

RESEARCH PAPER

Molecular mechanism of allosteric modulation at GPCRs: insight from a binding kinetics study at the human A₁ adenosine receptor

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BACKGROUND AND PURPOSE

Many GPCRs can be allosterically modulated by small-molecule ligands. This modulation is best understood in terms of the kinetics of the ligand–receptor interaction. However, many current kinetic assays require at least the (radio)labelling of the orthosteric ligand, which is impractical for studying a range of ligands. Here, we describe the application of a so-called competition association assay at the adenosine A₁ receptor for this purpose.

EXPERIMENTAL APPROACH

We used a competition association assay to examine the binding kinetics of several unlabelled orthosteric agonists of the A₁ receptor in the absence or presence of two allosteric modulators. We also tested three bitopic ligands, in which an orthosteric and an allosteric pharmacophore were covalently linked with different spacer lengths. The relevance of the competition association assay for the binding kinetics of the bitopic ligands was also explored by analysing simulated data.

KEY RESULTS

The binding kinetics of an unlabelled orthosteric ligand were affected by the addition of an allosteric modulator and such effects were probe- and concentration-dependent. Covalently linking the orthosteric and allosteric pharmacophores into one bitopic molecule had a substantial effect on the overall on- or off-rate.

CONCLUSION AND IMPLICATIONS

The competition association assay is a useful tool for exploring the allosteric modulation of the human adenosine A₁ receptor. This assay may have general applicability to study allosteric modulation at other GPCRs as well.

Abbreviations

BC-1, (2-amino-4-((4-(4-(trifluoromethyl)phenyl)piperazin-1-yl)methyl)thiophen-3-yl)(4-chlorophenyl)methanone; CCPA, 2-chloro-N⁶-cyclopentyladenosine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHO-hA₁R, Chinese hamster ovary cells stably expressing the human adenosine A₁ receptor; CPA, N⁶-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; *k*₁, the association rate constant of the

radioligand; k_2 , the dissociation rate constant of the radioligand; k_3 , the association rate constant of the unlabelled ligand; k_4 , the dissociation rate constant of the unlabelled ligand; LUF5519, N^6 -(4-methoxyphenyl)adenosine; LUF5834, 2-amino-4-(4-hydroxyphenyl)-6-(1*H*-imidazol-2-ylmethyl-sulfanyl)-pyridine-3,5-dicarbonitrile; LUF6232, N^6 -[2-amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-4-butyloxy-4-phenyl]-adenosine; LUF6234, N^6 -[2-amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-5-pentyloxy-4-phenyl]-adenosine; LUF6258, N^6 -[2-amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridin-6-yl-9-nonyloxy-4-phenyl]-adenosine; LUF7160, N^6 -(4-nonyloxyphenyl) adenosine; LUF7161, N^6 -(4-pentyloxyphenyl)adenosine; NECA, *N*-5'-ethylcarboxamidoadenosine; PD81,723, (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)-phenyl]methanone; RT, residence time.

Table of Links

TARGET	LIGAND	
Adenosine A ₁ receptor	CCPA	DPCPX
	CPA	NECA

This Table lists key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Adenosine receptors are a subfamily within the class A of GPCRs, which includes four subtypes: A₁, A_{2A}, A_{2B} and A₃ (Fredholm *et al.*, 2011). These receptors are expressed in many tissues and play important roles in numerous physiological processes (Gao and Jacobson, 2007). Adenosine receptors can be activated by their endogenous ligand adenosine or by a range of synthetic low MW agonists that share the same binding site as the natural ligand, which is referred to as the orthosteric site (Gao *et al.*, 2005). Adenosine receptors also possess an allosteric site, which is a binding pocket topographically distinct from the orthosteric site (Göblyös and Ijzerman, 2011). Currently, many GPCRs, including the adenosine receptor subfamily, have been reported to have one or several allosteric binding sites that interact with an allosteric modulator (Gregory *et al.*, 2007; Keov *et al.*, 2011; Müller *et al.*, 2012; Davie *et al.*, 2013; Gao and Jacobson, 2013; Wang and Lewis, 2013). Such a binding event is believed to result in receptor conformational changes, which, in turn, affect the binding of the orthosteric ligand, its potency and/or level of activation (Christopoulos and Kenakin, 2002; Melancon *et al.*, 2012). Moreover, the extent of allosteric modulation also depends upon the nature of the orthosteric ligand, so-called probe dependency (Christopoulos, 2002; Keov *et al.*, 2011). These complexities add difficulties to a full understanding of the concept and mechanism of allosteric modulation.

Recently, an increasing amount of evidence suggests that ligand–receptor binding kinetics is an overlooked key factor in the broader concept of a drug's mechanism of action (Copeland *et al.*, 2006; Zhang and Monsma, 2010; Guo *et al.*, 2014). Insight in the ligand–receptor binding kinetics may further our knowledge of the molecular mechanism of GPCR allosterism and improve our understanding of this concept (May *et al.*, 2010). However, current kinetic studies of GPCR

allosterism mostly rely upon the 'probe' dissociation experiment, that is, determining the change in the dissociation rate of a radiolabelled orthosteric ligand (Kostenis and Mohr, 1996; Christopoulos and Kenakin, 2002; De Amici *et al.*, 2010). This radiolabelling process is both labour-intensive and, importantly, only practical for high affinity ligands. As a consequence, the investigation of GPCR allosterism from a kinetic point of view has been limited. Therefore, it is highly desirable to measure the binding kinetics of label-free orthosteric ligands in the absence or presence of an allosteric modulator.

To determine the binding kinetics, we followed a recently validated competition association assay on the human adenosine A₁ receptor in our laboratory (Guo *et al.*, 2013), based upon the mathematical model described by Motulsky and Mahan (1984). In the current study, we examined the association and dissociation rates of several adenosine A₁ receptor agonists in the absence or presence of different allosteric modulators (Figure 1). Among these compounds, CCPA (2-chloro- N^6 -cyclopentyladenosine) and NECA (*N*-5'-ethylcarboxamidoadenosine) are ribose-containing A₁ receptor agonists (Lohse *et al.*, 1988; Müller, 2001), whereas LUF5834 [2-amino-4-(4-hydroxyphenyl)-6-(1*H*-imidazol-2-ylmethyl-sulfanyl)-pyridine-3,5-dicarbonitrile] is a A₁ receptor agonist without the ribose moiety (Beukers *et al.*, 2004). The allosteric modulators, PD81,723 (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)-phenyl]methanone and BC-1 [i.e. compound **8j** by Romagnoli *et al.* (2008), 2-amino-4-((4-(4-(trifluoromethyl)phenyl)piperazin-1-yl)methyl)thiophen-3-yl)(4-chlorophenyl)methanone] are allosteric enhancers of the A₁ receptor (Bruns and Fergus, 1990; Romagnoli *et al.*, 2008). The binding kinetics of three 'bitopic' ligands (synthesized in-house), namely LUF6232 { N^6 -[2-amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-4-butyloxy-4-phenyl]-adenosine}, LUF6234 { N^6 -[2-amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno-[2,3-*c*]pyridin-6-yl-5-pentyloxy-4-phenyl]-adenosine} and LUF6258

