Non-ribose ligands for the human adenosine A1 receptor

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CHAPTER 7

[^{3}H]LUF5834, a new non-adenosine radioligand for the adenosine A_1 receptor, revealing 3 binding sites for DPCPX on the A_1 receptor

Recently, non-adenosine ligands for the adenosine receptors were synthesized. One of these ligands, LUF5834, a high affinity full agonist for the A_1 receptor, was radioactively labeled. Here we report on the characterization of [^{3}H]LUF5834 as a new radioligand for the A_1 receptor. Radioligand binding studies were performed in the absence and presence of the allosteric enhancer PD81,723 and GTP on CHO cells stably expressing the A_1 receptor. [^{3}H]LUF5834 recognized two binding sites in kinetic and saturation studies. K_d-values of 0.16 ± 0.07 nM and 1.69 ± 0.05 nM were determined. This new radioligand [^{3}H]LUF5834 recognized the same number of receptors as [^{3}H]DPCPX. Surprisingly, unlabeled LUF5834 showed a one site displacement curve with an IC_{50}-value of 5.4 $\pm$ 1.3 nM. PD81,723 (10 μM) and GTP (1mM) were not able to shift its affinity. CPA yielded a two site displacement curve with IC_{50}-values of 4.8 $\pm$ 1.9 nM and 339 $\pm$ 150 nM, respectively. PD81,723 was able to increase only the $IC_{50,low}$-value of CPA. GTP did not affect the IC_{50}-values, but lowered the fraction of receptors in the high affinity state. DPCPX showed a three site displacement curve with IC_{50}-values of 1.2 $\pm$ 1.4 pM, 0.26 $\pm$ 0.24 nM and 127 $\pm$ 44 nM respectively. PD81,723 decreased all affinities, GTP on the other hand only shifted the percentages of receptors in the direction of the high affinity states. In conclusion, [^{3}H]LUF5834 recognizes two binding sites with very high affinity. Moreover, the displacement of [^{3}H]LUF5834 by DPCPX reveals not two, but three binding sites.

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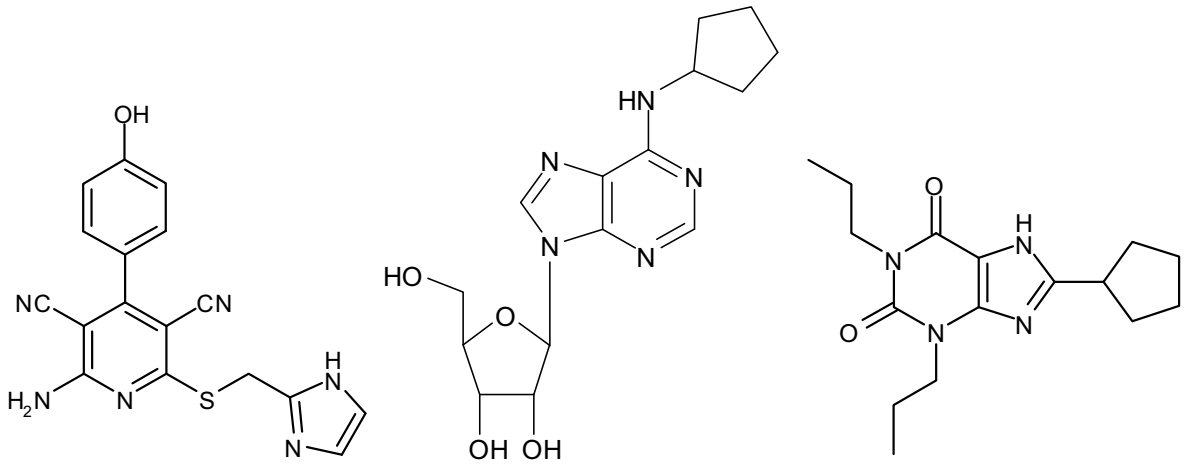
Introduction

Recently, Rosentreter *et al.*[1,2] patented a new class of adenosine receptor ligands, the 2-amino-4-(3,4 substituted phenyl)-6-(2-hydroxyethylsulfanyl)-pyridin-3,5-dicarbonitriles. This class shows no structural similarity to adenosine, the endogenous ligand for the adenosine receptors. However, it was observed that several compounds of this class display a significant affinity and efficacy towards different adenosine receptor subtypes[1-4]. Until then, only modified analogues of adenosine substituted at different positions had been introduced as potent and selective ligands for the adenosine receptors. Especially substitutions at the N^6- and C2 positions of adenosine yielded potent adenosine A_1 agonists, e.g. N^6-cyclopentyladenosine (CPA), N^6-2-chloro-cyclopentyladenosine (CCPA) and N^6-cyclohexyladenosine (CHA)[5].

One of the new, non-adenosine compounds, LUF5831, appeared to be a partial agonist with an affinity of 18 $\pm$ 1 nM for the adenosine A_1 receptor[6]. From this study it also appeared that allosteric modulators such as PD81,723 and GTP seem to have no or much less effect on the binding of LUF5831 than they have on the binding of the traditional adenosine derivative CPA[6]. Another non-adenosine compound, LUF5834, was characterized as a full agonist for the human adenosine A_1 receptor with a very high affinity, comparable to the $K_{i,\ high}$-value of CPA, and as a partial agonist for the A_{2B} receptor with high affinity[3]. For these reasons, LUF5834 was chosen as the non-ribose ligand to radioactively label with ^{3}H.

In this study, we describe the characterization of the new, non-adenosine agonist radioligand for the human adenosine A_1 receptor. Displacement experiments were performed, and the effect of PD81,723 and GTP on the displacement of a non-adenosine-like agonist (LUF5834), adenosine-like agonist (CPA), and an inverse agonist (DPCPX) was studied (see Figure 7.1 for the chemical structures of the compounds). Surprisingly, [^{3}H]LUF5834 revealed three binding sites in the displacement with DPCPX. Finally, the influence of G proteins on the different affinity states of the receptor was studied using Sf9 Insect cell homogenates, expressing the human adenosine A_1his_{10} receptor but lacking functionally active endogenous G_i proteins.



LUF5834 CPA DPCPX

Figure 7.1. Structures of LUF5834, CPA and DPCPX.

