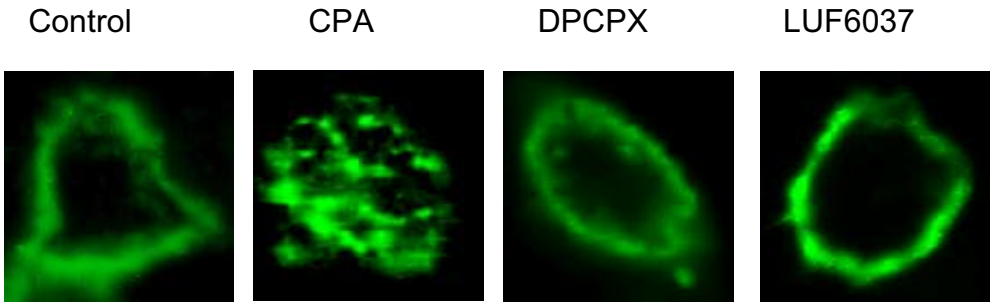

Figure 6.8. Confocal fluorescent pictures of internalized human adenosine A_1YFP receptors in CHO cells. The cells were incubated for 16 hours with CPA, DPCPX or LUF6037, each at a concentration of 4 μM. Pictures were taken from a representative transsection of the cell.

Apparently, LUF6037 is able to inhibit cAMP production as effectively as the full agonist CPA, but differs from CPA with respect to its sensitivity to PD81,723 and its ability to induce receptor internalization.

Discussion

Fuelled by a number of patent disclosures[2,3], we recently synthesized LUF6037. This compound is structurally unrelated to the endogenous ligand adenosine, the most striking feature being the absence of a ribose moiety, which had always been thought to be responsible for 'agonistic' behaviour and activation of the receptor[17,18]. In this study, LUF6037 was tested on its ability to bind, activate and induce internalization of the adenosine A_1 receptor.

Affinity of LUF6037 for the human adenosine A_1 receptor

Previously we reported on a structural analogue of LUF6037, LUF5831[6]. LUF5831 recognized a single state/site of the human adenosine A_1 receptor and was a partial

agonist. In line with the full agonism displayed by LUF6037 in the cAMP experiments (see below), LUF6037 recognized two states of the receptor, a high and a low affinity state just like the reference full agonist CPA. However, LUF6037 was almost 100-fold more potent than CPA, one of the typical high affinity reference agonists in this research field.

Effect of the allosteric modulator PD81,723 on the affinity of LUF6037

PD81,723 has been classified as an allosteric enhancer as it increases the affinity of CPA for the human adenosine A_1 receptor [10,13,15,19]. More precisely, addition of this allosteric enhancer at a concentration of 10 μM resulted in a leftward shift of the [^{3}H]DPCPX displacement curve affecting the low, but not the high affinity state/site of the receptor[6].

On the contrary, PD81,723 did not have an allosteric effect on LUF6037. The affinity of LUF6037 was not affected by the presence of PD81,723, indicating that the change in receptor conformation induced by PD81,723 is unable to enhance the binding of this non-adenosine compound to the human adenosine A_1 receptor. One explanation could be that the non-adenosine ligands bind to a different binding site, insensitive to PD81,723, since the binding of the partial agonist LUF5831 was not sensitive to PD81,723 either[6]. The ability of the non-adenosine ligands to displace [^{3}H]DPCPX binding, just like the adenosine derivatives, suggests however that these ligands share – at least partly – the same binding site. These findings also show the general importance of defining the object of allosteric enhancement. In this case, PD81,723 is an allosteric enhancer of CPA binding, not of LUF6037 binding, despite the agonistic nature of both CPA and LUF6037.

Effect of temperature on affinity of the affinity of LUF6037

Thermodynamic experiments can be used to characterize ligand binding at GPCRs[20]; for review see Borea et al.[21]. In these studies interaction of an agonist with the adenosine A_1 receptor appeared entropy driven whereas the binding of antagonist was essentially enthalpy driven. Partial agonists display intermediate behaviour both for adenosine derivates [14,21,22] and for the non-adenosine ligand LUF5831[6]. Based on these criteria LUF6037 should be classified as a full agonist as the thermodynamic experiments at 25 ºC and 0 ºC demonstrated that the interaction with the high affinity state is fully entropy driven. Indeed the interaction of LUF6037 largely mimics CPA with respect to interaction with both the low and high affinity sites, although the ΔH^o (enthalpy) values are quite different.

