Allosteric modulation, thermodynamics and binding to wild-type and mutant (T277A) adenosine A1 receptors of LUF5831, a novel nonadenosine-like agonist

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Allosteric modulation, thermodynamics and binding to wild-type and mutant (T277A) adenosine A$_1$ receptors of LUF5831, a novel nonadenosine-like agonist

[1]Laura H. Heitman, [1]Thea Mulder-Krieger, [1]Ronald F. Spanjersberg, [1]Jacobien K. von Frijtag Drabbe Künzel, [2]Alessandro Dalpiaz & *,[1]Adriaan P. IJzerman

[1]Division of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands and [2]Department of Pharmaceutical Chemistry, Ferrara University, Via Fossato di Mortara 19, Ferrara I-44100, Italy

1 The interaction of a new nonribose ligand (LUF5831) with the human adenosine A$_1$ receptor was investigated in the present study.

2 Radioligand binding experiments were performed in the absence and presence of diverse allosteric modulators on both wild-type (wt) and mutant (T277A) adenosine A$_1$ receptors. Thermodynamic data were obtained by performing these assays at different temperatures. In addition, cyclic adenosine monophosphate (cAMP) assays were performed.

3 The presence of allosteric modulators had diverse effects on the affinity of LUF5831, N^6-cyclopentyladenosine (CPA), a full agonist, and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), an inverse agonist/antagonist, for the adenosine A$_1$ receptor. PD81,723, for example, increased the affinity of CPA, while the affinity of LUF5831 was decreased. However, the affinity of DPCPX was decreased even more. In addition, LUF5831 was shown to have an affinity for the mutant (T277A) adenosine A$_1$ receptor ($K_i = 122 \pm 22$ nM), whereas CPA's affinity was negligible. The results of temperature-dependent binding assays showed that the binding of LUF5831 was entropy driven, in between the behaviour of CPA binding to the high- and low-affinity states of the receptor, respectively.

4 The inhibition of the forskolin-induced production of cAMP through activation of the wt adenosine A$_1$ receptor showed that LUF5831 had a submaximal effect ($37 \pm 1\%$) in comparison to CPA ($66 \pm 5\%$). On the mutant receptor, however, neither CPA nor LUF5831 inhibited cAMP production.

5 This study indicates that the nonribose ligand, LUF5831, is a partial agonist for the adenosine A$_1$ receptor.

Abbreviations: ADA, adenosine deaminase; cAMP, cyclic adenosine monophosphate; CHO, Chinese hamster ovary; CPA, N^6-cyclopentyladenosine; 8-CPT, 8-cyclopentyltheophylline; [^{3}H]DPCPX, 8-cyclopentyl-[^{3}H]1,3-dipropylxanthine; ΔG^0, standard free energy; GPCR, G-protein-coupled receptor; GTP, guanosine-5′-triphosphate; ΔH^0, standard enthalpy; K_H, equilibrium binding dissociation constant for high-affinity state of the receptor; K_L, equilibrium binding dissociation constant for low-affinity state of the receptor; PD81,723, 2-amino-4,5-dimethyl-3-thienyl-[3(trifluoromethyl)-phenyl]methanone; R$_H$, fraction of receptors in high-affinity state; ΔS^0, standard entropy; wt, wild type

Introduction

Extracellular adenosine plays an important physiological role and mediates a large variety of effects, for example, on the cardiovascular, immune, and central nervous systems (Ralevic & Burnstock, 1998). These effects are mediated by adenosine receptors, which belong to the large family of membrane-bound G-protein-coupled receptors (GPCRs) (Fredholm *et al.*, 2001). The adenosine receptors have been subclassified into four subtypes, A$_1$, A$_{2A}$, A$_{2B}$ and A$_3$, according to their molecular, biochemical and pharmacological properties. The adenosine A$_1$ and A$_3$ receptors are coupled to the enzyme adenylate cyclase in an inhibitory fashion *via* a G$_i$ protein, whereas the A$_{2A}$ and A$_{2B}$ receptors stimulate this enzyme *via* a G$_s$ protein.

So far, agonists for the adenosine receptors have all been derivatives of the endogenous ligand adenosine. Chemical modification of the adenosine structure (Figure 1), particularly at the N^6, C2, and C5′ positions, has proven feasible with N^6-cyclopentyladenosine (CPA, Figure 1), a selective, high-affinity agonist for the adenosine A$_1$ receptor, as a reference example (Müller, 2001). The ribose moiety was thought necessary for agonistic behaviour, as manipulation of this

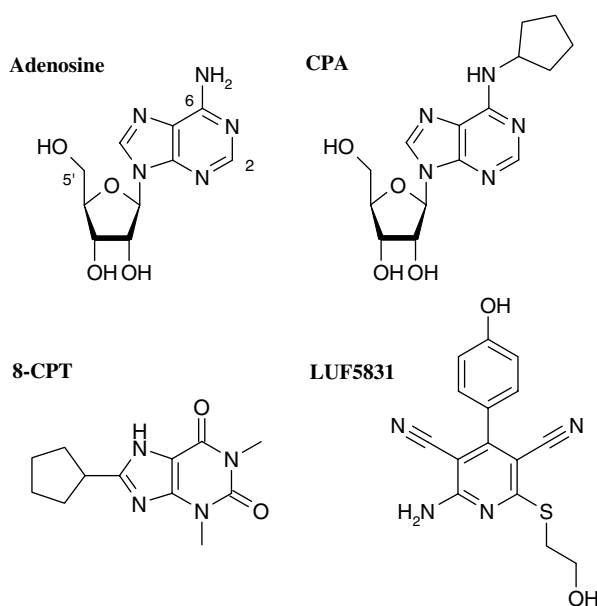*Author for correspondence; E-mail: ijzerman@lacdr.leidenuniv.nl