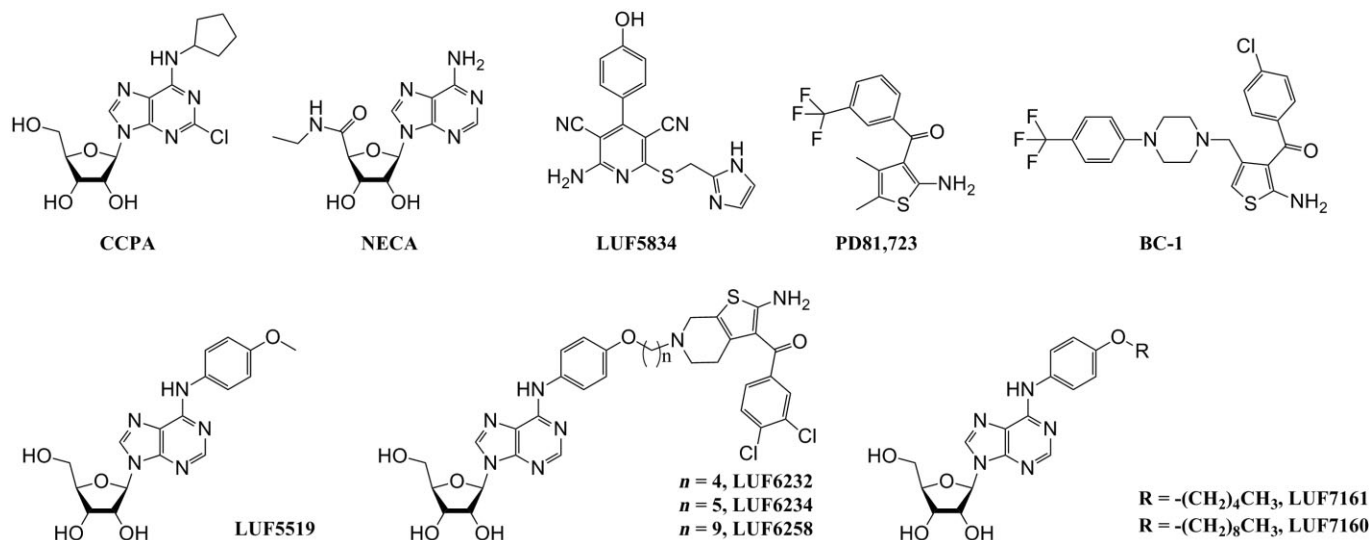


Figure 1

Chemical structures of compounds used in the present study. CCPA, NECA and LUF5519 are ribose-containing adenosine A₁ receptor agonists, whereas LUF5834 is a non-ribose agonist (Lohse *et al.*, 1988; Müller, 2001; Beukers *et al.*, 2004); PD81,723 and BC-1 are allosteric enhancers for adenosine A₁ receptor agonists (Romagnoli *et al.*, 2008). LUF6232, LUF6234 and LUF6258 are in-house synthesized bitopic ligands for the adenosine A₁ receptor with four-, five- and nine-carbon linker, respectively (Narlwar *et al.*, 2010). LUF7161 and LUF7160 are newly synthesized monovalent ligands with five-atom linker and nine-atom linker respectively.

[N⁶-[2-amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno[2,3-c]-pyridin-6-yl-9-nonyloxy-4-phenyl]-adenosine] (Figure 1, Narlwar *et al.*, 2010), were also investigated, as they are useful tools for the exploration of A₁ receptor allosteric modulation (Lane *et al.*, 2013). These bitopic ligands share the same orthosteric and allosteric pharmacophores, mimicking LUF5519 [A₁ receptor agonist, N⁶-(4-methoxyphenyl)adenosine] and PD81,723 [2-amino-4,5-dimethyl-3-thienyl]-[3-(trifluoromethyl)-phenyl] methanone], respectively, which are covalently linked via a spacer with different lengths, that is, four-, five- and nine-carbon atoms respectively.

Methods

Cell culture and membrane preparation

Cell culture and membrane preparation were performed as reported previously (Guo *et al.*, 2013).

Radioligand displacement assays

Membrane aliquots containing 5 µg of protein were incubated in a total volume of 100 µL assay buffer [50 mM Tris-HCl (pH 7.4), supplemented with 5 mM MgCl₂ and 0.1% (w/v) CHAPS] at 25°C for 1 h. The displacement experiments were performed using 11 concentrations of competing ligands with 2.6 nM [³H]-DPCPX in the absence or presence of 10 µM of PD81,723 or BC-1. In such experiments, CCPA and NECA were also tested in the presence of 1 mM GTP. For the determination of the allosteric potency of PD81,723 and BC-1, 100 nM (IC₈₀ value) CCPA was used in the presence of increasing concentrations of these two allosteric modulators

to examine the potentiated [³H]-DPCPX displacement. Non-specific binding was determined in the presence of 100 µM N⁶-cyclopentyladenosine (CPA) and represented less than 10% of the total binding. Incubations were terminated and samples were obtained as described previously (Guo *et al.*, 2013).

Radioligand association and dissociation assays

Association experiments were performed by incubating membrane aliquots containing 5 µg of protein in a total volume of 100 µL of assay buffer at 25°C with 2.6 nM [³H]-DPCPX. The amount of radioligand bound to the receptor was measured at different time intervals during a total incubation of 45 min in the absence or presence of 1, 10 and 33 µM PD81,723 or BC-1. For the dissociation assay, membrane aliquots containing 5 µg of protein were allowed to reach equilibrium for 1 h with the same amount of radioligand. After the pre-incubation, radioligand dissociation was started by adding 10 µM of DPCPX at different time points in the absence or presence of 1, 10 and 33 µM PD81,723 or BC-1. The amount of radioligand still bound to the receptor was measured at various time intervals for a total of 1 h. Incubations were terminated and samples were obtained as described previously (Guo *et al.*, 2013).

Radioligand competition association assay

The binding kinetics of unlabelled ligands was quantified at 25°C using the competition association assay based upon the theoretical framework by Motulsky and Mahan (1984). This method has been recently adopted and validated on the adenosine A₁ receptor in our laboratory (Guo *et al.*, 2013),

which enables accurate determination of unlabelled ligands' binding kinetics. In the present study, we followed the same method. In brief, we used a concentration of approximately 1-, 3- and/or 10-fold K_i of the unlabelled ligand with or without the co-incubation of 1, 10 or 33 μM PD81,723 or BC-1. The experiment was initiated by adding membrane aliquots containing 5 μg of protein in a total volume of 100 μL assay buffer at different time points for a total incubation of 1 h, except for LUF6232, LUF6234 and LUF6258, which were incubated for 2 h given their slow kinetic profiles. Incubations were terminated and samples were obtained as described previously (Guo *et al.*, 2013).