Methods

Cell culture

Chinese hamster ovary (CHO) cells stably expressing the human adenosine A_1 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, 50 µg/mL streptomycin, 50 IU/mL penicillin and 0.2 mg/mL neomycin (G418), respectively. The cells were maintained in a humidified atmosphere at 37 ºC and 5% CO_2, and subcultured twice weekly (ratio 1:20).

Spodoptera frugiperda (Sf9) cells were cultured in Insect Xpress medium containing 50 µg/mL streptomycin and 50 IU/mL penicillin at 27 ºC. The cells were subcultured twice weekly (1:5). Confluent Sf9 Insect cells were infected with baculovirus, containing the human adenosine A_1his_{10} construct. Six to seven days post infection, the infected Sf9 Insect cells expressing the human adenosine A_1his_{10} receptor were harvested by centrifugation (5 min, 1300 rpm) and the cell pellets were stored at – 80 ºC.

Membrane preparation

Membranes of CHO cells stably expressing the human adenosine A_1 receptor were prepared as previously described[7]. Membrane protein concentrations were measured using the BCA (bicinchonic acid) method with BSA as a standard[8].

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, 0.1% CHAPS, ADA (1U/mL) pH 7.4, at 25 ºC

for 60 min in the absence or presence of 1 mM GTP or 10 μM PD81,723. Displacement experiments were performed using 24 concentrations of cold ligand in the presence of 2.6 nM [^{3}H]LUF5834. Nonspecific binding was determined in the presence of 10 μM CPA and represented approximately 10% of the total binding. Saturation experiments were carried out using nine to 21 different concentrations of [^{3}H]LUF5834, [^{3}H]DPCPX or [^{3}H]LUF5834/[^{3}H]DPCPX ranging from 0.1 to 12 nM. In kinetic studies, the association of the radioligand [^{3}H]LUF5834 (2.6 nM) was initiated by addition of the membrane preparation (10 μg) to the radioligand. To study the dissociation of [^{3}H]LUF5834, membranes were preincubated with [^{3}H]LUF5834 (2.6 nM) at 25 ºC for 60 min. Dissociation of [^{3}H]LUF5834 was then initiated by the addition of LUF5834 (1 μM), DPCPX (1 μM) or CPA (10 μM). Incubations were terminated by dilution with ice-cold 50 mM Tris-HCl buffer. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/C filters using a Brandel harvester. Filters where subsequently washed three times with ice-cold buffer, or six times for saturation experiments. Filter-bound radioactivity was measured by scintillation spectrometry (Tri-Carb 2900TR, Perkin Elmer) after addition of 3.5 mL Packard Emulsifier Safe. Experiments were performed at least in triplicate, unless otherwise stated.

Data analysis

Data of radioligand binding experiments were analyzed using the non-linear regression curve fitting program Prism v. 4.0.2 (GraphPad, San Diego, CA, USA). k_{on} and k_{off} values were obtained by computer analysis of the association and dissociation data. Both association and dissociation experiments showed a two site binding profile for [^{3}H]LUF5834.

Saturation curves were fitted to one and two state/site binding models. For [^{3}H]DPCPX and [^{3}H]DPCPX/[^{3}H]LUF5834, a one site binding curve was favoured. For [^{3}H]LUF5834 saturation however, a two site binding hyperbola was preferred. Radioligand displacement curves were fitted to one, two and three state/site binding models. The LUF5834 displacement curve was best fitted to a one state/site binding model, CPA data were best fitted to a two state/site binding model. For DPCPX however, a three state/site binding model was preferred. IC_{50}-values were taken instead of calculated K_i-values, since we did not attempt to attribute K_d-values to each affinity state, especially in the case of the three site binding model.

Three site equation

Since the datapoints of the DPCPX displacement curves did not fit a two site displacement curve very well, a three site competition equation was incorporated in

Prism to check whether the datapoints would fit better to a three site model. Analogous to two site competition, a three site competition equation was written:

$$Y = Bottom + PART1 + PART2 + PART3$$
$$PART1 = Span \times \frac{Fraction1}{1+10^{(X-LogIC_{50-1})}}$$
$$PART2 = Span \times \frac{Fraction2}{1+10^{(X-LogIC_{50-2})}}$$
$$PART3 = Span \times \frac{1-(Fraction1+Fraction2)}{1+10^{(X-LogIC_{50-3})}}$$
$$Span = Top - Bottom$$

with Y: the binding of the radioligand, and X: the log concentration of unlabeled (competing) ligand. Fraction 1 is the fraction of all sites that have affinity 1, and the same applies to fractions 2 and 3. Log $IC_{50,1}$, Log $IC_{50,2}$ and Log $IC_{50,3}$ are the Log IC_{50}-values for the three sites.

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) and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) 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] 2-amino-4-(4-hydroxyphenyl)-6-(1*H*-imidazol-2ylmethylsulfanyl)-pyridine-3,5-dicarbonitrile ([^{3}H]LUF5834 – specific activity 25 Ci/mmol) was labeled by Sibtech, Inc. (Newington, CT, U.S.A.). The radiolabeled ligand was purified over a Kromasil C18 column with a gradient of 10% to 50% MeCN/H_2O/0.1% TFA. TLC on SiO_2 was developed with CH_2Cl_2:MeOH:NH_4OH (10:1.5:0.25). TLC on C18 was developed with MeOH:0.1M NH_4OAc pH 3.5 (3:1). G418 (neomycin) was obtained from Stratagene (Cedar Creek, U.S.A.). Guanosine triphosphate (GTP) was purchased from Acros Organics (Geel, Belgium). (2-Amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone (PD81,723) and LUF5834 were synthesized in our laboratory as described by Van der Klein et al.[9] and Chang et al.[4], respectively. Chinese hamster ovary (CHO) cells stably expressing the human adenosine A_1 receptor were obtained from A. Townsend – Nicholson[10]. Sf9 insect cells were obtained from BD-Biosciences (Alphen aan den Rijn, the Netherlands). Insect Xpress Protein Free Insect Cell Growth Media were

obtained from Cambrex (Verviers, Belgium), Dulbecco's Modified Eagle's Medium and F-12 Ham medium were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands). All other chemicals were of analytical grade and obtained from standard commercial sources.