Figure 1 Chemical structures of adenosine (endogenous ligand), CPA (full agonist), 8-CPT (inverse agonist) and LUF5831.

moiety yielded a lower intrinsic activity at the adenosine A$_1$ receptor (Siddiqi *et al.*, 1995; Van Calenbergh *et al.*, 1997; Soudijn *et al.*, 2003).

Recently, however, a series of novel ligands for the adenosine receptor, all pyridine-3,5-dicarbonitriles and thus structurally unlike adenosine, has been described in patent literature, some of them claimed to be agonists (Rosentreter *et al.*, 2001; 2003). We decided to follow-up on these findings and started a synthetic program from which LUF5831 (Figure 1) emerged (Chang *et al.*, 2005).

For the present study, we decided to analyse the pharmacological properties of LUF5831 in radioligand-binding studies and second messenger assays, always in comparison to the reference full agonist and adenosine-look-alike CPA. Radioligand-binding studies were performed on the wild-type (wt) human adenosine A$_1$ receptor in the absence or presence of allosteric modulators and on a mutant (T277A) A$_1$ receptor. This mutant receptor recognizes antagonists/inverse agonists, but has a remarkably low affinity for agonists such as CPA (Townsend-Nicholson & Schofield, 1994; Dalpiaz *et al.*, 1998). The temperature dependence of the ligand–receptor interaction was also studied, yielding the thermodynamic terms ΔG^0, ΔH^0 and ΔS^0 for the binding of both compounds. In addition, the effects of both CPA and LUF5831 on the inhibition of adenylate cyclase were determined as a measure of intrinsic activity.

Methods

Cell culture

Chinese hamster ovary (CHO) cells expressing the wt human adenosine A$_1$ receptor were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% bovine calf serum, streptomycin (50 μg ml^{-1}), penicillin (50 IU ml^{-1}) and G418 (0.2 mg ml^{-1}) at

37°C in 5% CO$_2$. CHO cells expressing the mutant T277A human adenosine A$_1$ receptor were grown as described above, but without G418. The cells were subcultured twice weekly at a ratio of 1:20 or 1:30 for the cells expressing the wt or the mutant receptors, respectively. For membrane preparation the cells were transferred to large 14-cm diameter plates (Dalpiaz *et al.*, 1998).

Membrane preparation

Cells were detached from the plates by scraping them into 5 ml PBS, collected and centrifuged at 700 × *g* (3000 r.p.m.) for 5 min. Pellets derived from 20 plates were pooled and resuspended in 20 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.4. An UltraThurrax was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 × *g* (31,000 r.p.m.) in a Beckman Optima LE-80 K ultracentrifuge at 4°C for 20 min. The pellet was resuspended in 10 ml of the Tris buffer and the homogenization and centrifugation step was repeated. Tris buffer (10 ml) was used to resuspend the pellet and the membranes were stored in 500 μl aliquots at −80°C. Membrane protein concentrations were measured using the BCA (bicinchoninic acid) method (Smith *et al.*, 1985).

Radioligand-binding assays

Membrane aliquots containing 10 μg (CHOhA$_1$-wt) protein were incubated in a total volume of 400 μl of 50 mM Tris-HCl, pH 7.4 at 25°C for 60 min in the presence or absence of GTP (guanosine-5′-triphosphate) (1 mM), PD81,723 (10 μM), SCH-202676 (10 μM) or NaCl (1 M). Membrane aliquots containing 20 μg (CHOhA$_1$-mutT277A) protein were incubated in a total volume of 200 μl of 50 mM Tris-HCl, pH 7.4 at 25°C for 60 min. Saturation experiments were carried out using nine different concentrations of [^{3}H]DPCPX ranging from 0.1 to 8.0 nM. Displacement experiments on CHOhA$_1$-wt cell membranes were performed using either 12 or 24 concentrations of cold ligand in the presence of 1.6 nM [^{3}H]DPCPX. Displacement experiments on CHOhA$_1$-mutT277A cell membranes were carried out with 1.3 nM [^{3}H]DPCPX. Nonspecific binding was determined in the presence of 10 μM CPA (CHOhA$_1$-wt) or 0.1 mM 8-CPT (CHOhA$_1$-mutT277A) and represented approximately 10% of the total binding. Incubations were terminated by dilution with ice-cold Tris-HCl buffer. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters using a Brandel harvester. Filters were subsequently washed three times with ice-cold buffer. Filter-bound radioactivity was measured by scintillation spectrometry (LKB Wallac, 1219 Rackbeta) after addition of 3.5 ml of Packard Emulsifier Safe.