Data analysis

All experimental data were analysed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Radioligand displacement curves were fitted to one- and two-state/site binding models. k_{obs} and k_{off} values of [^3H]-DPCPX in the absence or presence of PD81,723 or BC-1 were obtained from the association and dissociation curves. Values for k_{on} were obtained by converting k_{obs} values using the following equation:

$$k_{\text{on}} = \frac{k_{\text{obs}} - k_{\text{off}}}{[\text{Radioligand}]} \quad (1)$$

Association and dissociation rates for unlabelled ligands were calculated by fitting the data in the competition association model using 'kinetics of competitive binding' (Motulsky and Mahan, 1984):

$$\begin{aligned} K_A &= k_1 \cdot [L] + k_2 \\ K_B &= k_3 \cdot [I] + k_4 \\ S &= \sqrt{(K_A - K_B)^2 + 4 \cdot k_1 \cdot k_3 \cdot L \cdot I} \\ K_F &= 0.5 \cdot (K_A + K_B + S) \\ K_S &= 0.5 \cdot (K_A + K_B - S) \\ Q &= \frac{B_{\text{max}} \cdot k_1 \cdot L}{K_F - K_S} \\ Y &= Q \cdot \left[\frac{k_4 \cdot (K_F - K_S)}{K_F \cdot K_S} + \frac{k_4 - K_F}{K_F} e^{(-K_F \cdot X)} - \frac{k_4 - K_S}{K_S} e^{(-K_S \cdot X)} \right] \end{aligned} \quad (2)$$

where X is the time, Y is the specific [^3H]-DPCPX binding (DPM), k_1 and k_2 are the k_{on} ($\text{M}^{-1} \cdot \text{min}^{-1}$) and k_{off} (min^{-1}) of [^3H]-DPCPX pre-determined in radioligand association and dissociation assay, respectively, in the absence or presence of different concentrations of PD81,723 or BC-1, L is the concentration of [^3H]-DPCPX used (M), B_{max} is the total binding (DPM) and I is the concentration unlabelled ligand (M). Fixing these parameters allows the following parameters to be calculated: k_3 , which is the k_{on} value ($\text{M}^{-1} \cdot \text{min}^{-1}$) of the unlabelled ligand, and k_4 , which is the k_{off} value (min^{-1}) of the unlabelled ligand. Ligand-receptor residence times (RT) were calculated using the following equation (Copeland, 2005):

$$\text{RT} = \frac{1}{k_{\text{off}}} \quad (3)$$

The derived association and dissociation rates of the unlabelled ligand were used to calculate the 'kinetic K_D ' using the following equation:

$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}} \quad (4)$$

Data simulations

Differential equations (see below, Equations 5–12) were used to follow the time (t)-wise changes in target 'AB' occupancy by a bitopic ligand, 'ab', or a monovalent ligand, 'c', based upon the thermodynamic model in Figure 2 (Vauquelin *et al.*, 2013). The abbreviation notions for different molecular complexes are in parentheses in Figure 2:

$$d[AB]/d(t) = k_{-a} \cdot [aAB] - k_{+a} \cdot [AB] \cdot [ab] + k_{-b} \cdot [ABb] - k_{+b} \cdot [AB] \cdot [ab] + k_{-c} \cdot [cAB] - k_{+c} \cdot [AB] \cdot [c] \quad (5)$$

$$d[aAB]/d(t) = k_{+a} \cdot [AB] \cdot [ab] - k_{-a} \cdot [aAB] + k_{-b} \cdot [aABb]/\alpha'_{ab} - \alpha_{ab} \cdot k_{+b} \cdot [aAB] \cdot [L] + k_{-b} \cdot [a'ABb']/\alpha'_{ab} - \alpha_{ab} \cdot k_{+b} \cdot [aAB] \cdot [ab] \quad (6)$$

$$d[ABb]/d(t) = k_{+b} \cdot [AB] \cdot [ab] - k_{-b} \cdot [ABb] + k_{-a} \cdot [aABb]/\alpha'_{ab} - \alpha_{ab} \cdot k_{+a} \cdot [ABb] \cdot [L] + k_{-c} \cdot [cABb]/\alpha'_{bc} - \alpha_{bc} \cdot k_{+c} \cdot [ABb] \cdot [c] + k_{-a} \cdot [a'ABb']/\alpha'_{ab} - \alpha_{ab} \cdot k_{+a} \cdot [aAB] \cdot [ab] \quad (7)$$

$$d[aABb]/d(t) = \alpha_{ab} \cdot k_{+a} \cdot [ABb] \cdot [L] - k_{-a} \cdot [aABb]/\alpha'_{ab} + \alpha_{ab} \cdot k_{+b} \cdot [aAB] \cdot [L] - k_{-b} \cdot [aABb]/\alpha'_{ab} \quad (8)$$

$$d[cAB]/d(t) = k_{+c} \cdot [AB] \cdot [c] - k_{-c} \cdot [cAB] + k_{-b} \cdot [cABb]/\alpha'_{bc} - \alpha_{bc} \cdot k_{+b} \cdot [cAB] \cdot [ab] \quad (9)$$

$$d[cABb]/d(t) = \alpha_{bc} \cdot k_{+b} \cdot [cAB] \cdot [ab] - k_{-b} \cdot [cABb]/\alpha'_{bc} + \alpha_{bc} \cdot k_{+c} \cdot [ABb] \cdot [c] - k_{-c} \cdot [cABb]/\alpha'_{bc} \quad (10)$$

$$d[a'ABb']/d(t) = \alpha_{ab} \cdot k_{+b} \cdot [aAB] \cdot [ab] - k_{-b} \cdot [a'ABb']/\alpha'_{ab} + \alpha_{ab} \cdot k_{+a} \cdot [aAB] \cdot [ab] - k_{-a} \cdot [a'ABb']/\alpha'_{ab} \quad (11)$$

$$[AB]_{\text{occ}} \text{ by } c = 100 \cdot ([cAB] + [cABb]) / ([AB] + [aAB] + [aABb] + [ABb] + [a'ABb'] + [cAB] + [cABb]) \quad (12)$$

in which k_{+a} and k_{+b} ($\text{M}^{-1} \cdot \text{min}^{-1}$) are the microscopic association rate constants of the bitopic ligand, ab , for a -A and b -B binding; k_{-a} and k_{-b} (min^{-1}) are the dissociation rate constants of the bitopic ligand, ab , for a -A and b -B unbinding; $[c]$ is the concentration of the monovalent ligand (M), representing the radioligand; k_{+c} and k_{-c} are the association rate and dissociation rate for c -A binding/unbinding. Both ab and c are assumed to be in large excess over the target sites. $[ab]$ is the total concentration (M) of the bitopic ligand and $[L]$ is the local concentration of the second pharmacophore to bind. $[AB]_{\text{occ}}$ by c is the sum of all c -bound species and is expressed as percentage of the total target population. The cooperativity factor (α), which is defined by how much binding affinities of two ligands increase (Lazareno and Birdsall, 1995), was also included in the thermodynamic model in Figure 2 and the data simulations. The cooperativity factor that affects the association process was named α ; the cooperativity factor that affects the dissociation process was named α' . The first pharmacophore of the bitopic ligand to bind (either 'a' or 'b') may trigger a conformational change of the target. This may modify the association rate constant that governs the binding of the second pharmacophore ('b' if 'a' was bound first and vice versa) and/or the dissociation rate constant of the first unbinding event (i.e. dissociation of 'a' or 'b' from the $aABb$ complex). Additionally, while the binding of 'a' and 'c' to 'A' is considered to be competitive, the c -A and b -B interactions may show cooperativity as well. Thus, the cooperativity factors were subdivided even further to yield α_{ab} and α_{bc} for association and α'_{ab} and α'_{bc} for dissociation (Vauquelin *et al.*,

