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**Characterization of [³H]LUF5834: a novel non-ribose high-affinity agonist
radioligand for the adenosine A₁ receptor**

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Abbreviations

Adenosine deaminase, ADA; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHAPS; Chinese hamster ovary, CHO; N⁶-cyclopentyladenosine, CPA; 1,3-dipropyl-8-cyclopentylxanthine, DPCPX; C8-butylamino-N⁶-cyclopentyladenosine, 8BCPA; C8-ethylamino-N⁶-cyclopentyladenosine, 8ECPA; 2-chloro-N⁶-cyclopentyladenosine, CCPA; G protein-coupled receptor, GPCR; Guanosine triphosphate, GTP; 2-amino-4-(4-hydroxyphenyl)-6-(1*H*-imidazol-2-ylmethylsulfanyl)-pyridine-3,5-dicarbonitrile, LUF5834.

Abstract:

The adenosine A₁ receptor is a promising therapeutic target for neurological disorders such as cognition deficits and is involved in cardiovascular preconditioning. Classically adenosine receptor agonists were all derivatives of adenosine, and thought to require a D-ribose moiety. More recently, however, the discovery of non-adenosine agonists for the human adenosine A₁ receptor (hA₁R) has challenged this dogma (Beukers et al. 2004). In this study we characterize the tritiated form of one of these compounds, [³H]LUF5834, as the first non-ribose partial agonist radioligand with nanomolar affinity for the hA₁R. Due to its partial agonist efficacy, [³H]LUF5834 labeled both G protein-coupled and uncoupled receptors with a similar high affinity. Using [³H]LUF5834 we performed competition binding experiments to characterize a range of A₁R ligands varying in efficacy from the full agonist CPA to the inverse agonist DPCPX. Surprisingly, in the control condition both agonists and inverse agonists displayed biphasic isotherms. With the addition of 1 mM GTP the high affinity isotherm of agonists or the low affinity isotherm of inverse agonists was lost revealing the mechanism of action of such inverse agonists at the A₁R. Consequently, [³H]LUF5834 represents a novel high affinity radioligand for the A₁R and may prove a useful tool to provide estimates of inverse agonist efficacy at this receptor.

1 Introduction

Adenosine is a ubiquitous local hormone that has been reported to play an important role in numerous tissues by acting mainly through four subtypes of adenosine receptors [1]. These receptors (A_1R , $A_{2A}R$, $A_{2B}R$ and A_3R) belong to the G protein-coupled receptor (GPCR) superfamily. This study focuses on the human adenosine A_1 receptor (hA_1R) which primarily couples to $G_{i/o}$ G proteins which mediate the inhibition of adenylate cyclase. The A_1R receptor is widely expressed, being present in the CNS and in peripheral systems such as cardiovascular tissue [1, 2]. The wide and abundant distribution of these receptors underlines their important role in the body and therefore they represent targets for useful therapeutic agents [3, 4]. For example the abundance of the A_1R in the atrioventricular node of the heart is the basis for clinical development of selective A_1R agonists as antiarrhythmic agents [5]. Similarly, activation of A_1Rs in the spinal cord may represent a novel treatment for neuropathic pain [6].

Historically, agonists for the adenosine receptors have all been derivatives of the endogenous ligand adenosine. For example chemical modification of the adenosine structure particularly at the N^6 , C2 and C5' positions have yielded selective high affinity agonists[7]. One such example is N^6 -cyclopentyladenosine (CPA), a high-affinity and selective agonist for the A_1R . Furthermore the ribose moiety was thought to be essential for the agonistic efficacy of adenosine receptor ligands [8]. However, recent research has challenged this dogma. Rosentreter et al. patented a new class of adenosine receptor ligands, the 2-amino-4-(3,4 substituted phenyl)-6-(2-hydroxyethylsulfanyl)-pyridine-3,5-dicarbonitriles [9, 10]. These compounds were demonstrated to have both a significant

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4 affinity and efficacy towards different adenosine receptor subtypes [9-12]. One of these
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6 compounds, LUF 5831, was shown to be a partial agonist with a high affinity of 18 nM
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8 for the hA₁R [13]. Accordingly, this compound displayed binding characteristics typical
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10 of a partial agonist such as an insensitivity to GTP in radioligand binding studies and the
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12 ability to bind to a G protein-uncoupled mutant of the hA₁ receptor (hA₁-T²⁷⁷A). Another
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14 high-affinity non-adenosine compound, LUF 5834, was characterized as an agonist at the
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16 hA₁R in a cAMP assay [11]. Furthermore LUF5834 showed a moderate (10 fold)
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18 selectivity for the hA₁R over the hA_{2A}R and hA_{2B}R and a higher selectivity (100 fold)
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20 over the hA₃R [11]. Consequently it was of interest to further characterize this ligand at
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22 the hA₁R. In this paper we confirm that LUF5834 indeed has a high (nanomolar) affinity
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24 for the hA₁R. Using [³⁵S] GTPγS binding experiments to measure hA₁R mediated G
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26 protein activation we demonstrate that that LUF5834 is a partial agonist as compared to
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28 the reference full agonist CPA. Given the high affinity of LUF5834 it was interesting to
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30 radioactively label this agonist with tritium. We demonstrate that [³H]LUF5834 is a high
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32 affinity radioligand for the hA₁R specifically labeling the hA₁R expressed in CHO cells
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34 with negligible non-specific binding. Furthermore, due to its high affinity and partial
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36 agonist nature [³H]LUF5834 was capable of labeling hA₁R both coupled and uncoupled
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38 to G proteins. This allowed the characterization of high and low affinity binding for both
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40 agonists and antagonists at the hA₁R. In summary then we describe a useful and novel
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42 high affinity radioligand for the hA₁R.
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2 Methods

2.1 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 bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Chemical Company (Rockford, IL, U.S.A.). [^3H] 1,3-dipropyl-8-cyclopentylxanthine ([^3H]DPCPX -specific activity 124 Ci/mmol) and [^3H]CCPA (2-chloro- N^6 -cyclopentyladenosine, 55 Ci/mmol) was purchased from NEN (Du Pont Nemours, 's Hertogenbosch, The Netherlands). [^3H] 2-amino-4-(4-hydroxyphenyl)-6-(1*H*-imidazol-2-ylmethylsulfanyl)-pyridine-3,5-dicarbonitrile ([^3H]LUF5834 – specific activity 25 Ci/mmol) was labeled by Sibtech, Inc. (Newington, CT, U.S.A.). Unlabelled LUF5834 was provided as the precursor (synthesized in-house at University of Leiden, Netherlands). Radiolabeling was performed using a halogenation - dehalogenation approach yielding the desired labeled product. The radiolabeled ligand was purified over a Kromasil C18 column with a gradient of 10% to 50% MeCN/H₂O/0.1% TFA. TLC on SiO₂ was developed with CH₂Cl₂:MeOH:NH₄OH (10:1.5:0.25). TLC on C18 was developed with MeOH:0.1M NH₄OAc pH 3.5 (3:1). Radiochemical purity was 98% . G418 (neomycin) was obtained from Stratagene (Cedar Creek, U.S.A.). Guanosine triphosphate (GTP) was purchased from Acros Organics (Geel, Belgium). LUF5834 was synthesized in our laboratory as described by Chang et al. (2005) [12]. C8-butylamino- N^6 cyclopentyladenosine (8BCPA) and C8-ethylamino-

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4 N^6 -cyclopentyladenosine (8ECPA) were synthesised as previously described [14].
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6 Chinese hamster ovary (CHO) cells stably expressing the hA₁R or the hA_{2B}R were
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8 obtained from Prof. Steve Hill (University of Nottingham, UK) or S.Rees (GSK,
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10 Stevenage, UK). HEK293 cells stably expressing the hA_{2A}R were kindly provided by Dr.
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12 J Wang (Biogen/IDEC, Cambridge, MA) All other chemicals were of analytical grade
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14 and obtained from standard commercial sources.
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23 *2.2 Cell culture*

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25 Chinese hamster ovary (CHO) cells stably expressing the human adenosine A₁ receptor
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27 (CHO-hA₁) or the human A_{2B} receptor (CHO-hA_{2B}) were cultured in a 1:1 mixture of
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29 Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10%
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31 newborn calf serum, 50 µg/mL streptomycin, 50 IU/mL penicillin and 0.2 mg/mL
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33 neomycin (G418). Human embryonic kidney cells (HEK293) stably expressing the
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35 hA_{2A}R were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10%
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37 newborn calf serum, 50 µg/mL streptomycin, 50 IU/mL penicillin and 0.2 mg/mL
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39 neomycin (G418). The cells were maintained in a humidified atmosphere at 37 °C and
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41 5% CO₂.
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50 *2.3 Membrane preparation*

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52 Membranes of CHO or HEK293 cells stably expressing the hA₁R, hA_{2A} or hA_{2B} were
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54 prepared as previously described [13]. Membrane protein concentrations were measured
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56 using the BCA (bicinchoninic acid) method with BSA as a standard.
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2.4 Radioligand-binding assays

For experiments using [^3H]LUF5834, membrane aliquots containing 10 μg (CHO-hA₁) 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 $^{\circ}\text{C}$ for 60 min in the absence or presence of 1 mM GTP. Displacement experiments were performed using 24 concentrations of unlabeled ligand in the presence of 2.6 nM [^3H]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 [^3H]LUF5834. In kinetic studies, the association of the radioligand [^3H]LUF5834 (2.6 nM) was initiated by addition of the membrane preparation (10 μg) to the radioligand. To study the dissociation of [^3H]LUF5834, membranes were preincubated with [^3H]LUF5834 (2.6 nM) at 25 $^{\circ}\text{C}$ for 60 min. Dissociation of [^3H]LUF5834 was then initiated by the addition of LUF5834 (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 were subsequently washed three times with ice-cold buffer, or six times in 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 three times in duplicate, unless otherwise stated. Experiments using [^3H]DPCPX were performed as above with non-specific binding determined using 100 μM CPA. Experiments using [^3H]CCPA

were performed as above using 30 μg of receptor per assay point and non-specific binding was determined using 10 μM DPCPX.