Results

Radioligand-binding assay

As a starting point for the set up of a radioligand binding assay for [^{3}H]LUF5834, the radioligand-binding assay for [^{3}H]DPCPX was taken. CHOhA_1-wt membrane protein (10 μg) was incubated in a total volume of 400 μl Tris-HCl buffer, 50 mM, pH 7.4 as described in the Methods section. On control CHO-K1 cell membranes, [^{3}H]LUF5834 did not display any specific binding. To maximize the window of specific binding to the CHOhA_1-wt membranes, the following parameters were varied: GF/B vs GF/C glass fiber filters, with or without pretreatment of the filters with 0.25% polyethylene imine (pei) for 1 hour. The effect of addition of 0.1% CHPAS or 0.1% BSA to the assay buffer was also verified. It appeared that GF/B and GF/C filters performed equally well; only the addition of 0.1% CHAPS increased the window of specific binding significantly (results not shown). It was therefore decided to use the radioligand-binding assay of [^{3}H]DPCPX plus 0.1% CHAPS added to the assay buffer. The samples were harvested over GF/C filters.

Kinetics of [^{3}H]LUF5834

To characterize the new, non-adenosine radioligand [^{3}H]LUF5834, kinetic association and dissociation experiments on membranes of CHO cells stably expressing the wt human adenosine A_1 receptor were performed.

Association of [^{3}H]LUF5834 reached a plateau in 30 min and appeared to be biphasic (Figure 7.2A). From these experiments, the k_{on} values and half lives were determined (Table 7.1). The $k_{on,1}$ value was 2.01 ± 0.26 nM^{-1} min^{-1} and $k_{on,2}$ value was 0.12 ± 0.03 nM^{-1} min^{-1}. The corresponding half lives were 0.35 ± 0.04 min and 6.01 ± 1.33 min.

Dissociation of [^{3}H]LUF5834 was achieved by adding CPA (10 μM) or LUF5834 (1 μM), see Figure 7.2B. In both cases, the dissociation of [^{3}H]LUF5834 appeared to be biphasic. Full dissociation, i.e. to levels of non-specific binding, was reached within 60 minutes. In table 7.1, the radioligand half-life and k_{off} values are represented.

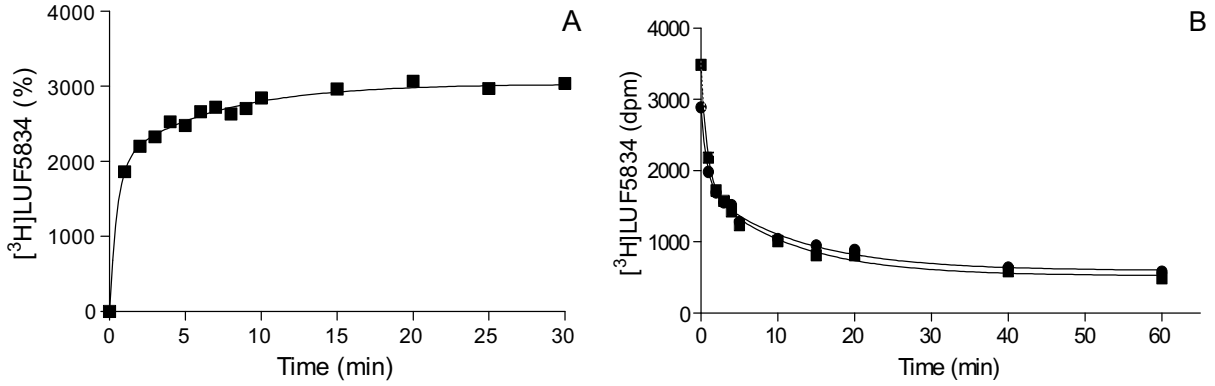


Figure 7.2. Association of [^{3}H]LUF5834 (A) to and dissociation of [^{3}H]LUF5834 (B) from the wt human adenosine A_1 receptor stably expressed on CHO cell membranes. Dissociation of [^{3}H]LUF5834 was achieved by adding CPA (10 μM,●) or LUF5834 (1 μM,■). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

Table 7.1. Association and dissociation kinetic parameters (± s.e.m.) of [^{3}H]LUF5834 binding to wt human adenosine A_1 receptors. Dissociation was induced with either CPA (10 μM) or LUF5834 (1 μM). Significant differences between ligands are marked with an asterisk. Values are means (± s.e.m.) of three separate assays each performed in duplicate.

Association

	$t_{1/2,\,1}$ (min)	$t_{1/2,\,2}$ (min)	$k_{on,1}$ (nM^{-1} min^{-1})	$k_{on,2}$ (nM^{-1} min^{-1})
[^{3}H]LUF5834	0.35 ± 0.04	6.01 ± 1.33	2.01 ± 0.26	0.12 ± 0.03

Dissociation

	$t_{1/2,1}$ (min)	$t_{1/2,2}$ (min)	$k_{off,1}$ (min^{-1})	$k_{off,2}$ (min^{-1})
CPA	0.35 ± 0.21	8.5 ± 0.60	2.60 ± 1.64	0.088 ± 0.006
LUF5834	0.50 ± 0.13	11.8 ± 5.55	1.43 ± 0.37	0.066 ± 0.031

As expected, the half lives for the two states were the same independent of the use of CPA or LUF5834 as the displacing ligand. Since these studies provide insight in ligand binding rather than activation, we cannot specify whether the two sites represent two states i.e. G protein dependent affinities or two different binding sites. The half lives for the high affinity states/sites, were 0.35 ± 0.21 min and 0.50 ± 0.13 min (CPA and LUF5834, respectively). The half lives for the low affinity states/sites, were 8.5 ± 0.60 min vs 11.8 ± 5.55 min. From the k_{on}- and k_{off} values, kinetic K_d values were calculated according to the formula: $K_d = k_{off} / k_{on.}$ This yielded a kinetic $K_{d,1}$ value of 0.71 nM and $K_{d,2}$ value of 0.55 nM.