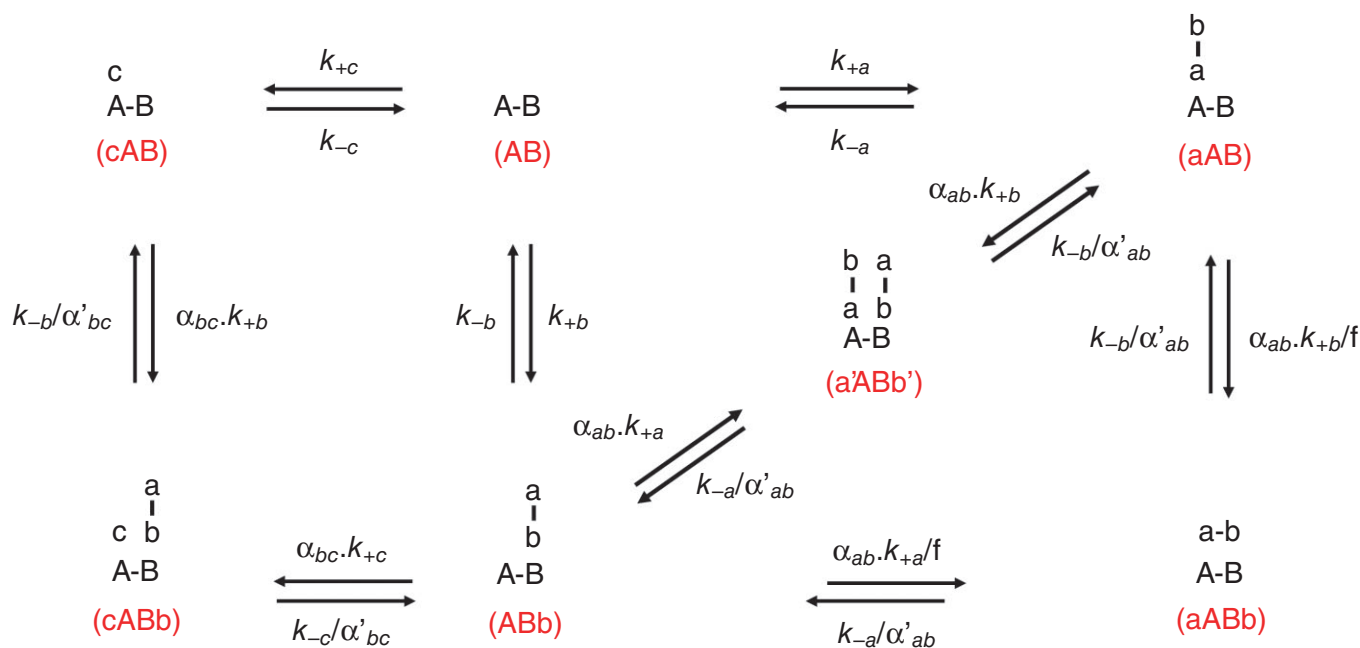


Figure 2

Schematic representation of the monovalent, 'c', and bitopic, 'ab', ligand–target site interactions. The bitopic ligand bears two distinct pharmacophores: 'a' and 'b'. 'AB' is the target with distinct binding sites 'A' and 'B'. 'a' and 'c' only bind to 'A' in a competitive manner and 'b' only binds to 'B'. The abbreviated notation for the free target and target complexes is in parentheses. k_{+a} , k_{+b} and k_{+c} (in $M^{-1} \cdot \text{min}^{-1}$) are the microscopic association rate constants and k_{-a} , k_{-b} and k_{-c} (in min^{-1}) are the microscopic dissociation rate constants for a-A, b-B and c-A binding respectively. α_{ab} and α_{bc} are cooperativity factors affecting the association process of different pharmacophores. α'_{ab} and α'_{bc} are cooperativity factors affecting the dissociation process of different pharmacophores.

2013). The macroscopic k_{on} and k_{off} value of a bitopic ligand can be theoretically calculated using the following equations (Vauquelin, 2013; Vauquelin *et al.*, 2013; Vauquelin and Charlton, 2013):

$$k_{\text{on}} = \frac{k_{+a} \cdot (\alpha_{ab} \cdot [L] \cdot k_{+b} + k_{-b}/\alpha'_{ab})}{(k_{-a} + \alpha_{ab} \cdot [L] \cdot k_{+b} + k_{-b}/\alpha'_{ab})} + \frac{k_{+b} \cdot (\alpha_{bc} \cdot [L] \cdot k_{+c} + k_{-c}/\alpha'_{bc})}{(k_{-b} + \alpha_{bc} \cdot [L] \cdot k_{+c} + k_{-c}/\alpha'_{bc})} \quad (13)$$

$$k_{\text{off}} = \frac{(k_{-a} \cdot k_{-b})}{(k_{-b} + \alpha'_{ab} \cdot (k_{-a} + \alpha_{ab} \cdot [L] \cdot k_{+b}))} + \frac{(k_{-b} \cdot k_{-c})}{(k_{-c} + \alpha'_{bc} \cdot (k_{-b} + \alpha_{bc} \cdot [L] \cdot k_{+c}))} \quad (14)$$

Data simulations were performed to mimic the binding of a radioligand (represented by the target occupancy of 'c') by solving Equations 5–12 during a period of 50 min, either alone or in the presence of increasing concentrations of 'ab'. The microscopic binding kinetics of the bitopic ligand 'ab' were defined as $k_{+a} = 1 \times 10^5 M^{-1} \cdot \text{min}^{-1}$, $k_{-a} = 1 \text{ min}^{-1}$, $k_{+b} = 1 \times 10^5 M^{-1} \cdot \text{min}^{-1}$, $k_{-b} = 1 \text{ min}^{-1}$ and $[L] = 0.29 \text{ nM}$. The binding kinetics of the monovalent ligand 'c' was defined as $k_{+c} = 1 \times 10^7 M^{-1} \cdot \text{min}^{-1}$, $k_{-c} = 0.3 \text{ min}^{-1}$ and $[c] = 100 \text{ nM}$. The cooperativity factors of 'ab' were set as (i) $\alpha_{ab} = 10$; (ii) $\alpha_{ab} = 0.1$; (iii) $\alpha'_{ab} = 10$; (iv) $\alpha'_{ab} = 0.1$. For simplicity, we kept $\alpha_{bc} = \alpha'_{bc} = 1$. The simulated data were collected for a total of 50 min and subsequently subjected to the competition association model using 'kinetics of competitive binding' (Motulsky and Mahan, 1984). The kinetics data obtained thereof were compared with the theoretically calculated values (by subjecting

the defined microscopic rate constants mentioned above into Equations 13 and 14) to explore the relevance of using the competition association assay for bitopic ligands' binding kinetics.