We performed similar studies on membranes expressing the $\text{hA}_{2\text{A}}\text{R}$ or $\text{hA}_{2\text{B}}\text{R}$ to assess the radioligand's potential to label these adenosine receptor subtypes as well.

2.5 hA_1R -mediated [^{35}S]GTP γS binding.

Membrane homogenates (CHO- hA_1 , 5 μg) were equilibrated in a 90 μL total volume of assay buffer (50 mM Tris, 200 mM NaCl, 10 mM MgCl_2 , pH 7.4) containing 3 μM GDP and a range of concentrations of ligand at 25 $^\circ\text{C}$ for 30 minutes. After this 10 μL of [^{35}S]GTP γS (final concentration 0.3 nM) was added and incubation continued for 45 minutes at 25 $^\circ\text{C}$. Incubation was terminated by rapid filtration through a 96 well Unifilter (PerkinElmer, NL) using a Filtermate Unifilter 96-well harvester (Perkin Elmer). Filters were washed three times with ice-cold assay buffer before drying. 25 μL of Microscint scintillation cocktail was added to each well, and plates were counted in a 1450 Trilux Microbeta liquid scintillation and luminescence counter (Perkin Elmer).

2.6 Data analysis

Data of radioligand binding experiments were analyzed using the non-linear regression curve fitting program Prism 5 (GraphPad, San Diego, CA, USA). Kinetic 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 [^3H]LUF5834. Saturation curves were fitted using either a one site isotherm or a one site isotherm with a variable Hill slope. For [^3H]DPCPX a one site binding curve was

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4 favored. For [³H]LUF5834 saturation however a one site isotherm with a variable Hill
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6 slope was favored and in three out of the seven experiments a two site binding equation
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8 could be fitted. Radioligand displacement curves were fitted to one and two state/site
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10 binding models. For the displacement of [³H]DPCPX by LUF5834 a one site model with
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12 a variable Hill slope was preferred using the equation;
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$$15 \quad Y = \frac{(top - bottom)x^{n_H}}{x^{n_H} + IC_{50}^{n_H}} \quad (1)$$

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17 where Y denotes the percent specific binding, top and bottom denote the maximal and
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19 minimal asymptotes respectively, x denotes the inhibitor potency (midpoint location)
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21 parameter and n_H denotes the Hill slope factor. Assuming simple competition IC₅₀ values
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23 were converted to K_i values using the Cheng and Prusoff (1973) equation.
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33 The CPA, DPCPX and N0840 displacement curves were best fitted to a two state/site
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35 binding model. For 8BCPA and 8ECPA one site binding isotherms were preferred. For
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37 experiments using [³H]LUF5834 IC₅₀-values were taken instead of calculated K_i-values,
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39 since we did not attempt to attribute K_d-values to each affinity state.
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45 In the functional [³⁵S]GTPγS assay, agonist concentration response curves were fitted to
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47 the following four-parameter Hill equation using Prism 5;
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$$53 \quad response = \frac{(top - bottom)}{1 + (10^{\log EC_{50} / x})^{n_H}} \quad (2)$$

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55 where top represents the maximal asymptote of the concentration response curves,
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58 bottom represents the lowest asymptote of the concentration-response curves, logEC₅₀
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represents the logarithm of the agonist EC_{50} , x represents the concentration of the agonist and n_H represents the Hill slope. Data shown are the mean \pm SEM of at least 3 separate experiments performed in duplicate. Comparisons between models were performed by applying the F-test in Prism 5. Unless otherwise stated values of $p < 0.05$ were taken as statistically significant.

3.0 Results

3.1 Characterization of LUF5834, a novel non-ribose agonist at the hA_1R

In a preliminary screen LUF5834 was shown to have a high (nanomolar) affinity for the hA_1R (Beukers et al, 2004). It was thought worthwhile to characterize this novel agonist more thoroughly. Firstly we tested the ability of 12 increasing concentrations of LUF5834 to displace [3H]DPCPX binding at the hA_1R expressed in CHO cell membranes (CHO- hA_1). As shown in **Figure 1A** and **Table 1** this displacement was best characterized by a monophasic curve with a variable Hill slope yielding a K_i value of 3nM and a Hill slope of 0.74. The affinity of LUF5834 and the Hill slope was unaffected

by the addition of 1 mM GTP. It should be noted that in three out of six experiments the data could be fit to a biphasic curve with a subnanomolar K_{iH} (0.3 nM) and a K_{iL} of 6 nM. These high and low affinity sites were only moderately affected by the addition of GTP, exhibiting 2 and 5- fold decreases in affinity respectively (K_{iH} of 0.7 nM and a K_{iL} of 18 nM), or when measured on pertussis toxin-treated membranes (K_{iH} of 0.7 nM and a K_{iL} of 34 nM). This relative insensitivity to GTP and pertussis toxin seems remarkable in terms of the observation that LUF5834 is a full agonist in a cyclic AMP assay. To assure ourselves that this effect was associated with the nature of the ligand rather than the receptor or indeed our experimental method, parallel experiments were performed on the same membranes using the typical agonist CPA as the competing ligand (**Figure 1B, Table 1**). As might be expected for a full agonist the displacement was best fit using a two site binding isotherm with a high affinity site ($K_{iH} = 7$ nM) and a low affinity site with a K_{iL} of 389 nM. However, in the case of CPA the high affinity site was lost when an equivalent experiment was performed in the presence of 1 mM GTP. Furthermore, the high affinity site was not observed when the assay was performed using CHO-hA₁ membranes derived from pertussis toxin-treated cells.

3.2 Characterization of [³H]LUF5834, a high affinity radioligand at the human A₁ adenosine receptor

The above data describing the characterization of LUF5834 underline its behavior as a novel high affinity agonist ligand for the hA₁R with characteristics different from the

reference 'ribose' agonist CPA. Therefore LUF5834 could be a potentially useful and novel type of radioligand. Consequently, LUF5834 was radiolabelled giving [^3H]LUF5834 with a specific activity of 25 Ci/mmol. The conditions employed for the binding of [^3H]DPCPX to CHO-hA₁ membranes were used as a basis for an assay for [^3H]LUF5834. Membrane protein (10 μg) was incubated in a total volume of 400 μL Tris-HCL buffer, 50 mM, pH 7.4 as described in Methods. On control, untransfected, CHO cell membranes, [^3H]LUF5834 did not display any specific binding (data not shown). The use of GF/B versus GF/C filters with or without pre-soaking the filters with 0.25 % poly(ethyleneimine) solution (PEI) for 1 hour had no effect on the 'window' of specific binding observed. However, the addition of 0.1 % CHAPS increased the window of specific binding (data not shown). All subsequent assays were therefore performed in the above conditions with the addition of 0.1 % CHAPS and reactions were terminated by fast flow filtration through GF/C filters. In identical assay conditions the ability of [^3H]LUF5834 to bind to the human A_{2A} receptor expressed in HEK293 cell membranes or to the human A_{2B} receptor expressed in CHO cell membranes was assessed using a final concentration of 20 nM [^3H]LUF5834. No specific binding was detected for the HEK-hA_{2A} membranes. For experiments with the CHO-hA_{2B} membranes a small window (200 dpm) of specific binding was detected. However, the relatively high amounts of radioligand and membranes required and the high non-specific binding (1000 dpm) observed in these experiments kept us from further characterization of this probe as a radioligand for hA_{2B}Rs (data not shown).

3.3 [^3H]LUF5834 demonstrates biphasic association and dissociation kinetics

As a starting point for the characterization of the novel radioligand [^3H]LUF5834, kinetic association and dissociation experiments were performed (**Figure 2**). As summarized in **Table 2**, both the association and dissociation of [^3H]LUF5834 were best fit by biphasic curves, yielding two values for both k_{obs} and k_{off} . It is interesting to note that the dissociation rate observed when using CPA as the displacing ligand was not significantly different from that observed when using LUF5834 (**Figure 2B**). This suggests that the binding sites of the typical agonist CPA and LUF5834 are at the very least overlapping, and the binding of CPA and LUF5834 is mutually exclusive. To extrapolate these results to determine from k_{obs} values both k_{on} values and a kinetic K_d is not straightforward. It is not possible to definitively state that the fast phase of association corresponds to the same site which is described by a slow phase of dissociation. Therefore, we decided to determine the affinity for both of these putative sites using radioligand saturation binding experiments.

3.4 Radioligand saturation binding experiments at hA_1 -CHO membranes using [^3H]LUF5834

Saturation experiments were performed with [^3H]LUF5834 on CHO- hA_1 membranes. The binding of [^3H]LUF5834 was best characterized using a one-site model but with a variable Hill slope (**Figure 3A, Table 3**). With this fit a K_d of 2.03 ± 0.52 nM and a B_{max} of 10.70 ± 0.76 pmol/mg was determined. The Hill slope was shown to be 0.78 ± 0.02

which is significantly lower than unity. This is graphically demonstrated in the Scatchard plot (**Figure 3Aii**) where a distinct deviation from linearity occurred. A Hill slope of less than unity can be explained by a negatively co-operative binding mechanism or suggests the presence of multiple binding sites. Interestingly the competition of LUF5834 versus DPCPX was also best fit by a curve with a Hill slope of 0.77. Indeed, in three out of the seven experimental repeats of saturation binding, the curve was well fit by a two site binding isotherm. The two sites were shown to have K_d values of 0.16 nM and 1.69 nM and gave B_{max} values of 1.7 pmol/mg and 7.9 pmol/mg respectively (**Table 3**). These values correspond well to the values of K_{iH} and K_{iL} determined for LUF5834 in competition binding experiments with [3 H]DPCPX (Table 1). This gave a combined B_{max} of 9.58 ± 0.32 pmol/mg protein, which is not significantly different from that determined using the one-phase curve with variable Hill slope. Parallel experiments using [3 H]DPCPX saturation binding on hA₁-CHO membranes experiments were also performed. In this case the data was best fit using a single site model with a K_d of 2.87 ± 0.39 nM and a B_{max} of 12.8 ± 1.7 pmol/mg protein (**Figure 3B i**). The total density of ligand binding sites observed for both [3 H]DPCPX and [3 H]LUF5834 was not significantly different. The monophasic nature of the curve is well demonstrated by the tight linear fit of the Scatchard plot in contrast to that of LUF5834 (**Figure 3B ii**). This demonstrates that the low Hill slopes and biphasic nature of [3 H]LUF5834 saturation isotherms are most likely associated with the nature of the ligand, as this was the only variable in the experiments. The biphasic association and dissociation kinetics also suggests the presence of two distinct binding sites labelled by [3 H]LUF5834 (**Table 3**).