For the other adenosine receptor subtypes radioligand-binding studies (A$_{2A}$ and A$_3$) and cAMP assays (A$_{2B}$) were performed essentially as described by Beukers *et al.* (2004).

cAMP assays

CHOhA$_1$-wt and CHOhA$_1$-mutT277A cells were harvested, using trypsol (0.25% in PBS containing 4.4 mM EDTA) and, after centrifugation at 700 × *g* for 5 min, resuspended in medium. The cells were plated in 24-well plates (400 μl/well, 2 × 10^5 cells/well) and grown overnight as a monolayer at 37°C

in 5% CO_2. The medium was removed and the cells were washed two times with $500\,\mu l$ DMEM, containing $50\,mM$ HEPES, pH 7.4. Subsequently, the cells were incubated with $250\,\mu l$ DMEM/HEPES, supplemented with adenosine deaminase (ADA) (final concentration $0.8\,IU\,ml^{-1}$), rolipram ($50\,\mu M$) and cilostamide ($50\,\mu M$). After 30 min of incubation at 37°C, $50\,\mu l$ of various ligands was added. CPA, 8-CPT and LUF5831 were tested at a concentration of 100 times their K_i values to determine their maximal effects at the CHOhA$_1$-wt receptor. For the CHOhA$_1$-T277A receptor, 8-CPT and LUF5831 were tested as described above. CPA, however, was tested at a concentration of $10^{-4}\,M$ due to its negligible affinity for this mutant receptor. After 10 min of incubation, $100\,\mu l$ forskolin (final concentration $10\,\mu M$) was added. The cells were incubated for an additional 15 min at 37°C, and the incubation was terminated by quick aspiration of the medium. The cells were lysed by the addition of $200\,\mu l$ ice-cold $0.1\,M$ HCl. The plates were stored at −20°C until further use.

The amount of cAMP was determined by competition with [³H]cAMP for protein kinase A (PKA)-binding protein. A final volume of $200\,\mu l$ containing $100\,\mu l$ PKA in buffer supplemented with bovine serum albumine (BSA) ($150\,mM$ K_2HPO_4, $10\,mM$ EDTA, 0.2% BSA, pH 7.5), $50\,\mu l$ [³H]cAMP in buffer, $50\,\mu l$ sample or cAMP standard (0–12 pmol) was incubated for 2.5 h on ice. The incubation was terminated by adding 2 ml of ice-cold Tris-HCl buffer ($50\,mM$, pH 7.4), and bound radioactivity was separated by rapid filtration through Whatman GF/C filters with a Brandel harvester. Filters were washed twice with 1 ml of buffer. Filter-bound radioactivity was measured by scintillation spectrometry (LKB Wallac, 1219 Rackbeta) after addition of 3.5 ml of Packard Emulsifier Safe.

Thermodynamic data determination

The values of thermodynamic terms (ΔG^0, ΔH^0 and ΔS^0) were obtained by measuring K_i values at 0°C and 25°C, followed by linear van 't Hoff plot regression (Dalpiaz *et al.*, 1998). The standard free energy, ΔG^0, was calculated according to $\Delta G^0 = -RT \ln K_A$, where $K_A = 1/K_i$. The van 't Hoff equation $\ln K_A = -\Delta H^0/RT + \Delta S^0/R$ gives a linear plot of $\ln K_A$ versus $1/T$. The standard enthalpy (ΔH^0) can be calculated from the slope, $-\Delta H^0/R$, and the standard entropy from the intercept, $\Delta S^0/R$ or as $\Delta S^0 = (\Delta H^0 - \Delta G^0)/T$, with $T = 298.15\,K$ and $R = 8.314\,JK^{-1}\,mol^{-1}$.

Data analysis

All binding data was analysed using the nonlinear regression curve-fitting program GraphPad Prism v. 4 (GraphPad Software Inc., San Diego, CA, U.S.A.). Inhibitory binding constants (K_i values) were derived from the IC$_{50}$ values according to the Cheng and Prusoff equation $K_i = IC_{50}/(1 + [C]/K_d)$ where $[C]$ is the concentration of the radioligand and K_d its dissociation constant (Cheng & Prusoff, 1973). The K_d values of [³H]DPCPX at CHOhA$_1$-wt and CHOhA$_1$-mutT277A membranes at different conditions were obtained by computer analysis of saturation curves. All values obtained are means of at least three independent experiments performed in duplicate.

Materials

Results

Allosteric modulation of affinity constants

Radioligand saturation experiments   Saturation experiments were performed with [³H]DPCPX on CHO cells expressing the wt adenosine A$_1$ receptor in the presence and absence of allosteric modulators. The binding of [³H]DPCPX was saturable and best characterized by a one-site competition model with a control K_d-value of 1.6 nM for the wt receptor (Table 1). From Table 1 it also follows that on CHOhA$_1$-wt cell membranes the K_d-value of [³H]DPCPX was not affected in the presence of GTP (1 mM). This value in the presence of PD81,723 ($10\,\mu M$) was increased approximately five-fold, whereas in the presence of SCH-202676 ($10\,\mu M$) or NaCl (1 M), the K_d-value was decreased, approximately 2- and 3-fold, respectively. The data in Table 1 was used to derive K_i rather than IC$_{50}$ values in the following paragraphs.