Materials

[³H]-1,3-dipropyl-8-cyclopentylxanthine ([³H]-DPCPX, specific activity 103 Ci·mmol⁻¹) was purchased from ARC, Inc. (St. Louis, MO, USA). Unlabelled DPCPX and CCPA were from Sigma (St. Louis, MO, USA). NECA was purchased from Sigma-Aldrich (Steinheim, Germany). CPA was obtained from Research Biochemicals Inc. (Natick, MA, USA). LUF5834, PD81,723 and BC-1 were prepared in-house following synthesis routes reported previously (Bruns and Fergus, 1990; Beukers *et al.*, 2004; Romagnoli *et al.*, 2008). LUF5519, LUF6232, LUF6234 and LUF6258 were also synthesized in-house as described by Narlawar *et al.* (2010). The synthesis of LUF7160 [*N*⁶-(4-nonyloxyphenyl) adenosine] and LUF7161 [*N*⁶-(4-pentyloxyphenyl)adenosine] was performed as described in the Supporting Information. CHAPS [3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate] was obtained from Carl Roth GmbH (Karlsruhe, Germany). GTP was purchased from Acros Organics (Geel, Belgium). Chinese hamster ovary cells stably expressing the human A₁ receptor (CHO-hA₁R) were obtained from Prof. Steve Hill (University of Nottingham, UK). All other chemicals were of analytical grade and obtained from standard commercial sources.

Results

Quantification of the affinity (K_i) of A_1 receptor ligands in the absence or presence of PD81,723, BC-1, GTP or a combination thereof in displacement experiments

To determine the affinities of several agonists at the A_1 receptor in the absence or presence of allosteric modulators, radioligand displacement experiments were performed. All compounds tested produced concentration-dependent inhibition of specific [3 H]-DPCPX binding (Figure 3) and their affinities are detailed in Table 1. Among the agonists tested, the binding of CCPA and NECA was preferably fitted in a two-site/state competition model, whereas the non-ribose agonist LUF5834 was best described in a one-site/state competition model. Upon the addition of 1 mM GTP, the binding curves of CCPA and NECA were best fitted with a one-site/state competition model with K_i values of 141 ± 28 and 505 ± 93 nM respectively (Table 1). In the presence of 10 μ M PD81,723, CCPA demonstrated a significant increase of both its high and low affinity ($K_H = 2 \pm 1$ nM, $K_L = 77 \pm 5$ nM). This effect was even more pronounced upon the addition of BC-1.

Notably, the two-site binding curve of CCPA was shifted to a one-site binding curve ($K_i = 4.5 \pm 0.3$ nM) in the presence of this allosteric modulator. Similar to CCPA, the affinity of NECA was increased in the presence of PD81,723 or BC-1 respectively. Both were mainly from a gain in affinity at the low-affinity binding site. Similar observations were carried out in the experiments with 1 mM GTP to solely examine agonist binding to the G protein-uncoupled form of the A_1 receptor. In this situation, the affinities of CCPA and NECA in the presence of PD81,723 (20 ± 2 and 115 ± 23 nM) increased approximately seven- and fivefold compared with their values in the absence of the same allosteric modulator respectively. In contrast to the ribose-containing agonists, that is, CCPA and NECA, the binding affinity of non-ribose agonist LUF5834 to the A_1 receptor was not significantly altered by the presence of either allosteric modulator.

Quantification of the potency of two allosteric modulators

The potencies of PD81,723 and BC-1 were investigated by examining the enhanced [3 H]-DPCPX displacement from the A_1 receptor by 100 nM CCPA (IC_{80}) in the presence of increasing concentrations of these two allosteric modulators. It

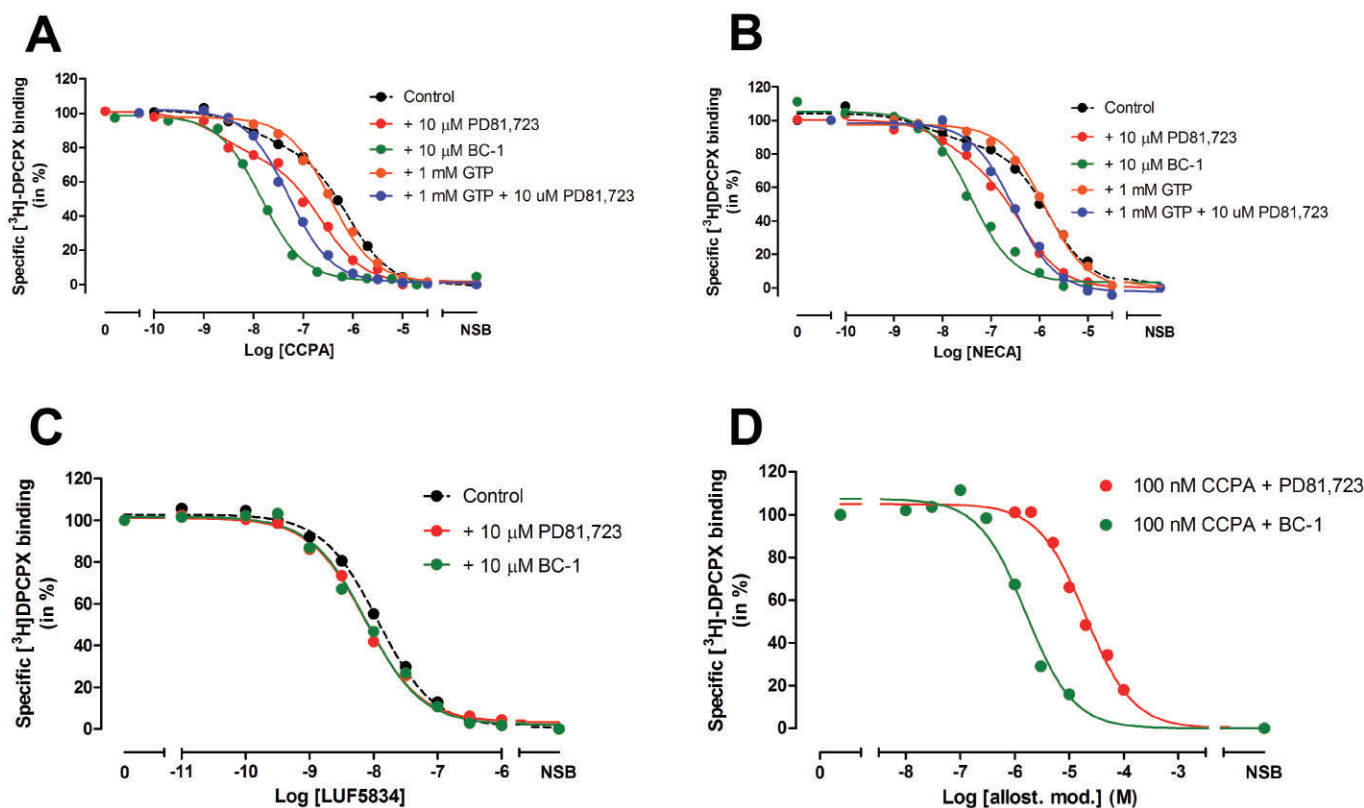


Figure 3

(A) Displacement of specific [3 H]-DPCPX binding from the adenosine A_1 receptor at 25°C by CCPA in the absence or presence of 10 μ M PD81,723, 10 μ M BC-1, 1 mM GTP or a combination thereof. (B) Displacement of specific [3 H]-DPCPX binding from the adenosine A_1 receptor at 25°C by NECA in the absence or presence of 10 μ M PD81,723, 10 μ M BC-1, 1 mM GTP or a combination thereof. (C) Displacement of specific [3 H]-DPCPX binding from the adenosine A_1 receptor at 25°C by LUF5834 in the absence or presence of 10 μ M PD81,723 or BC-1. (D) Displacement of specific [3 H]-DPCPX binding from the adenosine A_1 receptor at 25°C by 100 nM CCPA (normalized as 100%) in the presence of increased concentrations of PD81,723 or BC-1. Representative graphs from one experiment performed in duplicate.