Efficacy of LUF6037

LUF6037 was tested on its ability to activate the human adenosine A_1 receptor. The second messenger cAMP was used as a read-out to determine the efficacy of LUF6037. In line with the displacement experiments and the thermodynamic analysis, LUF6037 was a very potent full agonist for the human adenosine A_1 receptor with a picomolar EC_{50} value, closely mimicking the value of $K_{i,H}$ obtained in the radioligand binding studies. Its effect at a single concentration of 30 nM could be reversed with the antagonist/inverse agonist DPCPX (100 nM), suggesting a competitive interaction between the two ligands.

Internalization of the human adenosine A_1 receptor by LUF6037

Stimulation of GPCRs with agonists often results in internalization of the receptor. In this process, the receptors are transported from the plasma membrane to endosomal compartments, thereby contributing to desensitization, a loss of functional response[23]. Several studies have confirmed that treatment with the agonist *R*-PIA, an adenosine derivative, induces internalization of the adenosine A_1 receptor [24,25,26]. Upon stimulation a decrease in number of membrane-bound receptors was found, but an increase of adenosine A_1 receptors in light vesicles, indicating that internalization was the underlying process of the desensitization of the receptor[24]. Recently, we engineered a yellow fluorescent protein (YFP) to the C-terminus of the human adenosine A_1 receptor. By making the receptor fluorescent we could easily detect and quantify internalization with confocal microscopy. In that study CPA dose-dependently promoted internalization of the human adenosine A_1 receptor upon incubation during at least 16 hrs. Moreover, addition of 10 μM PD81,723 did not accelerate the process of internalization, but rather lowered the minimal concentration of CPA needed to induce internalization[10].

Since LUF6037 behaved as a full agonist in the radioligand binding as well as the second messenger studies we also evaluated the ability of LUF6037 to induce internalization of the human adenosine A_1YFP receptor. In contrast to CPA, LUF6037 was not able to induce receptor internalization. These findings differ from more commonly observed results such as reported by Schlag et al.[27] in their research on the 5-HT_{2C} receptor, who observed internalization levels corresponding to agonist intrinsic activity.

Receptor activation versus internalization

Apparently the high affinity and efficacy displayed by LUF6037 do not necessarily lead to internalization of receptors after exposure to this ligand. Although LUF6037 acts as a full agonist, effectuating down-stream inhibition of adenylyl cyclase, thereby decreasing cAMP formation, β-arrestins necessary to induce internalization are most

probably not attracted by the ligand-receptor complex. In that case, the broad spectrum of signaling molecules recruited upon β-arrestin activation as recently reviewed by Lefkowitz and Shenoy[28] will also be by-passed.
Complex signaling behaviour, also coined "collateral efficacy", in which receptor activation, phosphorylation and internalization are not always causally related, has been recently reviewed by Maudsley et al.[29] and Kenakin[30]. For instance agonists may effectuate receptor phosphorylation but are unable to either activate the second messenger system or to induce receptor internalization as observed with the angiotensin analogue [$Sar^{1,}$ Ile^{4}, Ile^{8}]AngII on the wild-type AT_{1A} receptor[31].
The opposite may also occur whereby the ligand mediates receptor internalization without activating the second messenger pathway. Such behaviour was reported for amino-truncated forms of parathyroid hormone[32] as well as for CCK analogues[33] that mediated internalization but not activation of their respective receptors. Keith et al.[34] demonstrated that both enkephalins and morphine activate δ- and μ-opioid receptors to inhibit adenylyl cyclase, but only enkephalins are able to induce rapid receptor internalization. Yu et al.[35] subsequently showed that a direct relationship existed between the efficacy of morphine and its analogues and their ability to induce phosphorylation and desensitization of the μ-opioid receptor. The $5\text{-}HT_{2C}$ receptor provides yet another example, in which the capacity of agonists to elicit desensitization was not related to their efficacy to activate signaling[36].
LUF6037 extends the list of examples of collateral efficacy in which a full agonist lacks the ability to induce receptor internalization.

In conclusion, a new agonist for the adenosine A_1 receptor with a structure completely different from the classic adenosine-like compounds was pharmacologically evaluated. This ligand, LUF6037, showed a very high affinity for the human adenosine A_1 receptor, with an affinity far extending beyond that of the reference full agonist CPA. Remarkably, the allosteric modulator PD81,723 was not able to enhance the affinity of LUF6037 for the human adenosine A_1 receptor. Whereas the thermodynamic interaction of LUF6037 with the receptor was characteristic of a full agonist and cAMP production could indeed be inhibited as much as with CPA, LUF6037 was unable to induce internalization of the human adenosine A_1YFP receptor. This finding, together with the inefficacy of PD81,723, suggest that LUF6037 interacts with the receptor binding pocket in a partially different way from the known adenosine-like agonists. The data show the first proof of separation between activation and internalization of the adenosine A_1 receptor by a full agonist with 100-fold higher affinity and 35-fold greater potency with respect to the reference agonist CPA. The ability of LUF6037 to fully activate the receptor without inducing internalization is a novel example of collateral efficacy. This

behaviour may be therapeutically advantageous as drug resistance due to receptor internalization, as seen with traditional full agonists, might be avoided.