Radioligand displacement assays   Displacement experiments of [³H]DPCPX on CHOhA$_1$-wt cell membranes by CPA, as a reference full agonist, and LUF5831 were carried out in the presence or absence of 1 mM GTP, $10\,\mu M$ PD81,723, $10\,\mu M$ SCH-202676 or 1 M NaCl. For CPA a two-state receptor

Table 1 K_d-values of [³H]DPCPX in the presence of buffer; 1 mM GTP; $10\,\mu M$ PD81,723; $10\,\mu M$ SCH-202676, 1 M NaCl or at 0°C at CHO human wild-type adenosine A$_1$ receptors

Membranes	[³H]DPCPX K_d (nM)[a]	Shift[b]
CHOhA$_1$-wt	1.6 ± 0.1	—
+ GTP	1.6 ± 0.3	1.0
+ PD81,723	8.8 ± 1.4	5.5
+ SCH-202676	0.79 ± 0.08	0.49
+ NaCl	0.55 ± 0.08	0.34
0°C	0.69 ± 0.05[c]	0.43

[a]Saturation of specific [³H]DPCPX binding at wild-type human adenosine A$_1$ receptors stably expressed on CHO cell membranes.
[b]The shift is defined as the ratio of K_d-values in the presence and absence of an allosteric modulator or at 0°C, respectively.
[c]Data from Dalpiaz *et al.* (1998).
Values are means ($\pm$ s.e.m.) of three separate assays each performed in duplicate.

Table 2 Affinities of CPA and LUF5831 at 25°C in the presence of buffer; 1 mM GTP; 10 μM PD81,723; 10 μM SCH-202676, 1 M NaCl or in buffer at 0°C at CHO human wild-type adenosine A₁ receptors, expressed as K_i values

Compound	K_H or K_i (nM)[a]	K_L (nM)[a]	% R_H	Shift[b]
CPA 25°C	2.2 ± 0.9	338 ± 24	34 ± 2	—
+ GTP	191 ± 24	—	—	—
+ NaCl	2.4 ± 0.3	309 ± 23	26 ± 4	1.1
+ PD81,723	2.3 ± 0.4	93 ± 22	40 ± 3	1.0
+ SCH-202676	225 ± 8	—	—	—
CPA 0°C	7.0 ± 3.0	128 ± 64	39 ± 10	3.2
LUF5831 25°C	18 ± 1	—	—	—
+ GTP	23 ± 4	—	—	1.3
+ NaCl	33 ± 5	—	—	1.8
+ PD81,723	32 ± 4	—	—	1.8
+ SCH-202676	25 ± 4	—	—	1.4
LUF5831 0°C	18 ± 1	—	—	1.0

[a]Displacement of specific [³H]DPCPX binding at human adenosine A₁ receptors stably expressed on CHO cell membranes. Only data are shown, which according to computer analysis of the human adenosine A₁ receptor-binding curves were statistically preferred under the different conditions.
[b]The shift is defined as the ratio of K_H-values (for CPA) or K_i-values (for LUF5831) in the presence and absence of an allosteric modulator, respectively.
Values are means (± s.e.m.) of three separate assays each performed in duplicate.

Figure 2 Effects of the allosteric modulators on the displacement of [³H]DPCPX binding from human adenosine A₁ receptors stably expressed on CHO cell membranes by CPA (a) or LUF5831 (b) in the absence or presence of GTP, PD81,723, SCH-202676 or NaCl. Representative graphs from one experiment performed in duplicate (see Table 2 for affinity values).

model with a higher (K_H) and a lower affinity (K_L) was sometimes statistically preferred over a one-state model, depending on the conditions applied. From Table 2 it follows that LUF5831 had a lower affinity (K_i value) for the A₁ receptor than the reference full agonist CPA (K_H value), 18 and 2.2 nM, respectively. Allosteric modulation of the adenosine A₁ receptor resulted in diverse effects on binding affinity of these two ligands. In Figure 2 representative binding curves under the different conditions for CPA and LUF5831 are depicted. We used more data points to assess the effects of LUF5831 than with CPA, to assure an unequivocal interpretation of the effects of this unusual nonribose compound. In the presence of GTP the curve of CPA was less shallow than under control conditions, changing the two-state receptor interaction into one state. For LUF5831, however, this shift in binding state was not observed. In particular, PD81,723 had a marked and divergent effect on the binding affinity of CPA and LUF5831. PD81,723 increased the affinity of CPA for the low-affinity receptors, while the apparent affinity of LUF5831 was decreased in the presence of PD81,723. SCH-202676 decreased the affinity of both compounds, of CPA more than that of LUF5831, while shifting the interaction of CPA with the receptor to a one-state model. In the presence of NaCl the binding affinity of CPA appeared unaffected. For LUF5831, however, the affinity was decreased approximately two-fold. Apparently, the addition of allosteric modulators in some cases resulted in a shift from two affinity-binding states to one for CPA. For LUF5831, two-state binding was not observed under any of the conditions. Lastly, we determined the affinity of CPA and LUF5831 for the mutant (T277A) A₁ receptor. We did not observe any displacement of [³H]DPCPX by CPA (1 μM). For LUF5831 we determined a K_i-value of 122 ± 22 nM
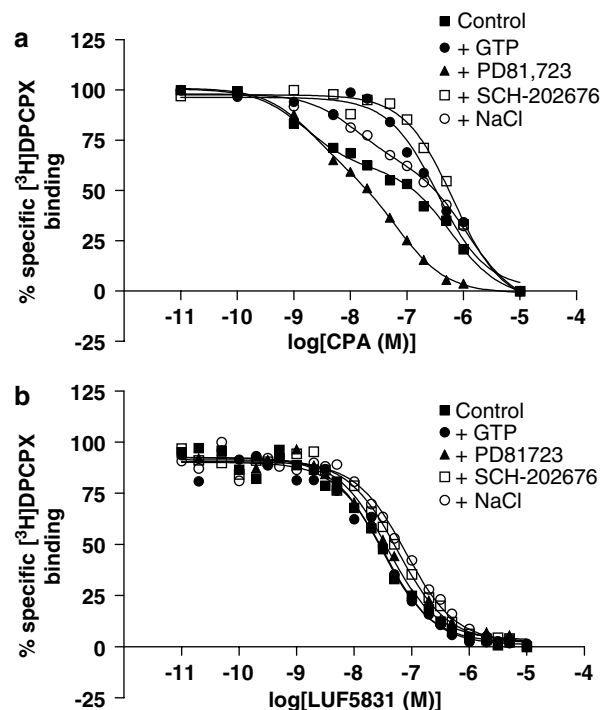
(one-state model) for this receptor, using a K_d value of 1.3 nM for [³H]DPCPX (Dalpiaz *et al.*, 1998).