Table 1[³H]-DPCPX displacement experiments by CCPA, NECA and LUF5834 on CHO-hA₁R membranes

Compound added	CCPA			NECA			LUF5834
	K _H (nM)	K _L or K _i (nM)	R _H (%)	K _H (nM)	K _L or K _i (nM)	R _H (%)	K _i (nM)
Control (none)	16 ± 1	338 ± 27	20 ± 4	4 ± 1	731 ± 94	18 ± 3	7.8 ± 1.5
+1 mM GTP	NA	141 ± 28	NA	NA	505 ± 93	NA	ND
+10 μM PD81,723	2 ± 1	77 ± 5	27 ± 7	6 ± 4	200 ± 78	26 ± 9	9.6 ± 2.6
+1 mM GTP, +10 μM PD81,723	NA	20 ± 2	NA	NA	115 ± 23	NA	ND
+10 μM BC-1	NA	4.5 ± 0.3	NA	8 ± 2	128 ± 27	10 ± 3	6.7 ± 1.5

Data are shown as mean ± SEM of three separate experiments each performed in duplicate. K_H and K_L are values for the high- and low-affinity states, respectively, and are shown for data that could be fitted to a two-state model; R_H is the fraction of receptors in the high affinity state and is shown in %. K_i values are shown for data that could be fitted to a one-state model. NA, not available; ND, not determined.

Table 2The association and dissociation rates of [³H]-DPCPX binding to CHO-hA₁R membranes in the absence or presence of 1, 10 or 33 μM PD81,723 or BC-1

Cmpd	k _{on} (M ⁻¹ ·min ⁻¹) ^a	k _{off} (min ⁻¹) ^b	K _D (nM) ^c
DPCPX	1.2 ± 0.1 × 10 ⁸	0.23 ± 0.02	1.9 ± 0.3
+1 μM PD81,723	1.2 ± 0.1 × 10 ⁸	0.24 ± 0.01	2.0 ± 0.1
+10 μM PD81,723	1.4 ± 0.1 × 10 ⁸	0.23 ± 0.01	1.6 ± 0.1
+33 μM PD81,723	1.6 ± 0.2 × 10 ⁸	0.27 ± 0.01	1.7 ± 0.1
+1 μM BC-1	1.5 ± 0.1 × 10 ⁸	0.14 ± 0.00***	0.93 ± 0.04***
+10 μM BC-1	2.8 ± 0.2 × 10 ⁸ ***	0.080 ± 0.004***	0.29 ± 0.02***
+33 μM BC-1	2.8 ± 0.3 × 10 ⁸ ***	0.076 ± 0.004***	0.27 ± 0.01***

Values are means ± SEM of three separate experiments each performed in duplicate. ^aAssociation [k_{on} (k₁)] of [³H]-DPCPX to CHO-hA₁R membranes at 25°C; k_{on} = (k_{obs} - k_{off})/[radioligand]. ^bDissociation [k_{off} (k₂)] of [³H]-DPCPX from CHO-hA₁R membranes at 25°C. ^cKinetic K_D = k_{off}/k_{on}. **P < 0.01, ***P < 0.001 compared with the values in the absence of an allosteric modulator; Student's *t*-test.

follows from Figure 3D that BC-1 had a 13-fold higher potency (EC₅₀ = 1.5 ± 0.2 μM) than PD81,723 (EC₅₀ = 19 ± 2 μM) for modulating CCPA binding to the A₁ receptor.

Quantification of the association [k_{on} (k₁)] and dissociation rate constants [k_{off} (k₂)] of [³H]-DPCPX in the absence or presence of PD81,723 or BC-1

Since knowledge of the association [k_{on} (k₁)] and dissociation rate constants [k_{off} (k₂)] of [³H]-DPCPX was necessary for subsequent competition association assays in the presence of an allosteric modulator, we performed a direct [³H]-DPCPX association and dissociation assay to determine DPCPX's binding kinetics in the absence or presence of 1, 10 and 33 μM PD81,723 or BC-1. As detailed in Table 2, [³H]-DPCPX displayed a fast association (1.2 ± 0.1 × 10⁸ M⁻¹·min⁻¹) and dissociation rate (0.23 ± 0.01 min⁻¹) in the absence of PD81,723, which values were not significantly affected by the presence of 1, 10 or 33 μM PD81,723 (*P* > 0.05, Student's *t*-test). In contrast, BC-1, a structural analogue of PD81,723 (Figure 1), in most cases significantly changed both the on- and off-rates

of [³H]-DPCPX at three different concentrations. These k₁ and k₂ values in the absence or presence of an allosteric modulator allowed us to further explore the corresponding k₃ and k₄ values of unlabelled ligands.

Validation and optimization of the competition association assay for the binding kinetics of unlabelled ligands on the A₁ receptor in the absence or presence of an allosteric modulator

In our previous work at the adenosine A₁ receptor, we have validated the competition association assay to test a ligand's binding kinetics (Guo *et al.*, 2013). Here, we adopted the method and examined the binding kinetics of unlabelled DPCPX in the absence or presence of two allosteric modulators (PD81,723 and BC-1). The obtained k_{on} (k₃) and k_{off} (k₄) values for DPCPX were compared with the k₁ and k₂ values in the radiolabelled probe, that is, [³H]-DPCPX, association and dissociation assay (Table 3). In the presence of 10 μM PD81,723, the k₁ (1.4 ± 0.1 × 10⁸ M⁻¹·min⁻¹) and k₂ (0.23 ± 0.01 min⁻¹) values of DPCPX corresponded to its k₃ (1.9 ± 0.1

Table 3

Comparison of the binding kinetics of DPCPX in the absence or presence of 10 μM PD81,723 or BC-1 determined by radioligand association and dissociation assay or by the competition association assay

	k_{on} ($\text{M}^{-1}\cdot\text{min}^{-1}$)		k_{off} (min^{-1})	
	k_1^{a}	k_3^{b}	k_2^{a}	k_4^{b}
DPCPX	$1.2 \pm 0.1 \times 10^8$	$1.7 \pm 0.4 \times 10^8$	0.23 ± 0.01	0.26 ± 0.01
+10 μM PD81,723	$1.4 \pm 0.1 \times 10^8$	$1.9 \pm 0.4 \times 10^8$	0.23 ± 0.01	0.30 ± 0.07
+10 μM BC-1	$2.8 \pm 0.2 \times 10^8$	$2.7 \pm 0.1 \times 10^8$	0.080 ± 0.004	0.072 ± 0.005

Values are means \pm SEM of three separate experiments each performed in duplicate. $^{\text{a}}k_{\text{on}}$ (k_1) and k_{off} (k_2) of unlabelled DPCPX in the absence or presence of 10 μM PD81,723 or BC-1 were determined in [^3H]-DPCPX (2.6 nM) association and dissociation assay. $^{\text{b}}k_{\text{on}}$ (k_3) and k_{off} (k_4) of unlabelled CCPA in the absence or presence of 10 μM PD81,723 or BC-1 were determined in [^3H]-DPCPX (2.6 nM) competition association assays.