Saturation

Saturation experiments were performed with [^{3}H]LUF5834 on membranes of CHO cells stably expressing the wt adenosine A_1 receptor (Figure 7.3). The binding of [^{3}H]LUF5834 was saturable and best characterized by a two-site model with a $K_{d,1}$ value of 0.16 ± 0.07 nM and a $K_{d,2}$ value of 1.69 ± 0.05 nM. Given the large s.e.m. of the $k_{off,2}$ value of LUF5834, the calculated kinetic K_d values are in the same range as the K_d values determined in the equilibrium saturation studies.

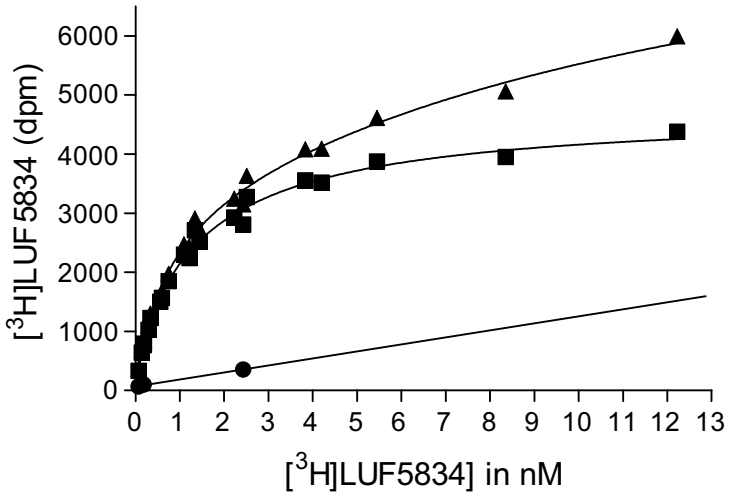


Figure 7.3. Equilibrium saturation curve of [^{3}H]LUF5834 on wt human adenosine A_1 receptor stably expressed on CHO cell membranes, total binding (▲), specific binding (■), non-specific binding (●). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

The corresponding B_{max} values were 1.68 ± 0.05 pmol/mg ($B_{max,1}$) and 7.82 ± 0.84 pmol/mg ($B_{max,2}$), respectively, yielding a total receptor density of 9.50 pmol/mg (Table 7.2). The non-specific binding accounted for approximately 10% of the total binding. In addition, saturation experiments with [^{3}H]DPCPX and a combination of [^{3}H]LUF5834/[^{3}H]DPCPX were performed to show that both radioligands share the same binding site on the receptor (Figure 7.4). The differences in dpm values can be explained by the fact that [^{3}H]DPCPX has a five-fold higher specific activity than [^{3}H]LUF5834. These saturation curves fitted best to a one-site model, with K_d values of 2.87 ± 1.03 nM and an apparent "mixed" K_d value of 2.30 ± 0.48 nM for the combined ([^{3}H]LUF5834/[^{3}H]DPCPX) saturation, respectively. The calculated B_{max} values were 12.8 ± 1.7 pmol/mg and 13.2 ± 1.1 pmol/mg respectively, comparable to the B_{max} value found with [^{3}H]LUF5834 saturation alone.

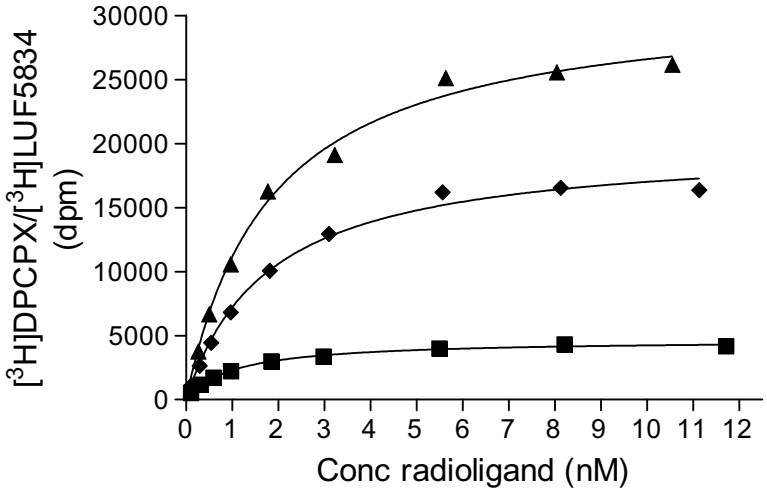


Figure 7.4. Saturation curves of [^{3}H]LUF5834 (■), [^{3}H]DPCPX (▲), or a combination of [^{3}H]LUF5834/[^{3}H]DPCPX (♦) on wt human adenosine A_1 receptor stably expressed on CHO cell membranes. Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

Table 7.2. K_d-values (± s.e.m.) of [^{3}H]LUF5834, [^{3}H]DPCPX and an apparent 'mixed' K_d-value for the combination of [^{3}H]LUF5834/ [^{3}H]DPCPX for the wt human adenosine A_1 receptor, stably expressed in CHO cell membranes. Corresponding B_{max}-values are also presented. Values are means (± s.e.m.) of three separate assays each performed in duplicate.

	K_d (nM)	$K_{d,1}$ (nM)	$K_{d,2}$ (nM)	B_{max} (pmol/mg)	$B_{max,1}$ (pmol/mg)	$B_{max,2}$ (pmol/mg)
[^{3}H]LUF5834		0.16 ± 0.07	1.69 ± 0.05		1.68±0.05	7.82±0.84
[^{3}H]DPCPX	2.87 ± 1.03			12.8±1.7		
[^{3}H]LUF5834/ [^{3}H]DPCPX	2.30 ± 0.48			13.2±1.1		

Displacement of [^{3}H]LUF5834

To determine the affinity of CPA, DPCPX and LUF5834, displacement experiments were performed with the new radioligand [^{3}H]LUF5834 on membranes of CHO cells stably expressing the wt human adenosine A_1 receptor (see Figure 7.5). LUF5834 recognized only one binding state/site with an IC_{50}-value of 5.4 ± 1.3 nM. CPA identified two binding states/sites with IC_{50}-values of 4.8 ± 1.9 nM and 339 ± 150 nM for the high and low affinity sites, respectively (see Table 7.3). DPCPX on the other hand recognized three binding states/sites, one with picomolar affinity (1.2 ± 1.4 pM), and the other two with nanomolar affinity (0.26 ± 0.24 nM and 127 ± 44 nM), respectively.