Thermodynamic experiments

Displacement experiments of [³H]DPCPX by CPA and LUF5831 on CHOhA₁-wt cell membranes were carried out at 0°C and 25°C. The K_d value of [³H]DPCPX at 0°C was determined to be 0.69 ± 0.05 nM (Dalpiaz *et al.*, 1998). The various displacement curves are shown in Figure 3. Apparently, a decrease in temperature had no effect on the curve and therefore on the affinity of LUF5831 (Table 2). The affinity of CPA decreased approximately three-fold for receptors in the high-affinity state (K_H), whereas the affinity for the low-affinity state of the receptors increased with a similar value (Table 2).

From the affinities shown in Table 2, the equilibrium binding association constants, K_A, were calculated. The temperature dependence of CPA and LUF5831 at the different affinity states is illustrated by Van 't Hoff plots, which are shown in Figure 4. The K_A value, the slope and the intercept of the Van 't Hoff plots were used to calculate the values of the thermodynamic terms (ΔG^0, ΔH^0 and ΔS^0) as described in Methods above (Table 3). The values of the equilibrium standard enthalpy, ΔH^0, and entropy, ΔS^0 show that: (1) the interaction of CPA with the high-affinity state of the receptor was endothermic and totally entropy driven; (2) the binding of CPA to the low-affinity state of the receptor was exothermic and essentially enthalpy driven; and (3) the binding of
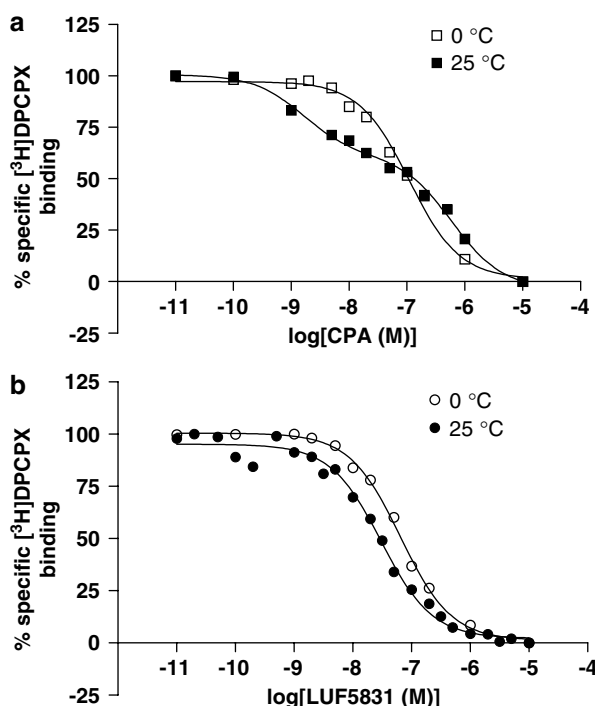
a

b

Figure 3 Effects of temperature on the displacement of [³H]DPCPX binding from human adenosine A₁ receptors stably expressed on CHO cell membranes by CPA (a) at 0 and 25°C or LUF5831 (b) at 0 and 25°C. Representative graphs from one experiment performed in duplicate (see Table 2 for affinity values).
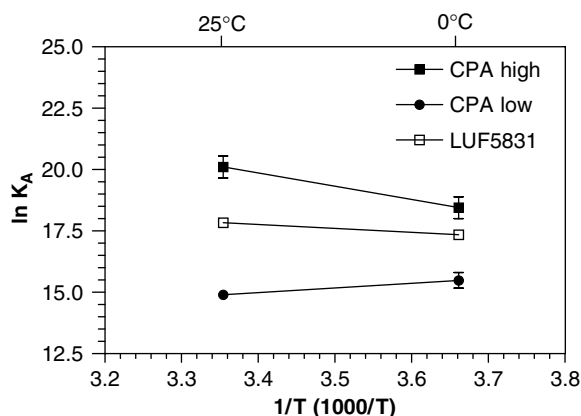

Figure 4 Van 't Hoff plots showing the effect of temperature on the equilibrium binding association constants, K_A, for CPA (high-affinity state), CPA (low-affinity state) and LUF5831. Graphs from three experiments performed in duplicate (see Table 3 for thermodynamic parameters).