3.5 Displacement of [³H]LUF5834 by the agonists LUF 5834 and CPA

For the experiments describing the displacement of [³H]LUF5834 by various hA₁R ligands a concentration of 2.6 nM was used. This corresponds to the K_d value determined by saturation analysis using a variable Hill slope plot. Using this concentration yields a window of specific binding of around 2000 dpm, a practical window for this type of experiment. However, we have also given considerable evidence that [³H]LUF5834 labels two distinct binding sites with sub-nanomolar and nanomolar affinities. In addition, in displacement experiments of [³H]DPCPX versus LUF5834, 1 mM GTP had negligible effect on either of these two sites suggesting that neither corresponds to a G protein-coupled state. Therefore, values of affinity for each of the displacing ligands are left as values of pIC₅₀, i.e. not converted to apparent pK_i values. Displacement of [³H]LUF5834 by unlabelled LUF5834 was best characterized by a monophasic curve with a high affinity binding site with an IC₅₀ value of 5 nM (**Figure 4A, Table 4**). As shown in figure **4A**, the addition of 1 mM GTP had negligible effect on the observed IC₅₀. However, in the presence of 1 mM GTP a reduction in total counts (**Figure 5Aii**) was observed. In contrast, the typical hA₁R agonist CPA displayed a biphasic displacement of [³H]LUF5834 with an IC_{50H} of 5 nM and an IC_{50L} of 340 nM (**Figure 4B, Table 4**). These values are consistent with the values of K_{ih} and K_{il} determined by competition of [³H]DPCPX. With the addition of 1 mM GTP, the affinity of CPA for the hA₁ receptor was not affected but the percentage of high affinity binding sites decreased from 56 % in the absence to 22 % in the presence of GTP (**Table 4**). However, when

[³H]LUF5834 was used at a concentration of 2.6 nM the addition of 1 mM GTP gave a decrease of approximately 30% of [³H]LUF5834 bound (**Figure 4Aii, 4Bii**). This would suggest then a fraction of bound [³H]LUF5834 is sensitive to GTP. Therefore it was of interest to investigate the ability of increasing concentrations of GTP to displace 2.6 nM [³H]LUF5834 binding at the hA₁R. As a comparison the effect of increasing concentrations of GTP on the binding of [³H]CCPA and [³H]DPCPX, a full agonist radioligand and an inverse agonist radioligand respectively, was also tested (**Figure 4C**). As might be expected, increasing concentrations of GTP had no significant effect on the binding of [³H]DPCPX, although a slight increase in ligand binding was observed (**Figure 4C**). Conversely the binding of [³H]CCPA was almost completely abolished with the addition of 1 mM GTP. Interestingly, increasing concentrations of GTP caused a maximum of 40 % displacement of LUF5834 binding (**Figure 4C**).

3.6 Displacement of specific [³H]LUF5834 binding by the partial agonists C8-ethylamino-N⁶-cyclopentyladenosine (8ECPA) or C8-butylamino-N⁶-cyclopentyladenosine (8BCPA)

It was interesting to observe that LUF5834, both in competition with [³H]DPCPX or indeed with [³H]LUF5834, displayed no shift in affinity or loss of high affinity binding with the addition of 1 mM GTP. We assessed the ability of two reference partial agonists C8-butylamino-N⁶-cyclopentyladenosine (8BCPA) or C8-ethylamino-N⁶-cyclopentyladenosine (8ECPA) to displace [³H]LUF5834 in the presence or absence of 1 mM GTP (**Figure 5, Table 4**). Unlike the full agonist CPA, the displacement of

[³H]LUF5834 by 8BCPA and 8ECPA was best fit using a monophasic isotherm. The addition of 1 mM GTP produced a negligible reduction in affinity and a modest reduction in affinity for 8BCPA and 8ECPA respectively ($pIC_{50GTP} - pIC_{50control} = 0.06$ and 0.16). In comparison the addition of 1 mM GTP caused a reduction in the percentage of receptors with a high affinity for the full agonist CPA. If we determine a value of affinity change for CPA in the presence and absence of GTP by subtracting the pK_i value determined with GTP from the pK_h value determined without it we observed a much more substantial change of 1.96.

3.7 Displacement of [³H]LUF5834 by the inverse agonists /antagonists DPCPX and N0840

Next, it was of interest to investigate the ability of the previously well characterized inverse agonist, DPCPX, to displace [³H]LUF5834 at the hA_1R . Interestingly this displacement was best characterized by a biphasic curve with an IC_{50H} of 8.5 nM and an IC_{50L} of 182 nM (**Figure 6A, Table 4**). With the addition of 1 mM GTP, the low affinity binding site was lost. Furthermore, when identical experiments were performed on CHO- hA_1 membranes from cells which had been pre-treated with pertussis toxin DPCPX displaced [³H]LUF5834 with a monophasic curve yielding an IC_{50} value equivalent to the IC_{50L} observed in the control experiment (**Figure 6A, Table 4**). We next characterized the ability of the putative neutral antagonist N0840 to displace [³H]LUF5834. If N0840 acted as a neutral antagonist we might expect to observe a monophasic displacement

curve that is not sensitive to the inclusion of 1 mM GTP. In reality, though, we observed a biphasic curve with an IC_{50L} of 8 μ M and an IC_{50H} of 455 nM (**Figure 6B, Table 4**).

With the addition of 1 mM GTP the low affinity site was lost demonstrating that this site is dependent on the coupling of the receptor to G protein. It is interesting to note the relative change in affinity with and without the presence of GTP as determined by $pIC_{50LGTP} - pIC_{50H}$ was smaller for N0840 than that of DPCPX (-0.92 and -1.33 respectively).

3.8 [35 S]GTP γ S binding experiments reveal LUF 5834 to be a partial agonist

To obtain an accurate measurement of ligand efficacy we investigated the ability of LUF5834 to stimulate hA₁R-mediated guanine nucleotide exchange using a [35 S]GTP γ S binding assay. [35 S]GTP γ S binding experiments were performed on membranes derived from the same CHO-hA₁R as used for all radioligand binding studies (**figure 7, Table 5**). As compared to the reference full agonist CPA which had a potency of 5 nM, LUF5834 was a partial agonist with a higher potency (3 nM) and an E_{max} of 70% of the maximal stimulation by CPA. Compared to LUF5834 the previously characterized partial agonists 8BPCA and 8ECPA demonstrated low potency at the hA₁R (2 μ M and 1 μ M, respectively). It is interesting to note that 8BCPA which showed a similar insensitivity to the addition of GTP in radioligand displacement assays to LUF5834 also demonstrated a similar efficacy in the [35 S]GTP γ S assay. DPCPX was shown to be an efficacious inverse agonist with a potency of 11 nM. In comparison N0840 had a much more modest

potency (20 μ M) and was an inverse agonist, although with a lower efficacy than DPCPX.

4 Discussion

The 2-amino-4-(substituted phenyl)-6-(substituted sulfanyl)-pyridin-3,5-dicarbonitriles show no structural similarity to adenosine. However, it has been observed that several compounds of this class display a significant affinity and efficacy towards the different adenosine receptor subtypes. LUF5834 (2-amino-4-(4-hydroxyphenyl)-6-(1*H*-imidazol-2-ylmethylsulfanyl)-pyridine-3,5-dicarbonitrile) has previously been characterized as a novel high affinity non-ribose agonist for the hA₁R (Beukers et al, 2004). We now demonstrate that, in more elaborate studies, the compound displayed two binding sites with a high sub-nanomolar and nanomolar affinity for this receptor, respectively. The high affinity of LUF5834 made it a suitable candidate for labeling with tritium to provide a new radioligand for the A₁R. Current adenosine A₁ receptor radioligands are confined to adenosine derivative full agonists such as [³H]CCPA or xanthine derivative inverse agonists such as [³H]DPCPX [15, 16]. Initial experiments demonstrated that [³H]LUF5834 could label A₁Rs with low non-specific binding. Furthermore, using the assay conditions described above, we have demonstrated that [³H]LUF5834 selectively labels A₁Rs over A_{2A}Rs and A_{2B}Rs, and that [³H]LUF5834 does not represent a radioligand for the latter receptors. In all saturation experiments the data was well fit using a single site binding model with a variable Hill slope and a K_d of approx. 2 nM and a B_{max} of approx, 10 pmol/mg. The Hill slope (0.78 \pm 0.02) was significantly less than unity which is suggestive of either negative co-operative binding or indeed the presence