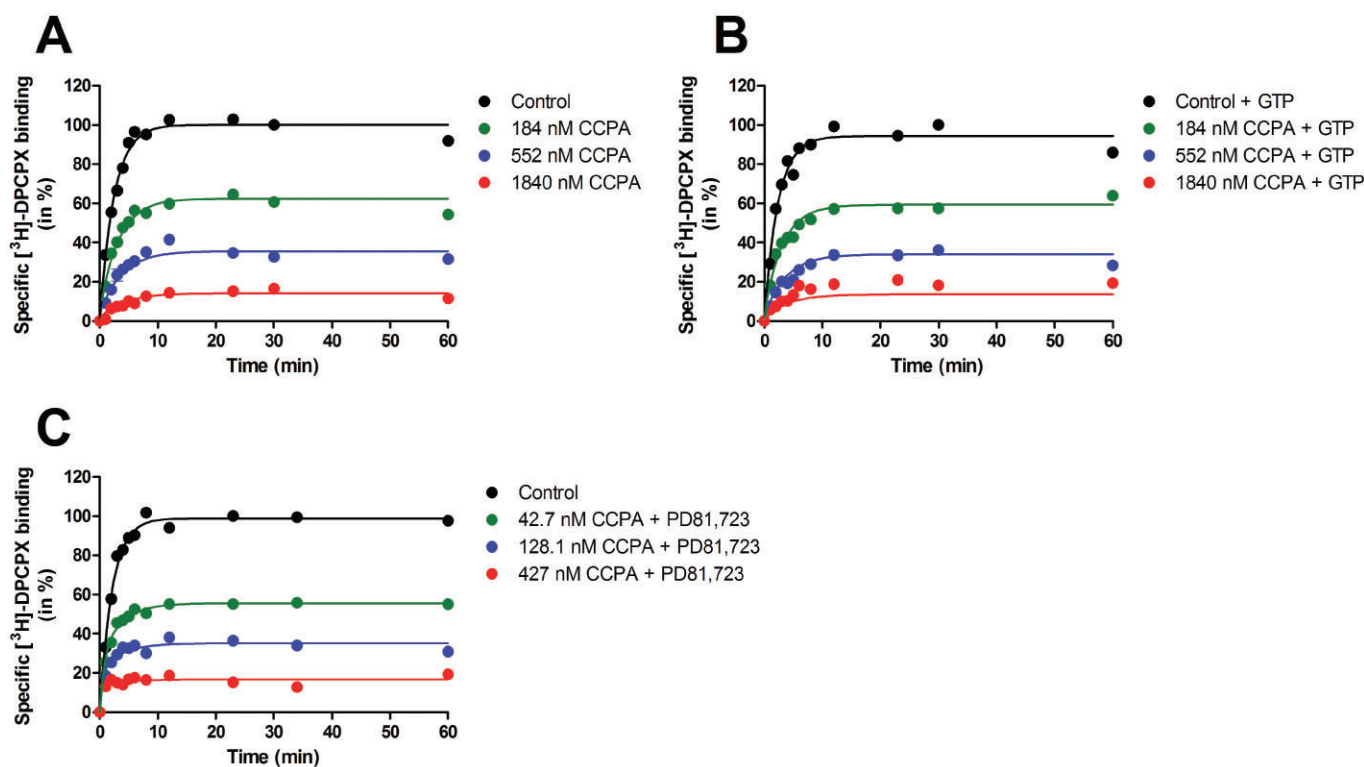


Figure 4

[^3H]-DPCPX competition association assay in the absence or presence of three different concentrations of unlabelled CCPA. (A) Control experiment. (B) Experiment in the presence of 1 mM GTP. (C) Experiment in the presence of 10 μM PD81,723. Representative graphs from one experiment performed in duplicate (see Table 4 for kinetic values).

$\times 10^8 \text{ M}^{-1}\cdot\text{min}^{-1}$) and k_4 ($0.30 \pm 0.07 \text{ min}^{-1}$) values. This held true for the presence of 10 μM BC-1 as well ($k_3 = 2.7 \pm 0.1 \times 10^8 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_4 = 0.072 \pm 0.005 \text{ min}^{-1}$), although the presence of BC-1 affected the on- and off-rates of the radiolabeled probe ($k_1 = 2.8 \pm 0.2 \times 10^8 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_2 = 0.080 \pm 0.004 \text{ min}^{-1}$). These findings convinced us that we could use this assay to measure the binding kinetics of unlabelled orthosteric ligands in the presence of an allosteric modulator.

Next, we determined the on- and off-rates of CCPA, either in the absence or presence of 1 mM GTP (Figure 4A and 4B).

The presence of 1 mM GTP did not significantly change the agonist's association and dissociation rate constants, which were $1.1 \pm 0.2 \times 10^7 \text{ M}^{-1}\cdot\text{min}^{-1}$ and $1.2 \pm 0.3 \text{ min}^{-1}$ (Table 4). Notably, the kinetic K_D values for CCPA ($109 \pm 12 \text{ nM}$ and 169 ± 40 ; Table 4) derived from their respective on- and off-rates were in agreement with CCPA's K_i value obtained from the equilibrium binding experiments in the presence of 1 mM GTP (i.e. $141 \pm 28 \text{ nM}$; Table 1). Thus, we decided not to add GTP in our system for further kinetic profiling of agonists. We also examined the binding kinetics of CCPA by analysing its

Table 4

The binding kinetics of unlabeled CCPA in the absence or presence of 10 μM PD81,723 or 1 mM GTP

Compound	k_{on} (M ⁻¹ ·min ⁻¹) ^a	k_{off} (min ⁻¹) ^a	K_D (nM) ^b
CCPA ^c	1.1 ± 0.2 × 10 ⁷	1.2 ± 0.3	109 ± 12
CCPA (184 nM) ^d	8.2 ± 2.0 × 10 ^{6e}	0.85 ± 0.23 ^e	104 ± 22 ^e
CCPA (552 nM) ^d	9.0 ± 2.5 × 10 ^{6e}	1.1 ± 0.3 ^e	122 ± 27 ^e
CCPA (1840 nM) ^d	6.7 ± 2.7 × 10 ^{6e}	0.91 ± 0.40 ^e	136 ± 47 ^e
CCPA + 1 mM GTP ^c	1.3 ± 0.4 × 10 ^{7f}	2.2 ± 0.6 ^f	169 ± 40 ^f
CCPA + 10 μM PD81,723 ^c	1.1 ± 0.1 × 10 ⁷	0.31 ± 0.04	28 ± 3
CCPA (43 nM) + 10 μM PD81,723 ^d	1.4 ± 0.2 × 10 ^{7g}	0.34 ± 0.06 ^g	24 ± 3 ^g
CCPA (128 nM) + 10 μM PD81,723 ^d	1.0 ± 0.1 × 10 ^{7g}	0.32 ± 0.03 ^g	32 ± 3 ^g
CCPA (427 nM) + 10 μM PD81,723 ^d	0.7 ± 0.1 × 10 ^{7g}	0.24 ± 0.03 ^g	34 ± 4 ^g