LUF5831 to the receptor was endothermic and again entropy driven.

Radioligand displacement assays at other human adenosine receptors

Additionally, affinity (A₂A and A₃) or potency (A₂B) of LUF5831 and CPA were determined at the other human adenosine receptor subtypes (Table 4). The affinity values were derived from a one-site competition analysis of the binding

Table 3 Thermodynamic parameters for CPA and LUF5831 binding at CHO human wild-type adenosine A₁ receptors, expressed as ΔG^0, ΔH^0 and ΔS^0

Compound	ΔG^0 (kJ mol⁻¹)	ΔH^0 (kJ mol⁻¹)	ΔS^0 (J mol⁻¹ K⁻¹)
CPA (high)	-50 ± 1	45 ± 17	318 ± 60
CPA (low)	-37 ± 1	-16 ± 9	71 ± 31
LUF5831	-44 ± 1	13 ± 11	193 ± 7

$\Delta G^0 =$ Gibbs energy, $\Delta H^0 =$ standard enthalpy and $\Delta S^0 =$ standard entropy. Values are given at 298.15 K and means ($\pm$ s.e.m.) are calculated from three separate assays performed in duplicate.

data. Both ligands, CPA and LUF5831, were shown to have the highest affinity for the adenosine A₁ receptor. Further experiments also showed that LUF5831 had a higher selectivity towards the adenosine A₁ receptor than CPA, except for the A₂B receptor (Table 4).

Inhibition of cAMP production via *the wt and mutant receptor*

CPA and LUF5831 were further tested in a cAMP assay on both CHOhA₁-wt and CHOhA₁-mutT277A cells. 8-CPT was also examined in this assay. Here, cAMP was produced upon stimulation with 10 μM forskolin (= 100% cAMP production) in order to examine agonist activation of the receptor. From Figure 5a it appears that CPA caused a stronger inhibition than LUF5831, with 66±5 and 37±1% reduction of cAMP levels, respectively. 8-CPT caused a substantial increase of cAMP levels, and is thus best classified as an inverse agonist in this system. We also determined the potency of both CPA and LUF5831. Both compounds inhibited the forskolin-induced cAMP production with EC₅₀ values of 7.8±0.9 and 102±14 nM for CPA and LUF5831, respectively (data not shown). While CPA, LUF5831 and 8-CPT all modulated cAMP production on the wt receptor, neither inhibition nor stimulation of the mutant receptor occurred (Figure 5b).

Discussion

The present study examines the affinity and activity of a new nonribose agonist, LUF5831, on the human adenosine A₁ receptor under a variety of conditions. First, radioligand saturation experiments were performed to obtain K_d values of [³H]DPCPX, a selective inverse agonist for the A₁ receptor, in the presence and absence of diverse allosteric modulators. In most cases the affinity of the radioligand was affected by the addition of allosteric modulators.

Next, radioligand displacement assays showed that on wt adenosine A₁ receptors LUF5831 was less potent than CPA, with a K_i value of 18 nM for LUF5831 and a K_H value of 2.2 nM for CPA. Further experiments in the absence and presence of GTP yielded the so-called GTP-shift of CPA and LUF5831 on CHOhA₁-wt cells, a measure for the intrinsic activity of a compound (Kent *et al.*, 1980; Ehlert, 1985). For both compounds a GTP-shift was observed, though in a different manner. For CPA the favoured mode of binding shifted towards a one-state competition model with a negligible fraction of high-affinity receptors. The shift of the

Table 4 Affinities of CPA and LUF5831 at the wild-type human adenosine A_{2A}, A_{2B} and A_3 receptor subtypes, expressed as K_i values or % displacement at $1\,\mu M$

			K_i (nM) *or % displacement at $1\,\mu M$*			
Compound	hA_{2A}[a]	A_{2A}/A_1[b]	hA_{2B}[c]	A_{2B}/A_1[b]	hA_3[d]	A_3/A_1[b]
CPA	131 ± 14	60	$203{,}000\pm97{,}000$[e]	92,272	281 ± 56[f]	128
LUF5831	37%	>185	780 ± 380	144	0%	>185