of two populations of receptor with distinct affinities for the receptor. Indeed, three out of the seven experiments could be well fit with a two binding site equation with values of K_d similar to those values of K_i determined using [3H]DPCPX competition experiments. Kinetic experiments demonstrated that both association and dissociation of [3H]LUF5834 were biphasic. The rate of dissociation observed remained unchanged when either the reference agonist CPA or the cold ligand LUF5834 was used as the displacing ligand. This indicates then that the binding site of LUF5834 overlaps greatly with that of the typical A_1 receptor agonist CPA, and provides evidence that LUF5834 is an orthosteric rather than an allosteric ligand. It is interesting to note that the number of receptors labeled by [3H]LUF5834 was similar to that labeled by the inverse agonist [3H]DPCPX. This is in stark contrast to a previous study using the radiolabelled agonist [3H]CCPA. In this study Kourounakis et al. found that [3H]CCPA labeled only 20% of the binding sites labeled by [3H]DPCPX [17]. This suggests then, that while [3H]DPCPX and [3H]LUF5834 can label both G protein-coupled and uncoupled receptors with high affinity, [3H]CCPA as a full agonist will only label G protein-coupled receptors. In agreement with the above conclusion [3H]CCPA binding in our hands was completely displaced by GTP whereas increasing concentrations of GTP could only partially displace bound [3H]LUF5834. This suggests that [3H]LUF5834 can label both G protein-coupled and -uncoupled receptors, an ability difficult to reconcile with LUF5834 being a full agonist as was shown in an initial study using a whole cell cAMP assay [11]. Similar experiments have been carried out for [3H]lisuride, [3H]S15535 and [3H]DOI, radiolabelled partial agonists at the $5HT_{1A}$, and $5HT_{2A}$ receptors, respectively [18-20]. In all cases displacement of these partial agonist radioligands by GTP analogues was

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4 incomplete. Guanine nucleotide exchange is a very early step in the signal transduction
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6 cascade, and hence is less subject to amplification or regulation by other cellular
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8 processes [21]. Making use of a [35 S]GTP γ S binding assay we demonstrated that, at the
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10 level of nucleotide exchange at the G protein, LUF5834 behaves as a partial agonist with
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12 greater potency for the hA₁R than that of the typical agonist CPA.
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18 The competition binding profile of [3 H]LUF5834 at the hA₁R was examined using a
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20 range of A₁R ligands. The competition between [3 H]LUF5834 and unlabeled LUF5834
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22 showed no shift in affinity in response to the addition of 1 mM GTP. The full agonist
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24 CPA displayed a biphasic curve with an IC_{50H} of 4.7 nM and an IC_{50L} of 338 nM. With
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26 the addition of 1 mM GTP or pre-treatment of CHO-hA₁ cells with pertussis toxin the
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28 high affinity (G protein-coupled) binding population decreased. This suggests that the
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30 high affinity site of CPA is dependent on G protein coupling and that in the case of the
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32 hA₁R and CPA this coupling is to member(s) of the G_{i/o} family of G proteins. This
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34 sensitivity to GTP of the reference agonist CPA was quite distinct from the relative
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36 nucleotide insensitivity of LUF5834. However, biphasic isotherms were not limited to
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38 full agonist ligands. DPCPX, shown to be a high-affinity inverse agonist in the [35 S]
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40 GTP γ S experiments, also displayed a biphasic curve with an IC_{50H} of 8.5 nM and an
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42 IC_{50L} of 182 nM. Interestingly, in the case of DPCPX the low affinity site was shown to
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44 represent the G protein-coupled receptor population and that this G protein coupling is
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46 exclusively mediated by G_{i/o} G proteins since this low affinity site was lost upon the
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48 addition of 1 mM GTP or by pretreatment of cells with pertussis toxin. Such behaviour
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50 by an inverse agonist has not been demonstrated before with other radioligands available
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for the A₁R. This is consistent with the action of DPCPX as an inverse agonist having a high affinity for and therefore stabilizing the uncoupled or inactive state of the receptor but having a lower affinity for the G protein-coupled state of the receptor in agreement with the ternary complex model [22, 23]. N0840 has been previously characterized as a neutral antagonist at the hA₁ adenosine receptor [24, 25]. However, in this study, [³⁵S]GTPγS experiments revealed N0840 to be a partial inverse agonist with modest potency. Consistent with this finding, competition binding experiments of [³H]LUF5834 versus N0840 reveal a biphasic curve with an IC_{50H} of 455 nM and an IC_{50L} of 8 μM. As for DPCPX, the low affinity binding site of N0840 disappeared with the addition of GTP. It should be noted however that the change in affinity with the addition of GTP was significantly smaller for N0840 than that observed for DPCPX ($pIC_{50LGTp} - pIC_{50H} = 0.92$ and -1.33 respectively). This more modest affinity change for N0840 compared to DPCPX is consistent with the partial inverse agonist efficacy of N0840 compared to that of DPCPX.

Consequently, competition studies using [³H]LUF5834 in the presence and absence of guanine nucleotides provide useful information as regards to the efficacy of antagonists and inverse agonists at the hA₁R receptor. Many studies have clearly shown the relationship between the observed change in binding affinity upon addition of guanine nucleotides and agonist efficacy [23, 26]. Indeed, such a relationship has even been demonstrated at the A₁R [25]. In this study we also saw a relationship between the efficacy of the ligand in the [³⁵S]GTPγS experiments and the affinity change with the addition of GTP in competition experiments. The changes of affinity for agonists and

inverse agonists were inversely related. Furthermore, affinity changes observed for partial agonists such as BCPA and ECPA and partial inverse agonists such as N0840 were more modest than those observed for the full agonist CPA and the efficacious inverse agonist DPCPX.

Several studies have used partial agonist or neutral antagonist radioligands to characterise both agonist and inverse agonists at the 5-HT_{1A} receptor [19, 27, 28]. It should be noted that such changes in affinity in the presence of GTP are not observed for inverse agonists at the hA₁R when the inverse agonist radioligand [³H]DPCPX is used, despite this ligand labelling populations of both G protein-coupled and -uncoupled receptor [25]. If the radioligand and 'cold' ligand have a similar efficacy they will be modulated by the addition of nucleotide to a similar extent, and therefore a change in affinity will not be observed. In agreement with this finding, in competition experiments of [³H]LUF5834 versus the partial agonist BCPA, no change in affinity was observed in the absence or presence of guanine nucleotide. This can be reconciled with both LUF5834 and BCPA having an identical efficacy in the [³⁵S]GTPγS assay. [³H]LUF5834 therefore allows the discrimination of high and low affinity sites for both inverse agonists and agonists as demonstrated by displacement studies using CPA and DPCPX.

In summary then we describe the first non-ribose partial agonist radioligand for the A₁R, LUF5834. This radioligand labels the hA₁ receptor with high, sub-nanomolar affinity, higher than any other radioligand available for that receptor. The high affinity of [³H]LUF5834, plus its partial agonist nature allows it to bind to both G protein-coupled

and -uncoupled receptors with similar high affinity. Competition experiments using [³H]LUF5834 revealed a population of receptors with a low affinity for inverse agonists such as DPCPX. To our knowledge this study is the first to reveal the affinity of A₁R inverse agonists such as DPCPX for the G protein-coupled population of receptors. There is increasing interest in the potential of adenosine A₁ receptor partial agonists, e.g. in the treatment of paroxysmal supraventricular tachycardia (PSVT) [4]. Similarly, blockade of A₁Rs may be useful in the treatment of neurological disorders such as cognitive decline [29]. [³H] LUF 5834 represents a new class of radioligand for the adenosine A₁ receptor and can be a versatile tool to allow estimations of efficacy for both agonists and antagonists at this increasingly attractive therapeutic target.

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Table 1

Estimates of affinity (K_i values) derived from the competition of [3 H]DPCPX with the agonists CPA and LUF5834 at the hA₁R expressed in CHO cell membranes.

Values represent the means \pm S.E.M. obtained from three to seven experiments conducted in duplicate. Values of pK_h and pK_i are expressed as $-\log$ values with the corresponding values of K_i shown in parentheses.

	LUF 5834	LUF 5834	LUF 5834	CPA	CPA	CPA
		+ GTP	+ PTX		+ GTP	+ PTX
pK_h				8.15 ± 0.39		
(K_h, nM)				(7 nM)		
pK_i or pK_l	8.59 ± 0.11	8.33 ± 0.10	8.47 ± 0.10	6.41 ± 0.07	6.44 ± 0.07	6.41 ± 0.08
(K_i or K_l, nM)	(3 nM)	(5 nM)	(4 nM)	(389 nM)	(363 nM)	(389 nM)
Hill Slope	0.74 ± 0.06	0.77 ± 0.06	0.79 ± 0.15			
% K_h				27 ± 6	0	0

Table 2

Association and dissociation kinetic parameters (\pm S.E.M.) of [^3H]LUF5834 binding to hA₁Rs stably expressed in CHO cell membranes. Dissociation was induced with the addition of a large excess of either CPA (10 μM) or LUF5834 (1 μM). Both association and dissociation curves were best fit using biphasic models. No significant difference was observed between the values of $k_{\text{off}1}$ or $k_{\text{off}2}$ when either LUF5834 or CPA was used as the displacing ligand (un-paired t test using Prism 5, where statistic significance was taken as $p < 0.05$)

Association

	$k_{\text{obs}1}, \text{min}^{-1} \cdot \text{M}^{-1}$	Half-life ₁ , min	$k_{\text{obs}2}, \text{min}^{-1} \cdot \text{M}^{-1}$	Half-life ₂ , min
[^3H]LUF5834	0.078 ± 0.03	14.37 ± 0.08	1.63 ± 0.24	0.45 ± 0.08

Dissociation

Isotopic ligand	$k_{\text{off}1}, \text{min}^{-1}$	Half-life ₁ , min	$k_{\text{off}2}, \text{min}^{-1}$	Half-life ₂ , min
LUF 5834	1.07 ± 0.37	0.93 ± 0.43	0.05 ± 0.01	15.83 ± 4.12
CPA	1.96 ± 1.24	0.98 ± 0.61	0.06 ± 0.02	12.77 ± 4.38

Table 3**Saturation binding of [³H]LUF5834 and [³H]DPCPX to hA₁-CHO membranes**

Saturation binding experiments were carried out using at least 12 concentrations of radioligand at hA₁-CHO membranes in duplicate at least three times. Data for the saturation binding of [³H]LUF5834 was best fit using a monophasic binding curve with a variable Hill slope. Three experiments from this series were also well fitted by a biphasic saturation curve. The saturation binding curve for [³H]DPCPX was best fitted by a monophasic curve (F-test using Prism 5, $p < 0.05$ was taken as statistically significant).