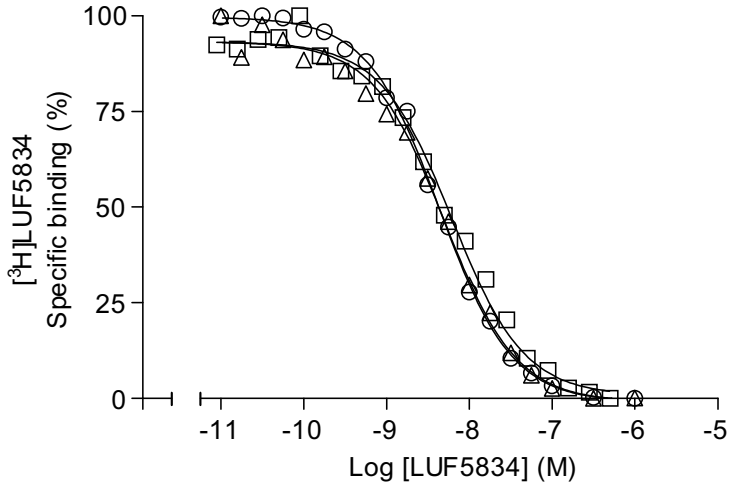


Figure 7.5. Displacement of [^{3}H]LUF5834 by LUF5834 from the wt human adenosine A_1 receptor stably expressed on CHO cell membranes, in the absence (○) or presence (Δ) of 1 mM GTP, or in the presence of 10 μM PD81,723 (□). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

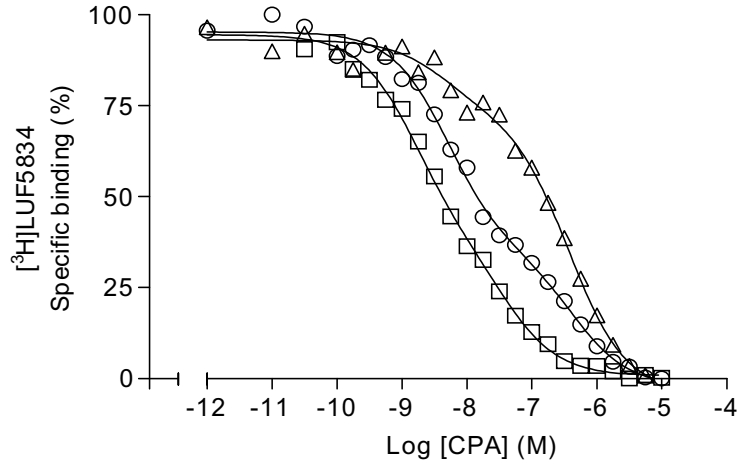


Figure 7.6. Displacement of [^{3}H]LUF5834 by CPA from the wt human adenosine A_1 receptor stably expressed on CHO cell membranes, in the absence (○) or presence (Δ) of 1 mM GTP, or in the presence of 10 μM PD81,723 (□). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

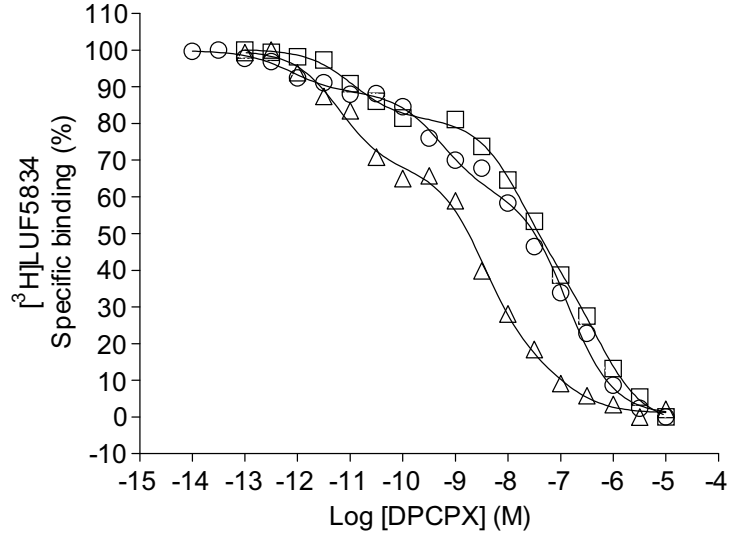


Figure 7.7. Displacement of [^{3}H]LUF5834 by DPCPX from the wt human adenosine A_1 receptor stably expressed on CHO cell membranes, in the absence (○) or presence (Δ) of 1 mM GTP, or in the presence of 10 μM PD81,723 (□). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

Table 7.3. Affinities of LUF5834, CPA and DPCPX for the wt human adenosine A_1 receptor in the absence or presence of either 10 μM PD81,723 or 1 mM GTP, expressed as IC_{50}-values (± s.e.m.). Significant differences with respect to the control values are marked with an asterisk. Values are means (± s.e.m.) of three separate assays each performed in duplicate, unless indicated otherwise.

	$IC_{50,high\ 2}$ (pM)	$IC_{50,high\ 1}$ (nM)	$IC_{50,low}$ (nM)	Fraction in%	Fraction in%	Fraction in%
LUF5834		5.4 ± 1.3				
LUF5834 + GTP		7.2 ± 0.8				
LUF5834 + PD		6.3 / 4.5[a]				
CPA		4.8 ± 1.9	339 ± 150		56 ± 6	44 ± 6
CPA + GTP		7.7 ± 2.9	440 ± 67		22 ± 5***	78 ± 5
CPA + PD		2.5 ± 0.8	36 ± 15*		51 ± 14	49 ± 6
DPCPX	1.2 ± 1.4	0.26 ± 0.24	127 ± 44	23 ± 13	29 ± 8	48 ± 11
DPCPX + GTP	2.6 ± 2.2	3.7 ± 1.4 *	120 ± 22	38 ± 4	51 ± 4*	11 ± 3**
DPCPX + PD	9.2 ± 3.4*	8.9 ± 7.2	573 ± 91**	22 ± 10	29 ± 11	49 ± 8

***: P < 0.0001, **: P < 0.005, *: P < 0.05, [a] n=2

Effects of the allosteric modulator PD81,723 and GTP

Radioligand binding experiments were performed in the absence and presence of 1 mM GTP or 10 μM of the allosteric modulator PD81,723 (see Figures 7.5-7.7 and Table 7.3). In contrast to CPA and DPCPX, LUF5834 was virtually insensitive to both GTP and PD81,723. The IC_{50}-values of LUF5834 in the presence of GTP and PD81,723 were 7.2 ± 0.8 nM and 6.3/4.5 nM, respectively, almost identical to the control value of 5.4 ± 1.3 nM.