[a]Displacement of specific [³H]ZM241385 binding at human adenosine A_{2A} receptors expressed on HEK293 cell membranes.
[b]The selectivity was calculated using the K_H values for CPA and LUF5831 at the adenosine A_1 receptor (see Table 2) and where % displacement is given, $>1\,\mu M$ was used as affinity value to calculate the selectivity.
[c]Activities (EC₅₀ value) on cAMP production in CHO cells expressing human A_{2B} receptors.
[d]Displacement of specific [¹²⁵I]AB-MECA binding at human adenosine A_3 receptors expressed on HEK293 cell membranes.
[e]Data from de Zwart *et al.* (1998).
[f]Data from van Tilburg *et al.* (1999).
Values are means ($\pm$s.e.m.) of three separate assays each performed in duplicate.
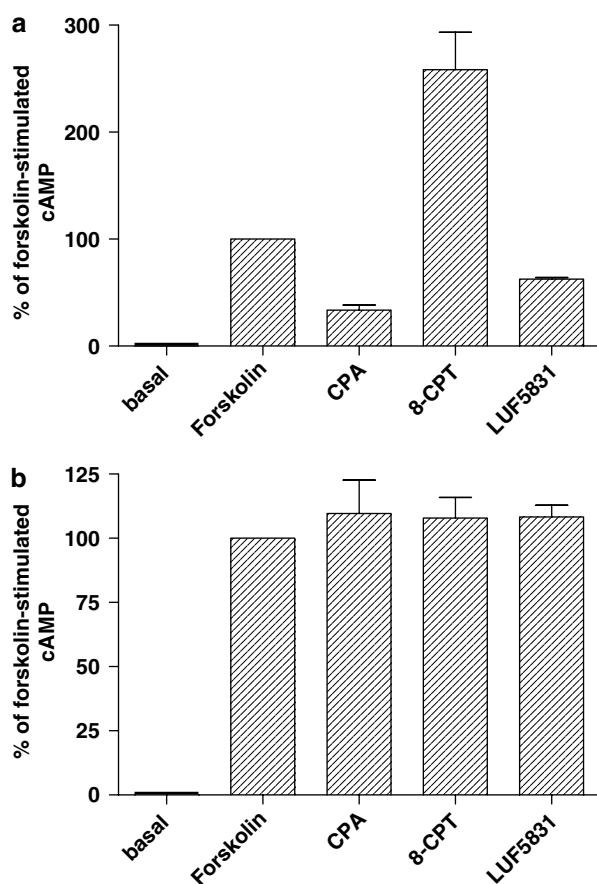

Figure 5 Bar graph representation of receptor activity of $1\,\mu M$ CPA, $3.3\,\mu M$ 8-CPT and $1\,\mu M$ LUF5831 on CHOhA₁-wt (a) or of $100\,\mu M$ CPA, $3.3\,\mu M$ 8-CPT and $13.5\,\mu M$ LUF5831 on CHOhA₁-mutT277A (b) cells expressed as a percentage of forskolin-induced cAMP production. Graph from three experiments performed in four-fold.

intrinsic activity on the wt A₁ receptor, indicative of their agonistic profile. There was no GTP effect on [³H]DPCPX binding (Table 1), suggestive of the radioligand's characteristics as antagonist/inverse agonist. All in all, the different binding modes of the ligands can be explained by the ternary complex model (Lefkowitz *et al.*, 1981).

Besides GTP, PD81,723 can also be used to discriminate between different classes of ligands (Kourounakis *et al.*, 2001). The effects of PD81,723 have been extensively studied, showing this compound acts selectively as an allosteric modulator for the adenosine A₁ receptor (Kollias-Baker *et al.*, 1994; 1997; Dennis *et al.*, 1996; Mizumura *et al.*, 1996). We showed that there is a significant difference between the shift in K_i values of CPA and LUF5831 in the presence of PD81,723. The affinity of the full agonist CPA for the low-affinity state of the receptor (K_L) increased three-fold, while we observed an almost two-fold decrease in the affinity of LUF5831 (Table 2). The affinity of the radioligand DPCPX decreased even more, approximately 5.5-fold, in the presence of PD81,723 (Table 1). This supports the findings that PD81,723 increases the affinity of agonists, but decreases the affinity of antagonists/inverse agonists (Kourounakis *et al.*, 2001). The shift of LUF5831 was in between that of an agonist and an antagonist/inverse agonist, indicating that this compound might be a partial agonist. Another difference between the effect of PD81,723 on the binding of CPA and LUF5831 is that for CPA the two-state competition model was maintained with approximately the same amount of high-affinity receptors. For LUF5831, however, data analysis was best with a one-state competition model, without the presence of high-affinity receptors. In earlier experiments, we already found that PD81,723 slows the dissociation rate of the [³H]CCPA (2-chloro-N^6-cyclopentyladenosine), a radiolabelled agonist, from the adenosine A₁ receptor, which results in a higher affinity of this compound for the receptor (Van der Klein *et al.*, 1999). Maybe PD81,723 somewhat accelerates dissociation of LUF5831 from the receptor, which would explain its lower affinity.

SCH-202676 has been shown to allosterically inhibit the binding of agonists and antagonists to various GPCRs (Fawzi *et al.*, 2001; Gao *et al.*, 2004; Lanzafame & Christopoulos, 2004; Van den Nieuwendijk *et al.*, 2004). Very recently we have demonstrated the compound to be a protein modifier rather than modulator (Göblyös *et al.*, 2005). In this study, the binding of CPA in the presence of SCH-202676 was best described by a one-state model with a lower apparent affinity.

binding model for CPA suggests the compound is an agonist, because the uncoupling of the G-protein from the receptors by GTP, and the concomitant disappearance of high-affinity receptors, induced a lower apparent affinity. Although the favoured binding model for LUF5831 was already one-state in the absence of GTP, the addition of GTP still caused a minor shift to a lower affinity. This experiment, therefore, showed that CPA, and LUF5831 to a lesser extent, are ligands with

On the contrary, the affinity of [^{3}H]DPCPX was increased two-fold in its presence. Therefore, it seems that SCH-202676 not only decreases the affinity of an agonist, but also increases the affinity of inverse agonists. The affinity of LUF5831 was slightly decreased, resembling neither full agonist nor antagonist/inverse agonist.