Radioligand	K_{D1}, nM	B_{max1}, pmol/mg	Hill slope	K_{D2}, nM	B_{max2}, pmol/mg
[³ H]LUF5834 n = 7	2.03 ± 0.52	10.70 ± 0.66	0.78 ± 0.02	-	-
[³ H]LUF5834 n = 3	0.16 ± 0.03	1.70 ± 0.02	-	1.69 ± 0.02	9.58 ± 0.32
[³ H]DPCPX	2.87 ± 1.03	12.8 ± 1.7	1	-	-

Table 4

Estimates of affinity (IC₅₀ values) derived from the competition of [³H]LUF5834 versus various A₁R agonists and antagonists, in the absence and presence of 1 mM GTP, at the hA₁R expressed in CHO cell membranes. Values represent the means \pm S.E.M. obtained from three to seven experiments conducted in duplicate. Values of pK_h and pK_i are expressed as $-\log$ values with the corresponding values of K_i shown in parentheses. The affinity change was calculated by subtracting the pIC_{50low} value determined in the presence of GTP by the pIC_{50high} value determined in its absence. When no pIC_{50high} could be determined then affinity change was calculated by subtracting the pIC_{50low} value determined in the presence of GTP by the pIC_{50low} value determined in its absence.

Compound	pIC _{50high}	pIC ₅₀ or pIC _{50 low}	Fraction High affinity, %	Affinity change
LUF5834		8.26 \pm 0.03 (5.4 nM)		
LUF5834 + GTP		8.18 \pm 0.04 (12.3 nM)		-0.08
CPA	8.32 \pm 0.06 (4.7 nM)	6.47 \pm 0.13 (338 nM)	56 \pm 2	
CPA + GTP	8.14 \pm 0.11 (7 nM)	6.36 \pm 0.04 (435 nM)	22 \pm 3	1.96
BCPA		4.94 \pm 0.07 (12 μ M)		
BCPA + GTP		4.89 \pm 0.11 (13 μ M)		0.06
ECPA		5.17 \pm 0.12 (6.7 μ M)		
ECPA + GTP		5.01 \pm 0.12 (9.8 μ M)		0.16
DPCPX	8.07 \pm 0.12 (8.5 nM)	6.74 \pm 0.14 (182 nM)	53 \pm 8	-1.33
DPCPX + GTP	8.34 \pm 0.13 (4.5 nM)		100	

DPCPX + PTX	7.96 ± 0.22 (10.9)		100	
N0840	6.34 ± 0.17 (455 nM)	5.10 ± 0.25 (7980 nM)	44 ± 11	-0.92
N0840 + GTP	6.00 ± 0.09 (995 nM)		100	

Table 5: The efficacy and potency of agonists, partial agonists and inverse agonists at the hA₁R in a [³⁵S] GTPγS binding assay. The ability of increasing concentrations of compound to stimulate or inhibit [³⁵S] GTPγS binding at CHO membranes expressing hA₁R was determined. E_{max} was determined as a percentage of the maximal stimulation exerted by the full agonist CPA ((maximal stimulation – basal stimulation)/(maximal CPA stimulation – basal stimulation)). Values of pEC₅₀ are expressed as –log values with the corresponding values of EC₅₀ shown in parentheses.

	pEC ₅₀ (EC ₅₀)	E _{max}
CPA	8.31 ± 0.02 (4.84 nM)	100
LUF 5834	8.48 ± 0.02 (3.29 nM)	70 ± 3
8BCPA	5.75 ± 0.04 (1.78 μM)	65 ± 1
8ECPA	6.12 ± 0.05 (0.79 μM)	94 ± 4
DPCPX	7.98 ± 0.14 (11 nM)	-18 ± 3
N0840	4.88 ± 0.3 (19.7 μM)	-12 ± 2

Figure 1

Displacement of [³H]DPCPX bound to the hA₁R expressed on CHO cell membranes by increasing concentrations of LUF5834 (A) or CPA (B) with or without the presence of 1 mM GTP or with treatment of the hA₁-CHO cells with pertussis toxin (PTX). Points shown are from representative experiments performed in duplicate and repeated on at least three independent occasions. Data was fit using non-linear regression isotherms. The curves for LUF5834 were best fit using a monophasic isotherm with a variable Hill slope both in the presence or absence of GTP or using membranes pre-treated with pertussis toxin. Contrastingly data from CPA experiments were best fit by biphasic isotherms in the control condition but monophasic isotherms in the presence of 1 mM GTP or on membranes pre-treated with pertussis toxin (F-test using Prism 5, statistical significance was regarded as $P < 0.05$).

Figure 2

Association (A) to and dissociation (B) of [³H]LUF5834 from the hA₁R stably expressed on CHO cell membranes. Dissociation of [³H]LUF5834 was achieved by adding CPA (10 μ M, ●) or LUF5834 (1 μ M, ■). Experiments were performed three times in duplicate. Data shown is from a representative experiment.

Figure 3

Saturation radioligand binding at the hA₁R expressed on CHO cell membranes for [³H]LUF5834 (A) or [³H]DPCPX (B). Experiments were performed at least three times in duplicate. Representative curves are shown for both experiments. Data from [³H]LUF5834 was best fit using non-linear regression to a monophasic curve but with a variable Hill slope (**Ai**). The deviation of the data from a simple monophasic curve with a Hill slope of unity is well demonstrated by the Scatchard plot (**Aii**). In contrast data for [³H]DPCPX was best fit using a monophasic curve with a Hill slope of unity (**Bi**), graphically demonstrated by the Scatchard plot (**Bii**).

Figure 4

Displacement of [³H]LUF5834 bound to the hA₁R expressed on CHO cell membranes by increasing concentrations of the agonists LUF5834 (A), CPA (B) or the nucleotide GTP (C). Points shown are from representative experiments performed in duplicate and repeated on at least three independent occasions. Data is shown normalised as a percentage of specific radioligand bound (i) or as raw counts (dpm) (ii). The curves for LUF5834 were best fit using biphasic isotherms independent of the presence or absence of GTP. Contrastingly data from CPA experiments were best fit by biphasic isotherms in the control condition but monophasic isotherms in the presence of 1 mM GTP (F-test using Prism 5, statistical significance was regarded as $P < 0.05$). The effect of increasing concentrations of GTP on the binding of [³H]LUF5834, the full agonist [³H]CCPA or the inverse agonist [³H]DPCPX was also tested (C). [³H]CCPA,

[³H]LUF5834 and [³H]DPCPX were used at concentrations of 1.6 nM, 2.6 nM and 2 nM respectively, values approximately equal to the respective K_d values at this receptor.

Figure 5

Displacement of [³H]LUF5834 bound to the hA₁R expressed on CHO cell membranes by increasing concentrations of the partial agonists 8-butylamino-CPA (8BCPA, A) or 8-ethylamino-CPA (8ECPA, B) in the absence or presence of 1mM GTP. Points shown are from representative experiments performed in triplicate and repeated on at least three independent occasions. Data was fit using non-linear regression isotherms. The curves for both DPCPX and N0840 experiments were best fit by monophasic isotherms both in the absence and the presence of 1 mM GTP (F-test using Prism 5, statistical significance was regarded as P < 0.05).

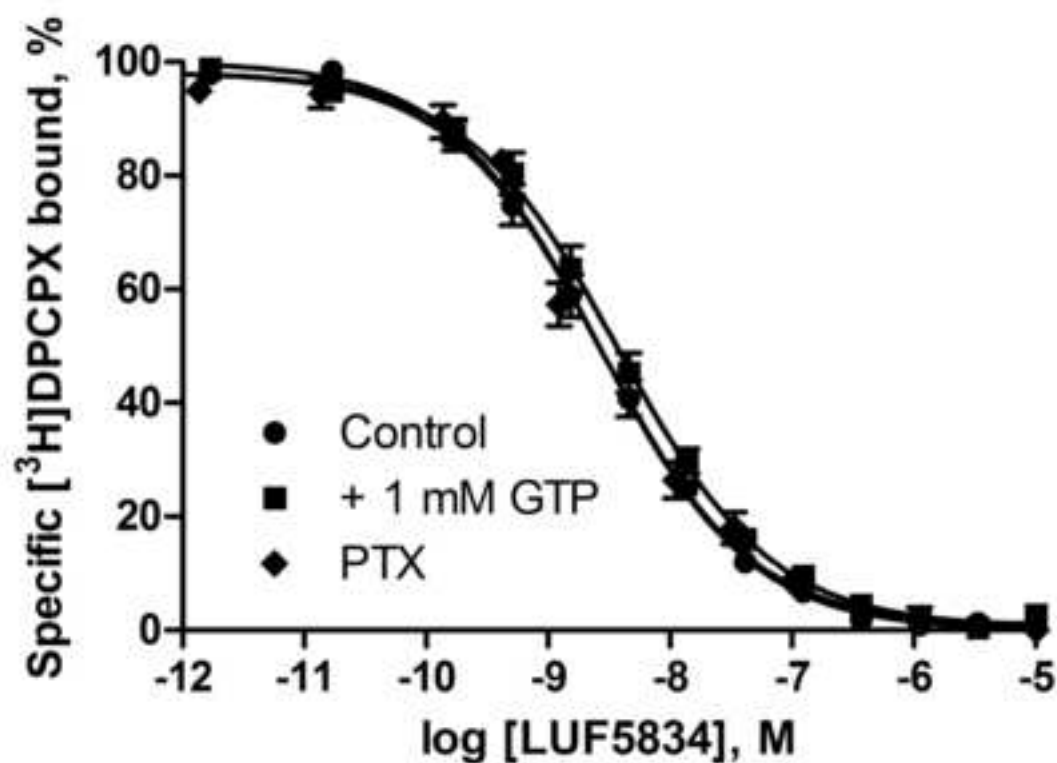
Figure 6

Displacement of [³H]LUF5834 bound to the hA₁R expressed on CHO cell membranes by increasing concentrations of the antagonists DPCPX (A) and N0840 (B). Points shown are from representative experiments performed in triplicate and repeated on at least three independent occasions. Data was fit using non-linear regression isotherms. The curves for both DPCPX and N0840 experiments were best fit by biphasic isotherms in the control condition but monophasic isotherms in the presence of 1 mM GTP (F-test using Prism 5, statistical significance was regarded as P < 0.05).