Addition of 1 mM GTP did not affect the affinity of CPA for either the high or low affinity states/sites. The $IC_{50,high}$ value was 4.8 ± 1.9 nM vs 7.7 ± 2.9 nM in the absence and presence of GTP, whereas the $IC_{50,low}$ value was 339 ± 150 nM vs 440 ± 67 nM, respectively. In contrast, the percentage receptors in the high affinity state decreased from 56 ± 6% to 22 ± 5% in the presence of 1 mM GTP.

Addition of 10 μM PD81,723 significantly lowered the $IC_{50,low}$ value to 36 ± 15 nM, whereas the $IC_{50,high}$ value remained unaltered, 2.5 ± 0.8 nM. The percentage receptors in the high affinity state was not affected by PD81,723 (51 ± 14%).

Like CPA, the affinities of DPCPX were not much affected by the addition of GTP. The $IC_{50,low}$-value and $IC_{50,high\ 2}$ values remained unaltered 127 ± 44 nM vs 120 ± 22 nM and 1.2 ± 1.4 nM to 2.6 ± 2.2 nM, respectively and the $IC_{50,high1}$ value increased from 0.26 ± 0.24 nM to 3.7 ± 1.4 nM (P<0.05). In contrast, GTP did increase the

percentage receptors in the two high affinity states from 23 ± 13% to 38 ± 4% and from 29 ± 8% to 51 ± 4%, respectively.

Whereas addition of GTP resulted in a shift from low to high affinity states, PD81,723 affected only the IC_{50}-values. PD81,723 increased the $IC_{50,high}$ values to 9.2 ± 3.4 pM ($P < 0.05$) and 8.9 ± 7.2 nM, respectively, and significantly increased the $IC_{50,low}$ value to 573 ± 91 nM ($P<0.005$). The percentage receptors in the high affinity states was unaffected, 22 ± 10% vs 23 ± 13% and 29 ± 11% vs 29 ± 8%, respectively.

Sf9 insect cell preparation

Displacement studies on a cell preparation of Sf9 insect cells expressing the human adenosine A_1his_{10} receptor were also performed. [^{3}H]LUF5834 was displaced by CPA or DPCPX, in the absence or presence of 1 mM GTP (Figure 7.8). In both cases, the addition of GTP did not shift the displacement curve. The CPA displacement curve showed only one binding site, with an affinity comparable to the $IC_{50,low}$-value found in the displacement experiment performed on wt hA_1-CHO membranes (360 nM vs 339 ± 150 nM, table 7.4). The DPCPX displacement curve showed two binding sites, with affinities of 36 pM and 21 nM respectively.

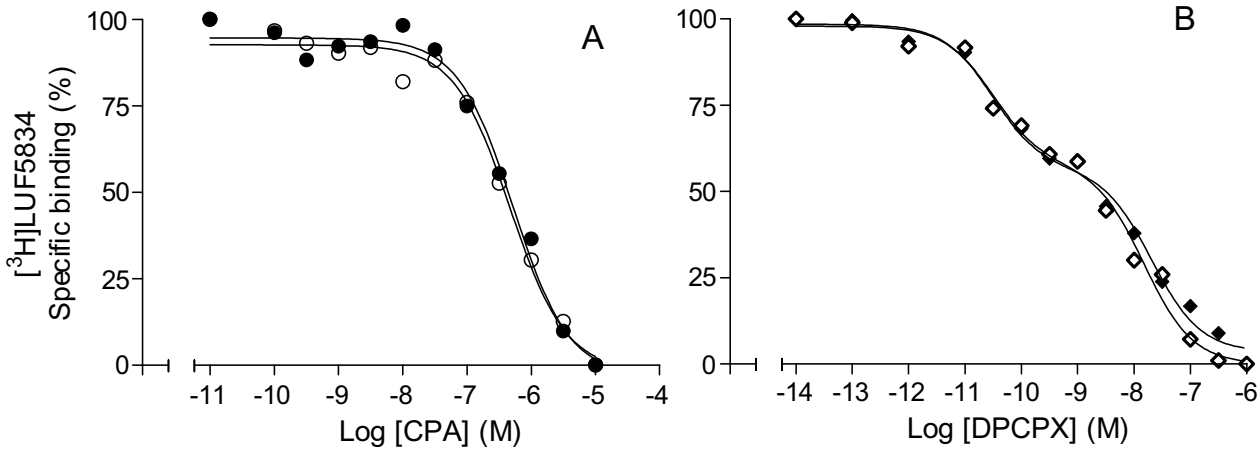


Figure 7.8. Displacement of [^{3}H]LUF5834 by CPA (A, ●) or DPCPX (B, ♦) from the human adenosine A_1his_{10} receptor expressed on Sf9 membranes, in the absence or presence of 1 mM GTP (○,◊). Experiments were performed three times, curves from a representative experiment performed in duplicate are shown.

Table 7.4. Affinities of CPA and DPCPX in the absence or presence of 1 mM GTP for the human adenosine A_1His_{10} receptor expressed in Sf9 Insect cells, expressed as IC_{50}-values. Two experiments (unless indicated otherwise) were performed, each in duplicate, yielding average IC_{50} values.

	IC_{50} (nM)	$IC_{50,high}$ (pM)	$IC_{50,low}$ (nM)	Fraction 1 (%)	Fraction 2 (%)
CPA	360				
CPA + GTP	504				
DPCPX		31*	21*	48	52
DPCPX + GTP		30	31	50	50

* n=1

Discussion

In this study, the new, non-ribose radioligand [^{3}H]LUF5834 was characterized for its kinetic, saturation and displacement properties on the human adenosine A_1 receptor. Also the effects of the allosteric modulators PD81,723 and GTP on the displacement of [^{3}H]LUF5834 were investigated. Finally, displacement studies of [^{3}H]LUF5834 on Sf9 Insect cell homogenates containing the human adenosine A_1 receptor were performed.