Lastly, we examined the so-called sodium shift at the adenosine A$_1$ receptor. On other GPCRs, for example, the α_2-adrenergic and D$_2$ and D$_4$ dopamine receptors, but also on adenosine receptors, it has been shown that sodium ions regulate ligand binding (Guyer *et al.*, 1990; Neve *et al.*, 1991; Schetz & Sibley, 2001). The presence of sodium ions reduces the affinity of agonists supposedly by a conformational change of the receptor. The extent of decrease in receptor affinity for agonists by sodium ions parallels agonist intrinsic activity in inhibiting adenylate cyclase (Tsai & Lefkowitz, 1978; Horstman *et al.*, 1990). However, this shift seems to be different from the GTP shift, because sodium ions do not entirely uncouple the receptors from the G-protein, like GTP does (Linden *et al.*, 1988). When the sodium shift of LUF5831 is compared with that of CPA and the radioligand DPCPX, it becomes clear that LUF5831 seems to behave more like an agonist than an antagonist/inverse agonist (Table 2). In addition, the amount of high-affinity receptors for CPA seems largely unaffected in the presence of NaCl. These findings indicate that the receptor indeed does not uncouple from the G-protein, which has already been reported (Linden *et al.*, 1988).

Other displacement experiments on a mutant (T277A) receptor showed that CPA lost all of its affinity for the receptor. This finding corresponds with earlier results (Townsend-Nicholson & Schofield, 1994; Dalpiaz *et al.*, 1998). Interestingly, LUF5831 still bound; its affinity was only reduced five-fold in comparison to the wt receptor. Apparently, Thr277 contributes largely to the binding of CPA and is far less important for the binding of LUF5831. It has been suggested earlier that Thr277 has a specific interaction with the ribose group (Dalpiaz *et al.*, 1998), which is lacking in LUF5831.

Discrimination between agonist and antagonist binding based on a thermodynamic evaluation has been reported for a number of GPCRs, including adenosine receptors (Borea *et al.*, 1991; for review see Borea *et al.*, 2004). Therefore, additional radioligand displacement studies were performed at 0°C to obtain thermodynamic parameters (ΔG^0, ΔH^0 and ΔS^0) of the binding of CPA and LUF5831. First of all, a lowering of the temperature during the binding experiments seemed to have little (CPA) or no effect (LUF5831) on the affinity of both ligands (Table 2). Comparison of CPA's affinity (K_H value) at 25°C and 0°C showed a decrease in affinity of approximately three-fold. The affinity of [^{3}H]DPCPX was increased two-fold (Table 1). The thermodynamic data thus obtained characterizes the binding process and therefore discriminates between full (partial/inverse) agonists and antagonists. On the A$_1$ receptor binding of an agonist has

been shown to be completely entropy driven, whereas the binding of an antagonist is essentially enthalpy driven (IJzerman *et al.*, 1994; Borea *et al.*, 1995). The thermodynamic values of enthalpy and entropy of the binding of a partial agonist are intermediate between a full agonist and an antagonist (Borea *et al.*, 1994; IJzerman *et al.*, 1994; Dalpiaz *et al.*, 1998). The slope of the line of LUF5831 in Figure 4 is between that of the slope of the lines corresponding to the high- and low-affinity binding state of CPA. This is yet another indication that LUF5831 acts as a partial agonist at the human adenosine A$_1$ receptor, which supports the results found with the displacement experiments in the presence of PD81,723 or SCH-202676. Partial agonists are thought to be a valuable addition to the current repertoire of medicines. Van Schaick *et al.* (1998) demonstrated that partial agonists for the adenosine A$_1$ receptor induce tissue selectivity of action. Partial agonists for the receptor caused a marked inhibition of lipolysis in freely moving rats with few cardiovascular effects. The full agonist CPA, however, produced pronounced effects in both tissues. Apparently, unwanted effects can be separated from desired effects with partial agonists.

We also examined the influence of the compound on cellular cAMP levels. Both compounds inhibited the forskolin-induced production of cAMP on the wt receptor. Here LUF5831 behaved as a partial agonist compared to CPA, the reference full agonist. The intrinsic activity of LUF5831 was approximately half that of CPA, while its EC$_{50}$ value was approximately 12-fold lower. The ratio of the EC$_{50}$ over the K_H values showed that the decrease in potency was higher for LUF5831 than for CPA, 18-fold and four-fold, respectively. On the mutant (T277A) receptor, however, both CPA and LUF5831 were unable to inhibit forskolin-induced cAMP production. This is particularly surprising for LUF5831, since this compound had appreciable affinity for the mutant. One explanation could be that LUF5831's interaction with the receptor involves at least some amino acids that are different from those in the adenosine/CPA-binding site, and that the threonine residue at position 277 plays a vital role in receptor activation irrespective of the chemical nature of the agonist.

In conclusion, diverse radioligand-binding assays, thermodynamic analysis and cAMP determinations have been performed to elucidate the binding characteristics of LUF5831 at the human adenosine A$_1$ receptor. The overall outcome of the experiments is that LUF5831 seems to be a partial agonist for this receptor. This is a surprising outcome, because the structure of this compound, unlike the endogenous ligand adenosine, does not include a ribose moiety. Until recently the 'dogma' was that a compound without a ribose moiety could not act as an agonist at the adenosine receptor. In the present study this dogma has been firmly challenged.

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