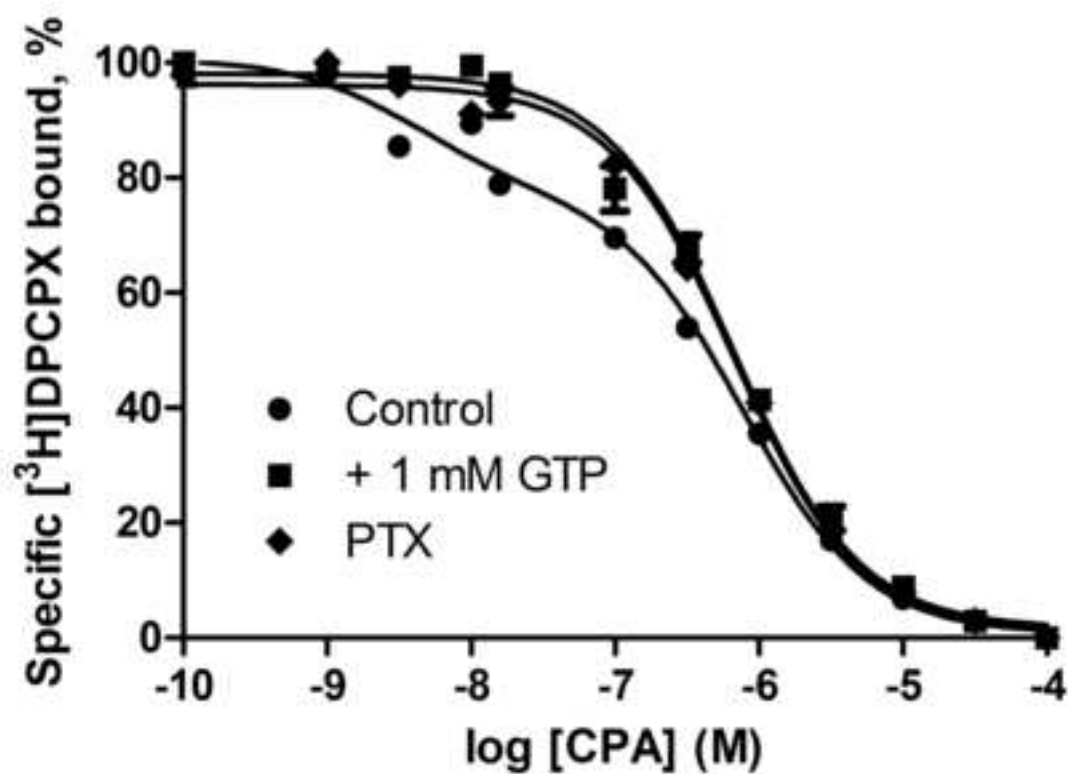
Figure 7

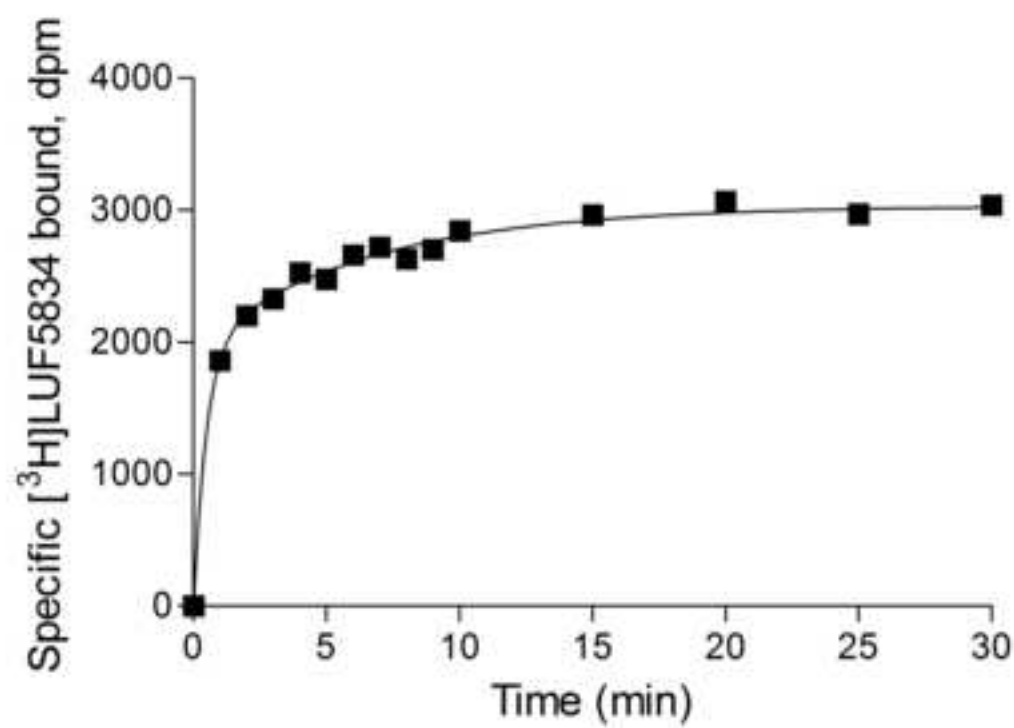
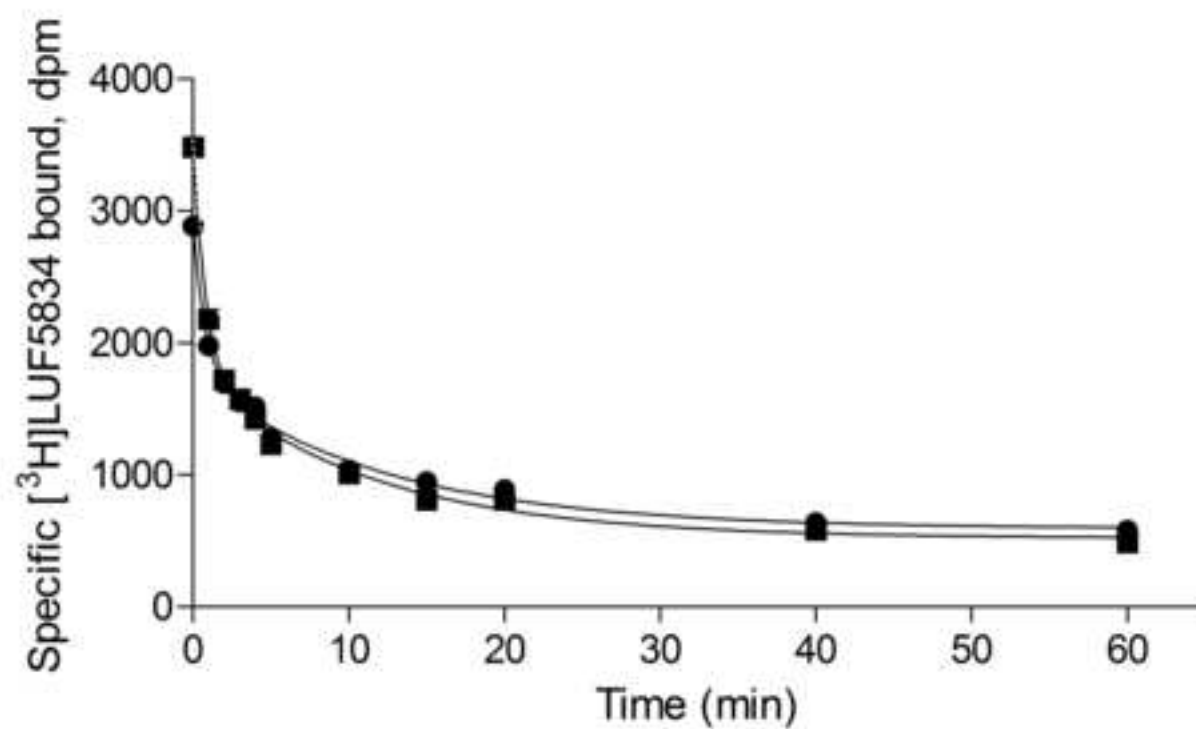
[³⁵S]GTP γ S binding experiments reveal LUF5834 to be a partial agonist at the hA₁R. The ability of increasing concentrations of the full agonist CPA, the partial agonists BCPA and ECPA, the inverse agonists DPCPX or N0840 and LUF5834 to stimulate or inhibit hA₁R-mediated nucleotide exchange was tested. A [³⁵S]GTP γ S assay was performed using CHO-hA₁ membranes. Experiments were performed in duplicate to N = 3. Data from a representative experiment are shown.