Kinetics of [^{3}H]LUF5834

Since LUF5834 had been shown to be a full agonist in cAMP experiments[3], the association and dissociation kinetics of [^{3}H]LUF5834 are best compared with other radiolabeled agonists. Characterization of ^{125}I-*N*^{6}-aminobenzyladenosine ([^{125}I]ABA) revealed a two site association. A $t_{1/2,1}$ of 1.85 min and a $t_{1/2,2}$ of 12.8 min were reported, five and two times slower, respectively than measured for [^{3}H]LUF5834[11]. [^{3}H]CCPA and [^{3}H]CHA, on the other hand, showed a one site association only, with a $t_{1/2}$ of 2.6 $\pm$ 0.3 min[12] and a $t_{1/2}$ value of 57.5 $\pm$ 14.4 min[13], respectively.

The $t_{1/2}$ for the dissociation of [^{3}H]CCPA was reported to be 10.8 $\pm$ 2.8 min[12], similar to the $t_{1/2,2}$ for the dissociation of [^{3}H]LUF5834. This is much faster than the k_{off} values reported for [^{3}H]CHA, 4.76 $\pm$ 0.26 min[13]. In conclusion, [^{3}H]LUF5834 has relatively fast kinetics among the agonist radioligands, and showed a two site association and dissociation profile.

Saturation

In line with the kinetic experiments, [^{3}H]LUF5834 also obeyed a two-site binding model in the saturation experiments. The $K_{d,1}$ value was 0.16 $\pm$ 0.07 nM and the $K_{d,2}$ value was 1.69 $\pm$ 0.05 nM. Other useful radioligands for the adenosine A_1 receptor also display affinities in the low nanomolar range, such as [^{3}H]DPCPX (2.87 $\pm$ 1.03 nM, (this study); 3.86 nM[14]; 1.6 $\pm$ 0.1 nM[6,13,15]; [^{3}H]CCPA (1.8 $\pm$ 0.2 nM)[15], [^{125}I]ABA, ($K_{d,1}$-value of 0.33 $\pm$ 0.16 nM and $K_{d,2}$-value of 10 $\pm$ 25 nM)[11]. The K_d-value of the

new, non-adenosine agonist radioligand [^{3}H]LUF5834 is thus in the same range of the K_d-values of the traditional agonist and inverse agonist radioligands for the human adenosine A_1 receptor. Additionally, the low non-specific binding of [^{3}H]LUF5834 makes it a suitable radioligand for binding studies.

The calculated B_{max}-values from the saturation curves (Figure 7.4) showed that both [^{3}H]DPCPX (12.8 ± 1.7 pmol/mg) and [^{3}H]LUF5834 (9.5 ± 0.9 pmol/mg) label approximately the same number of binding sites, despite the fact that LUF5834 is an agonist. The calculated B_{max} value from the combined [^{3}H]LUF5834/[^{3}H]DPCPX saturation experiment was 13.2 ± 1.1 pmol/mg, thus, no additional sites appeared occupied by [^{3}H]LUF5834 in the combined [^{3}H]LUF5834/[^{3}H]DPCPX saturation experiments. To compare, Kourounakis *et al.*[15] found that [^{3}H]CCPA when compared to [^{3}H]DPCPX labeled only 20% of the receptor binding sites. A putative explanation is that agonist radioligands such as [^{3}H]CCPA in low concentrations only label the receptors in the active, high affinity R* conformation. In contrast, the very high affinities of [^{3}H]LUF5834 (0.16 nM and 1.69 nM) allow full receptor occupancy in the concentration range studied (0-12 nM).

Displacement of [^{3}H]LUF5834

The displacement of [^{3}H]LUF5834 by LUF5834 followed a one site model with an IC_{50}-value of 5.4 ± 1.3 nM, equal to the affinity of LUF5834 displacing [^{3}H]DPCPX as the radioligand, 2.6 ± 0.3 nM[3]. The affinity of LUF5834 for the human adenosine A_1 receptor is slightly higher, but in the same range as the affinity of its close analogue LUF5831, 18 ± 1 nM[6]. The reason that only one K_i value is observed for LUF5834 in the displacement experiments versus two K_d values in the saturation experiments may be that the percentage receptors in the high affinity state was rather low (<20%). In addition, there were many more data points in the saturation curve around the $K_{d,1}$ value (10 points between 0 – 1 nM) than in the displacement curve (4 points). The displacement of [^{3}H]LUF5834 by CPA yielded a two site binding curve with IC_{50}-values of 4.8 ± 1.9 nM and 339 ± 150 nM, similar to the affinities found in displacement studies with [^{3}H]DPCPX as a radioligand, 2.2 ± 0.9 nM and 338 ± 24 nM[6]. LUF5834 thus has an affinity for the human adenosine A_1 receptor comparable to the $K_{i,high}$–value of CPA (5.4 ± 1.3 nM vs 2.2 ± 0.9 nM).

The displacement curve of DPCPX showed the most striking features. Not two, but three sites were observed. An $IC_{50,low}$- value of 127 ± 44 nM, and two $IC_{50,high}$-values were determined, one with nanomolar affinity (0.26 ± 0.24 nM) and the other one in the picomolar range (1.2 ± 1.4 pM). So far, a $K_{i,\ high}$ value of DPCPX with an affinity in the picomolar range, has never been observed before. Chang *et al.*[4] reported a K_i-value of 6.1 ± 1.6 nM and de Ligt *et al.*[16] reported a K_i-value of 2.4 ± 0.1 nM for DPCPX, which is in the same range as the $IC_{50,high\ 1}$-value. The percentage receptors

in the two high affinity states were 23 ± 13% and 29 ± 8% respectively, and 48 ± 11% of the receptors were in the low affinity state.

Effect of the allosteric modulator PD81,723 and GTP

Addition of 10 μM PD81,723, an allosteric enhancer[17,18], resulted in an increase in the affinity of CPA for the human adenosine A_1 receptor[6,7,15,19].