References

1. Müller CE (2000). Adenosine receptor ligands-recent developments part I. Agonists. *Curr Med Chem* 7:1269-1288.
2. Rosentreter U, Kraemer T, Shimada M, Huebsch W, Diedrichs N, Krahn T, Henninger K, Stasch J-P. Substituted 2-thio-3,5-dicyano-4-phenyl-6-aminopyridines and their use as adenosine receptor-selective ligands. WO patent 2003/03008384.
3. Rosentreter U, Kramer T, Shimada M, Hubsch W, Diedrichs N, Krahn T, Henninger K, Stasch J-P. Substituted 2-thio-3,5-dicyano-4-phenyl-6-aminopyridines and their use as adenosine receptor-selective ligands. US patent 2004/0176417.
4. Beukers MW, Chang LC, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Spanjersberg RF, Brussee J and IJzerman AP (2004). New, non-adenosine, high-potency agonists for the human adenosine A_{2B} receptor with an improved selectivity profile compared to the reference agonist N-ethylcarboxamidoadenosine. *J Med Chem* 47:3707-3709.
5. Chang LCW, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Spanjersberg RF, Roerink SF, van den Hout G, Beukers MW, Brussee J and IJzerman AP (2005). A series of ligands displaying a remarkable agonistic-antagonistic profile at the adenosine A_1 receptor. *J Med Chem* 48:2045-2053.
6. Heitman LH, Mulder-Krieger T, Spanjersberg RF, von Frijtag Drabbe Künzel JK, Dalpiaz A and IJzerman AP (2006). Allosteric modulation, thermodynamics and binding to wild-type and mutant (T277A) adenosine A_1 receptors of LUF5831, a novel non-adenosine like agonist. *Br J Pharmacol* 147:533-541.
7. van der Klein PA, Kourounakis AP and IJzerman AP (1999). Allosteric modulation of the adenosine A_1 receptor. Synthesis and biological evaluation of novel 2-amino-3-benzoylthiophenes as allosteric enhancers of agonist binding. *J Med Chem* 42:3629-3635.
8. Leurs R, Smit MJ, Menge WMBP and Timmerman H (1994). Pharmacological characterization of the human histamine H_2 receptor stably expressed in chinese hamster ovary cells. *Br J Pharmacol* 112:847-854.
9. Townsend-Nicholson A and Shine J (1992). Molecular cloning and characterisation of a human brain A_1 adenosine receptor cDNA. *Brain Res Mol Brain Res* 16:365-370.
10. Klaasse EC, van den Hout G, Roerink SF, de Grip WJ, IJzerman AP and Beukers MW (2005). Allosteric modulators affect the internalization of human adenosine A_1 receptors. *Eur J Pharmacol* 522:1-8.
11. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem* 150:76-85.
12. Klaasse EC, de Ligt RAF, Roerink SF, Lorenzen A, Milligan G, Leurs R and IJzerman AP (2004). Allosteric modulation and constitutive activity of fusion proteins between the adenosine A_1 receptor and different 351Cys-mutated $G_i\alpha$-subunits. *Eur J Pharmacol* 499:91-98.
13. Bruns RF and Fergus JH (1990). Allosteric enhancement of adenosine A_1 receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol Pharmacol* 38:939-949.
14. Dalpiaz A, Townsend-Nicholson A, Beukers MW, Schofield PR and IJzerman AP (1998). Thermodynamics of full agonist, partial agonist, and antagonist binding to wild-type and mutant adenosine A_1 receptors. Biochem Pharmacol 56:1437-1445.
15. Kourounakis A, Visser C, de Groote M and IJzerman AP (2001). Differential effects of the allosteric enhancer (2-amino-4,5-dimethyl-trienyl)[3-trifluoromethyl) phenyl]methanone (PD81,723) on agonist and antagonist binding and function at the human wild-type and a mutant (T277A) adenosine A_1 receptor. *Biochem Pharmacol* 61:137-144.
16. Cheng Y and Prusoff WH (1973). Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* 22:3099-3108.
17. Lohse MJ, Klotz KN, Diekmann E, Friedrich K and Schwabe U (1988). 2',3'-Dideoxy-N^6-cyclohexyladenosine: an adenosine derivative with antagonist properties at adenosine receptors. *Eur J Pharmacol* 156:157-160.
18. Siddiqi SM, Jacobson KA, Esker JL, Olah ME, Ji XD, Melman N, Tiwari KN, Secrist JA III, Schneller SW, Cristalli G, Stiles GL, Johnson CR and IJzerman AP (1995). Search for new