1 A)



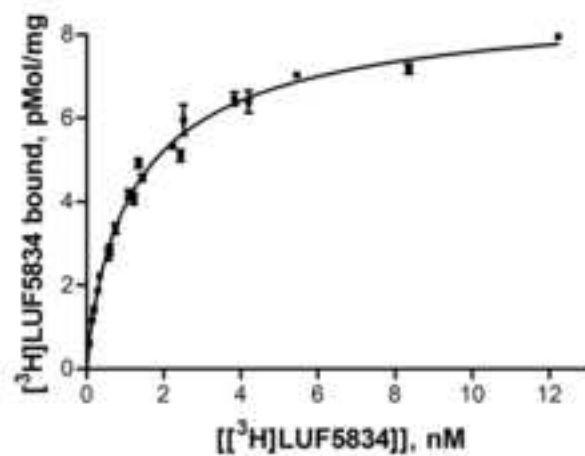
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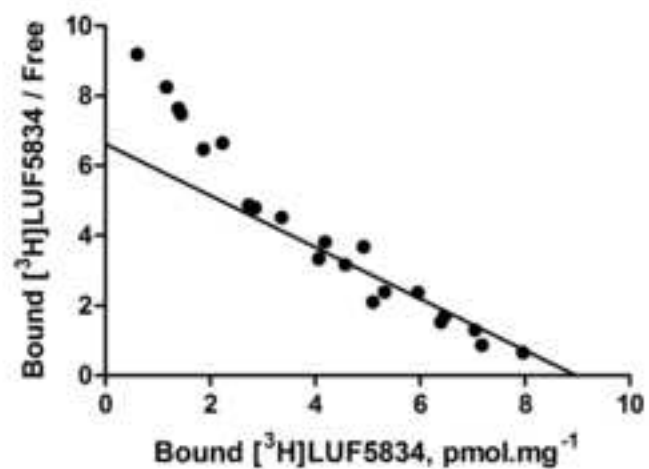
2 A)**B)**

3 A)

i

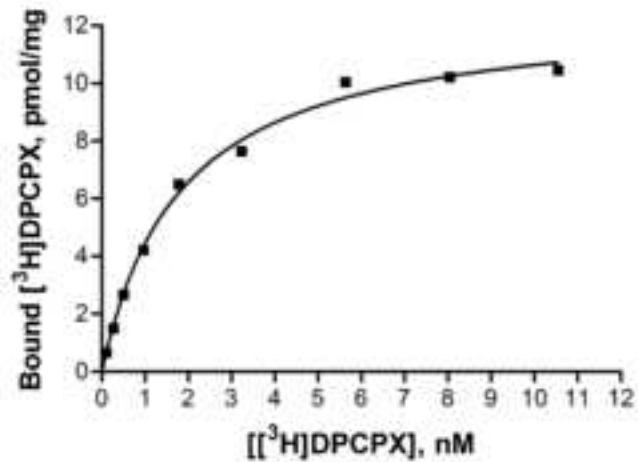


ii

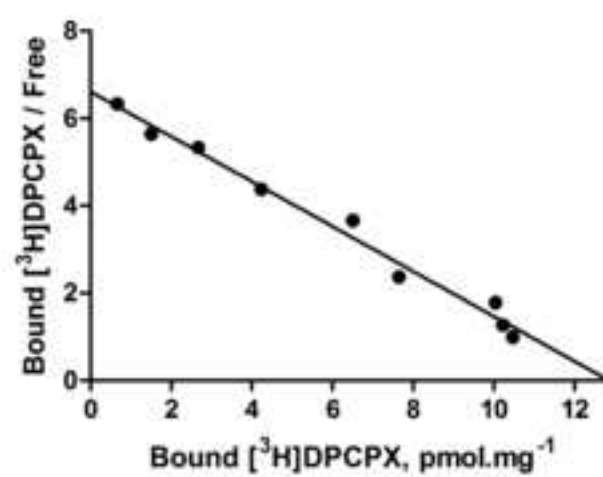


B)

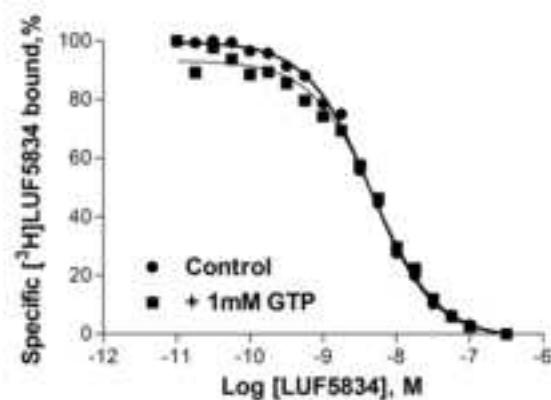
i



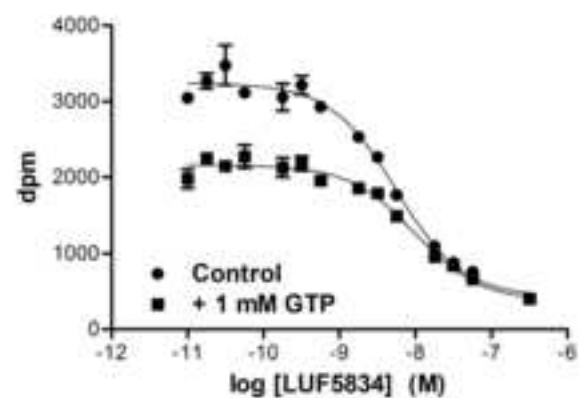
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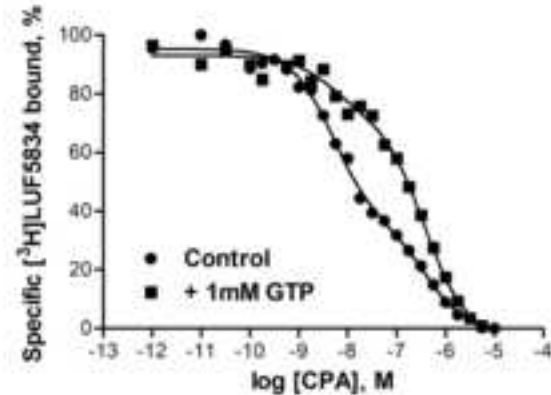
4 A) i