On the contrary, PD81,723 did not have an allosteric effect on the displacement of [^{3}H]LUF5834 by LUF5834. Similar results were previously reported for the displacement of [^{3}H]DPCPX by a structural analogue of LUF5834, LUF5831[6]. The affinities of LUF5834 and LUF5831 were apparently 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 these non-adenosine compounds to the human adenosine A_1 receptor. A possible explanation could be that non-adenosine ligands bind to a different binding site, insensitive to PD81,723. However, the ability of the non-adenosine ligands to displace [^{3}H]DPCPX binding, just like the adenosine derivatives, suggests that these ligands share – at least partly – the same binding site. The presence of 1 mM GTP did not shift the displacement curve of LUF5834 either. Apparently, the affinity of LUF5834 was not affected by the addition of GTP, which had also been observed for LUF5831[6]. Probably, the non-ribose agonists LUF5834 and LUF5831 have no prevalence for one of the two receptor conformations, R or R*.

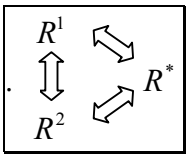
The addition of 10 μM PD81,723 in the [^{3}H]LUF5834/CPA displacement experiments only affected the $IC_{50,low}$-value of CPA; a ten-fold leftward shift was observed (339 ± 150 nM vs 36 ± 15 nM). The percentage receptors in the high affinity state was not affected by the presence of PD81,723. Heitman *et al.*[6] reported comparable data i.e. a 3.5-fold leftward shift of the low affinity site for CPA and no effect on the percentage receptors in the high affinity state. The addition of 1 mM GTP did not alter the affinities of CPA for the receptor, it only decreased the percentage receptors in the high affinity state from 56 ± 6% to 22 ± 5%. Heitman *et al.*[6] observed an even more pronounced effect in the experiments with [^{3}H]DPCPX; the receptors in the high affinity state were completely shifted to the low affinity state in the presence of 1 mM GTP, resulting in a one site displacement curve. As can be seen in the CPA curve, GTP decouples the receptor from its G protein, thereby decreasing the amount of receptors in the high affinity state (R*).

For all three affinities of DPCPX, the addition of 10 μM PD81,723 resulted in a decrease of affinity; for the $IC_{50,\ high\ 2}$-value and the $IC_{50,low}$-value this decrease was significant. However, the percentage receptors in the different affinity states remained unaltered.

In concert with the results obtained for CPA, the affinity of DPCPX for the different receptor states was not influenced by the addition of 1 mM GTP. The percentage receptors in the high affinity states, however, were significantly increased.

PD81,723 is usually viewed upon as an allosteric enhancer of agonist binding. However, our data in concert with the findings of Heitman et al.[6] show that PD81,723 is an allosteric enhancer of CPA binding, but not of LUF5834 or LUF5831 binding, despite the agonistic nature of both CPA and the non-ribose LUF compounds. In addition, PD81,723 lowered the affinity of the inverse agonist DPCPX for the receptor. Hence, it is not justified to coin PD81,723 as an allosteric enhancer per se, as is generally done.

GTP on the other hand, did not affect the affinities of the ligands for the receptor in any of the cases. It only shifted the equilibrium between the active (R*) and the inactive (R) state of the receptors. Moreover, according to the results obtained with the DPCPX displacement experiments, the R-state of the receptor seems to be divided in two high affinity sites for DPCPX, R^1 and R^2. The ratio between the two high affinity sites R^1 and R^2 is almost 1:1 (23 $\pm$ 13% vs 29 $\pm$ 8%), and increase both with more or less the same percentage (ca. 20%) upon addition of 1 mM GTP to the system. These observations point to the fact that these high affinity states may not be induced by differences in receptor conformation caused by e.g. accessory proteins or allosteric modulators which would be expected to affect the affinity, but are much more determined by the interactions between radioligand and/or displacing ligand and the uncoupled receptor, which may favour or induce certain receptor conformations. Following these observations, we propose an equilibrium of three receptor states, differing from the two state (R $\leftrightarrow$ R*) model, whereby the inactive, R state, of the receptor is subdivided in two states, R^1 and R^2.



Effect of G protein

All three states are recognized by the inverse agonist DPCPX, but only two of these by the agonist ligands. It has been shown before that the G_i-protein is involved in the active conformation, R*, of the adenosine A_1 receptor[6,20]. Interestingly, Sf9 insect cell membranes hardly contain G_i-protein. Figler *et al.*[21] showed that the addition of both $G_i\alpha$ and $G_i\beta\gamma$ subunits was necessary to regain the high affinity binding of recombinant bovine adenosine A_1 receptor in these cell membranes. We therefore

performed displacement experiments on an Sf9 insect cell membrane preparation expressing the his-tagged human adenosine A_1 receptor. The ligand pharmacology for the tagged receptor is identical to the wt human adenosine A_1 receptor (data not shown).

CPA's displacement curve was monophasic, and addition of 1 mM GTP had no effect on CPA's affinity (504 nM). Obviously, no receptors in the active conformation (R*) were present in this Sf9 insect cell preparation.

For DPCPX, two rather than three binding sites were found. The R*-conformation, the low affinity state of the receptor for DPCPX, did not seem to be present in the Sf9 Insect cell preparation. The addition of 1 mM GTP had no effect on the affinity of DPCPX, again proof of two receptor conformations despite the absence of G proteins.

Conclusion

In conclusion, a new, non-adenosine, agonist radioligand has been characterized for the human adenosine A_1 receptor. This radioligand, [^{3}H]LUF5834, recognized two high-affinity binding sites with a mere 10-fold difference in K_d values. Therefore it occupied all the receptor binding sites present on the cell membrane in saturation studies, comparable to the high affinity inverse agonist radioligand [^{3}H]DPCPX. Displacement experiments revealed that LUF5834 is not sensitive to PD81,723 or GTP addition. In addition, an 'extra' third site was revealed in displacement experiments with the inverse agonist DPCPX. This extra third site represents most probably a subdivision of the R conformation of the receptor into two additional states, R^1 and R^2. Based on these results, an extended receptor equilibrium was proposed for the different receptor states.

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