purine- and ribose-modified adenosine analogues as selective agonists and antagonists at adenosine receptors. *J Med Chem* 38:1174-1188.
19. Musser B, Mudumbi RV, Liu J, Olson RD and Vestal RE (1999). Adenosine A_1 receptor-dependent and -independent effects of the allosteric enhancer PD81,723. *J Pharmacol Exp Ther* 288:446-454.
20. Borea PA, Varani K, Malaguti V and Gilli G (1991). Receptor binding at two different temperatures to discriminate agonist and antagonist behaviour of adenosine A_1 receptor ligands in rat brain. *J Pharm Pharmacol* 43:866-868.
21. Borea PA, Varani K, Dalpiaz A, Capuzzo A, Fabbri E and IJzerman AP (1994). Full and partial agonistic behaviour and thermodynamic binding parameters of adenosine A_1 receptor ligands. *Eur J Pharmacol* 267:55-61.
22. IJzerman AP, van der Wenden EM, von Frijtag Drabbe Künzel JK, Mathôt RA, Danhof M, Borea PA and Varani K (1994). Partial agonism of theophylline-7-riboside on adenosine receptors. *Naunyn Schmiedebergs Arch Pharmacol* 350:638-645.
23. Koenig JA and Edwardson JM (1997). Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol Sc.* 18:276-287.
24. Ramkumar V, Olah ME, Jacobson KA and Stiles GL (1991). Distinct pathways of desensitization of A_1- and A_2-adenosine receptors in DDT1 MF-2 cells. *Mol Pharmacol* 40:639-647.
25. Ruiz A, Sanz JM, Gonzalez Calero G, Fernandez M, Andres A, Cubero A and Ros M (1996). Desensitization and internalization of adenosine A_1 receptors in rat brain by in vivo treatment with *R*-PIA: involvement of coated vesicles. *Biochim Biophys Acta* 10:168-174.
26. Ciruela F, Saura C, Canela EI, Mallol J, Lluis C and Franco R (1997). Ligand-induced phosphorylation, clustering, and desensitization of A_1 adenosine receptors. *Mol Pharmacol* 52:788-797.
27. Schlag BD, Lou Z, Fennell M and Dunlop J (2004). Ligand dependency of 5-hydroxytryptamine $_{2C}$ receptor internalization. *J Pharmacol Exp Ther* 310:865-870.
28. Lefkowitz RJ and Shenoy SK (2005). Transduction of receptor signals by -arrestins. *Science* 308:512-517.
29. Maudsley S, Martin B and Luttrell LM (2005). The origins of diversity and specificity in G protein-coupled receptor signaling. *J Pharmacol Exp Ther* 314:485-494.
30. Kenakin T (2005). New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nature Rev* 4:919-927.
31. Thomas WG, Qian H, Chang CS and Karnik S (2000). Agonist-induced phosphorylation of the angiotensin II (AT_{1A}) receptor requires generation of a conformation that is distinct from the inositol phosphate-signaling state. *J Biol Chem.* 275:2893-2900.
32. Sneddon WB, Syme CA, Bisello A, Magyar CE, Rochdi MD, Parent JL, Weinman EJ, Abou-Samra AB and Friedman PA (2003). Activation-dependent parathyroid hormone receptor internalization is regulated by NHERF1 (EPB50). *J Biol Chem* 278:43787-43796.
33. Roettger BF, Ghanekar D, Rao R, Toledo C, Yingling J, Pinon D and Miller LJ (1997). Antagonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. *Mol Pharmacol* 51:357-362.
34. Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ and von Zastrow M (1996). Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* 271:19021-19024.
35. Yu Y, Zhang L, Yin X, Sun H, Uhl GR and Wang JB (1997). Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. *J Biol Chem.* 272:28869-28874.
36. Stout BD, Clarke WP and Berg KA (2002). Rapid desensitization of the serotonin$_{2C}$ receptor system: effector pathway and agonist dependence. *J Pharmacol Exp Ther* 302:957-962.