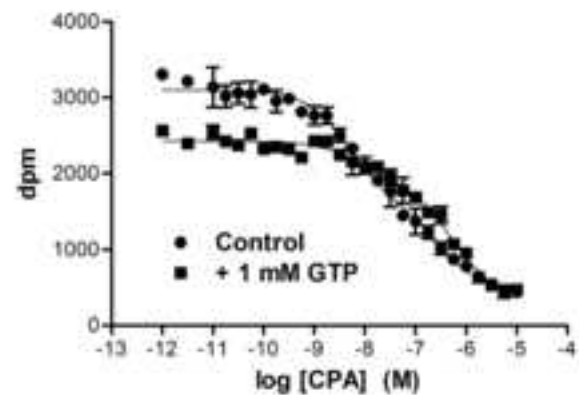
ii



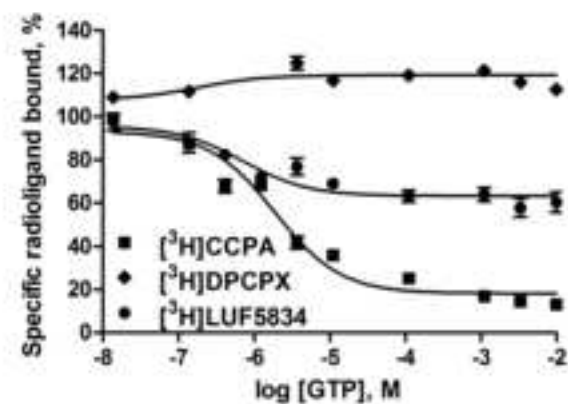
B) i

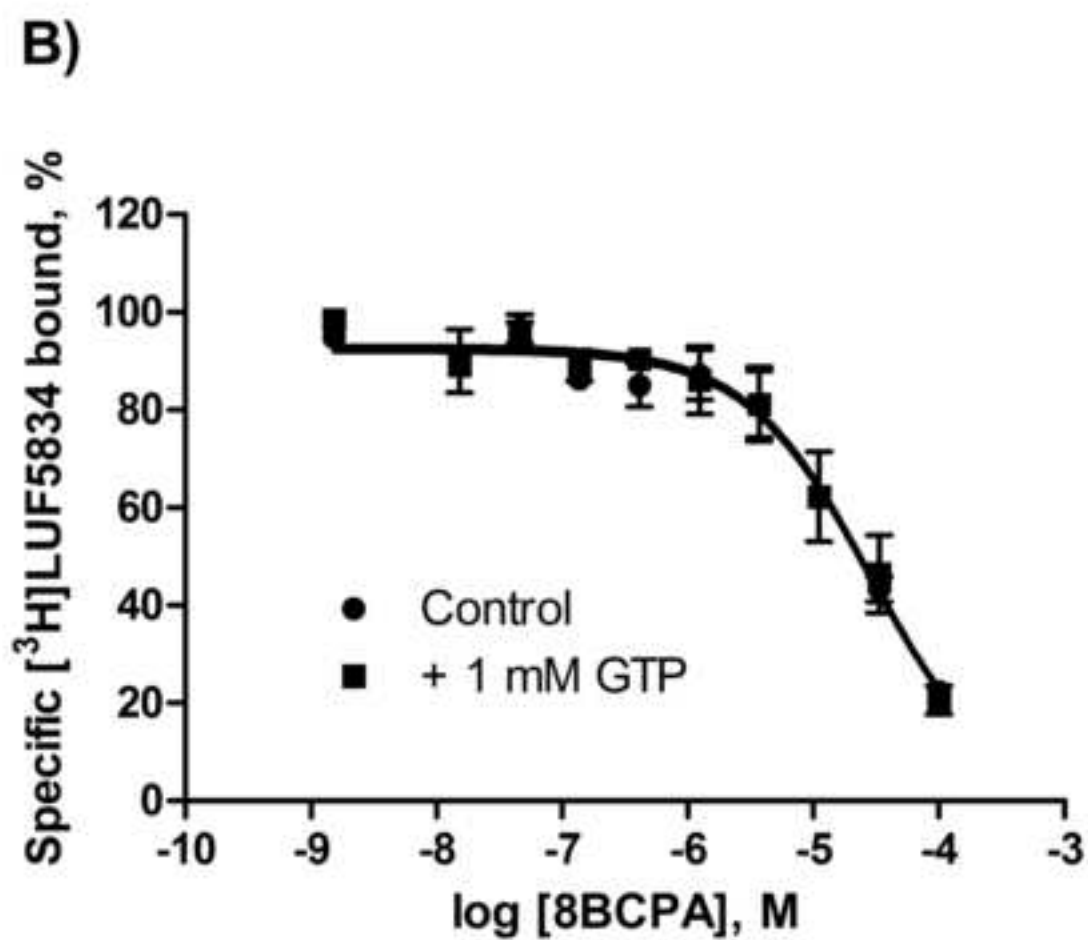
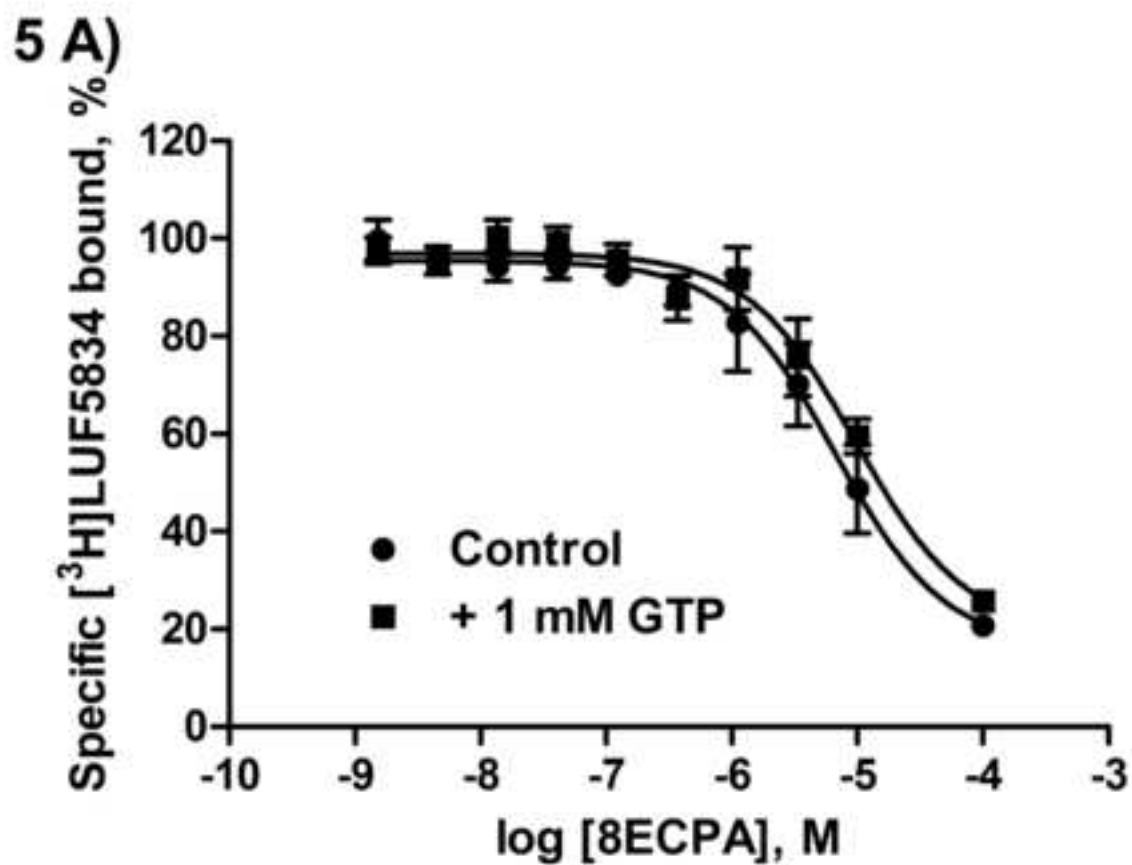


ii

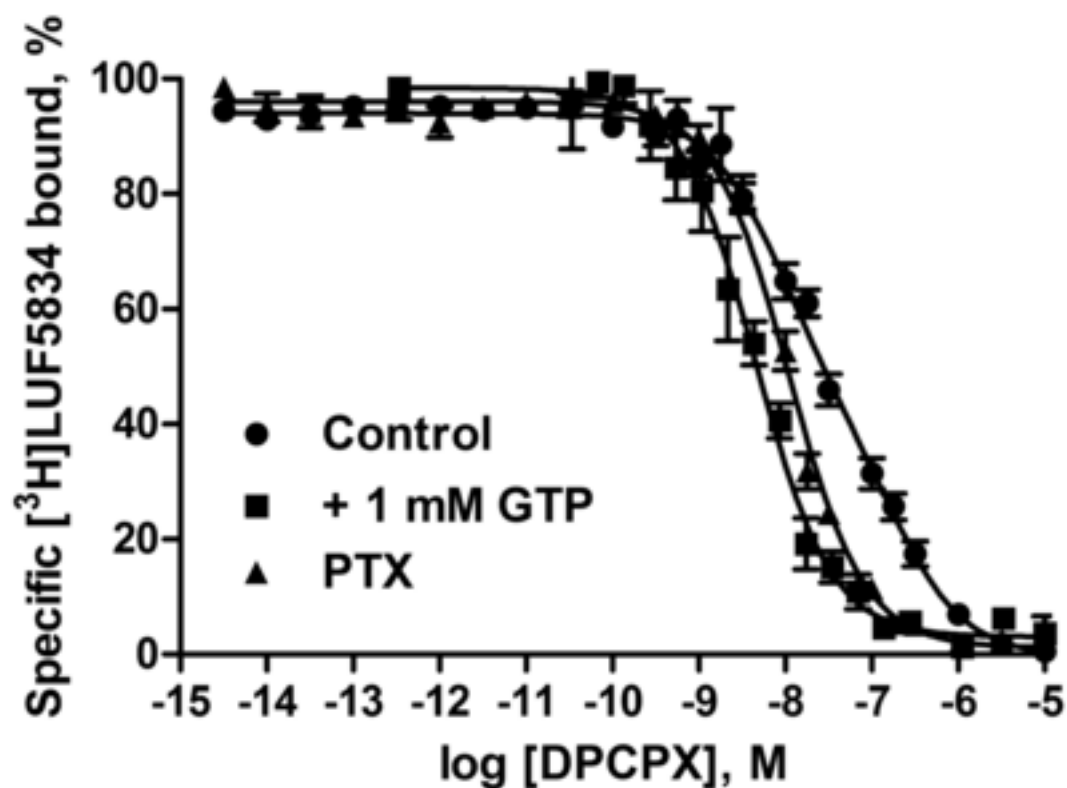


C)

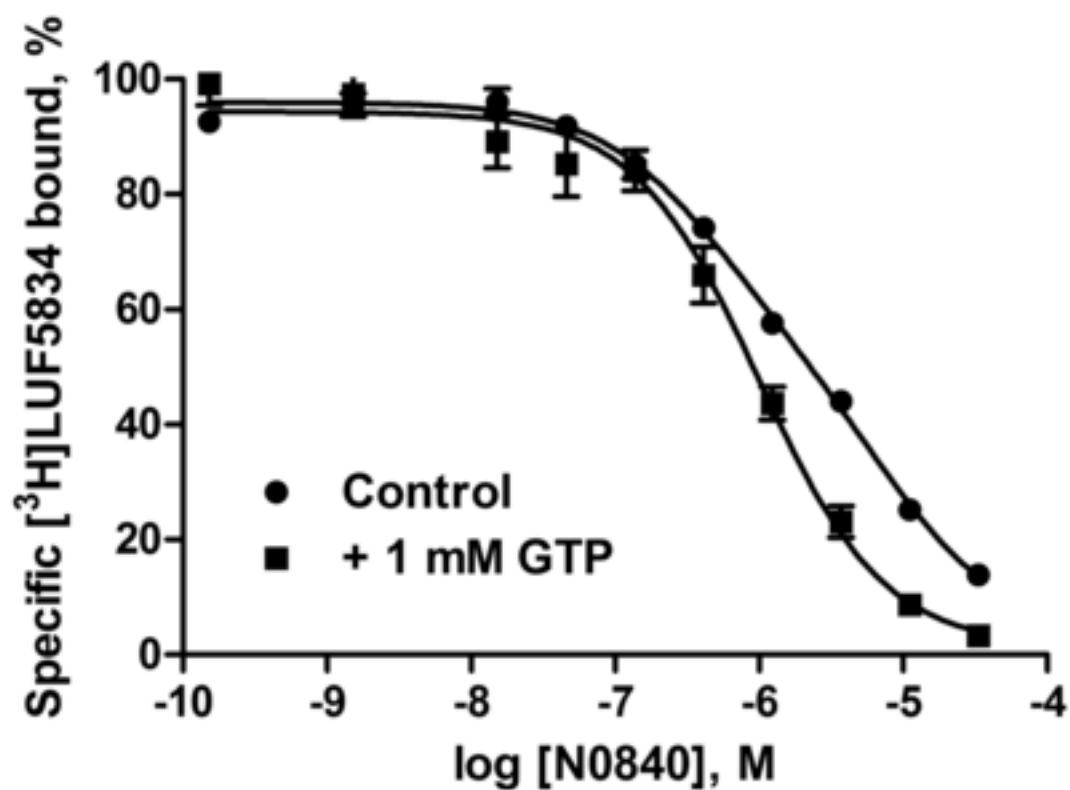




6 A)



B)



7)