Values are means ± SEM of three separate experiments each performed in duplicate. ^a k_{on} (k_3) and k_{off} (k_4) of unlabelled CCPA in the absence or presence of 10 μM PD81,723 or 1 mM GTP were determined in [³H]-DPCPX (2.6 nM) competition association assays. ^bKinetic $K_D = k_{off}/k_{on}$; k_{on} (k_3) and k_{off} (k_4) values of unlabelled CCPA were generated from [³H]-DPCPX (2.6 nM) competition association assay at 25°C. ^cData obtained by globally analysing three concentrations of unlabelled CCPA. ^dData obtained by analysing a single concentration of unlabelled CCPA. ^eNot significantly different from its respective values obtained by globally analysing three concentrations of unlabelled CCPA; $P > 0.05$; Student's *t*-test. ^fNot significantly different from its respective values in the absence of 1 mM GTP; $P > 0.05$; Student's *t*-test. ^gNot significantly different from data obtained by globally analysing three concentrations of unlabelled CCPA in the presence of 10 μM PD81,723; $P > 0.05$; Student's *t*-test.

data at three different concentrations (i.e. 184, 552 or 1840 nM), given that the agonist is known to occupy both high- and low-affinity binding sites. It follows from Table 4 that the on- and off-rates derived by analysing a single concentration were not statistically different from one another or from the values obtained by global, simultaneous analysis of three concentrations of unlabelled CCPA ($P > 0.05$). This held also true when we further included 10 μM PD81,723 (Table 4; Figure 4C). Thus, a single agonist concentration was used in the following experiments.

Quantification of the binding kinetics of unlabelled agonists on the A₁ receptor in the absence or presence of an allosteric modulator by using the competition association assay

We examined the binding kinetics of three A₁ receptor agonists (CCPA, NECA and LUF5834) in the absence or presence of two allosteric modulators (PD81,723 and BC-1). Several observations were made. Firstly, both allosteric modulators affected the affinity of the orthosteric ligands, by influencing their binding kinetics (Table 5; Figure 5). Taking CCPA as an example, its dissociation rate decreased almost fourfold in the presence of 10 μM PD81,723 ($k_{off} = 0.32 \pm 0.03 \text{ min}^{-1}$), whereas its association rate was not much affected. As a result, the affinity ($K_D = 131 \pm 21 \text{ nM}$) of CCPA was increased over fourfold in the presence of PD81,723 (32 ± 3 nM). In comparison, 10 μM BC-1 lowered CCPA's off-rate by 100-fold ($k_{off} = 0.011 \pm 0.03 \text{ min}^{-1}$) and raised its on-rate by 3.5-fold ($k_{on} = 3.5 \pm 0.2 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$). Overall, this resulted in a strongly increased A₁ receptor affinity of $0.31 \pm 0.05 \text{ nM}$. This finding correlated to our above-mentioned statement that BC-1 is a more potent A₁ receptor allosteric modulator than PD81,723, yet with more detailed kinetic information. Secondly, the allosteric effect was concentration dependent. It follows from

Table 5 and Figure 5 that BC-1 slowed down the dissociation process of CCPA by 19- to 105-fold at low concentrations (i.e. 1 μM or 10 μM), whereas at a relatively high concentration (i.e., 33 μM), it further decreased CCPA's off-rate by another twofold. A similar trend was observed for PD81,723, although the effect was smaller in comparison to that of BC-1. Thirdly, A₁ receptor allosteric modulation was 'probe-dependent', that is, the on- and off-rates of different orthosteric ligands were influenced to varying degrees in the presence of the same allosteric modulator. For instance, in the presence of 10 μM PD81,723, the dissociation rates of ribose-containing agonists (CCPA and NECA) were decreased by approximately four- to fivefold, whereas the dissociation rate of the non-ribose agonist (LUF5834) was not significantly affected ($0.92 \pm 0.09 \text{ min}^{-1}$ vs. $0.78 \pm 0.08 \text{ min}^{-1}$, $P = 0.31$). Similarly, in the presence of the more potent allosteric modulator BC-1, all three orthosteric ligands showed varying changes in their dissociation rates. A significant difference was observed for the residence times of LUF5834 and CCPA, that is, 34- or 200-fold increased to 29 ± 3 and $172 \pm 50 \text{ min}^{-1}$ respectively. Furthermore, it is interesting to note that an opposite effect on the non-ribose agonist's (LUF5834) association process was observed compared with the ribose-containing ligands in the presence of 10 μM BC-1. CCPA and NECA had a 3.6- and 1.8-fold increase in their on-rates, respectively, whereas for LUF5834, a five-fold decreased k_{on} value was observed ($4.2 \pm 0.4 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$).

Quantification of the binding kinetics of bitopic ligands for the A₁ receptor in the absence or presence of an allosteric modulator by using the competition association assay

The binding kinetics of three A₁ receptor bitopic ligands, synthesized in-house, were investigated in the competition

Table 5

List of binding kinetic variables (association rate, dissociation rate, residence time (RT) and kinetic K_D) for CCPA, NECA and LUF5834 in the absence or presence of an allosteric modulator (PD81,723 or BC-1) at CHO-hA₁R membranes, derived from competition association assays

Cmpd	k_{on} ($M^{-1}\cdot min^{-1}$) ^a	k_{off} (min^{-1}) ^a	RT (min) ^b	K_D (nM) ^c
CCPA	$9.6 \pm 1.8 \times 10^6$	1.2 ± 0.2	0.86 ± 0.17	131 ± 21
+1 μM PD81,723	$1.9 \pm 0.5 \times 10^7$	0.77 ± 0.03	1.3 ± 0.1	$43 \pm 7^*$
+10 μM PD81,723	$1.0 \pm 0.1 \times 10^7$	$0.32 \pm 0.03^*$	$3.1 \pm 0.3^*$	$32 \pm 3^{**}$
+33 μM PD81,723	$3.4 \pm 0.8 \times 10^7^*$	$0.20 \pm 0.02^{**}$	$5.0 \pm 0.4^{***}$	$5.9 \pm 1.9^{**}$
+1 μM BC-1	$1.9 \pm 0.1 \times 10^7^*$	$0.063 \pm 0.005^{**}$	$16 \pm 1^{***}$	$3.3 \pm 0.2^{**}$
+10 μM BC-1	$3.5 \pm 0.2 \times 10^7^{***}$	$0.011 \pm 0.003^{**}$	$91 \pm 14^{**}$	$0.31 \pm 0.05^{**}$
+33 μM BC-1	$3.5 \pm 0.3 \times 10^7^{**}$	$0.0058 \pm 0.0029^{**}$	$172 \pm 50^*$	$0.17 \pm 0.05^{**}$
NECA	$0.9 \pm 0.4 \times 10^6$	0.47 ± 0.09	2.1 ± 0.5	522 ± 146
+10 μM PD81,723	$3.0 \pm 0.3 \times 10^6^*$	$0.21 \pm 0.02^*$	$4.8 \pm 0.3^{**}$	$70 \pm 6^*$
+10 μM BC-1	$1.6 \pm 0.2 \times 10^6$	$0.025 \pm 0.005^{**}$	$40 \pm 5^{**}$	$16 \pm 2^*$
LUF5834	$2.0 \pm 0.2 \times 10^8$	0.92 ± 0.09	1.1 ± 0.1	4.6 ± 0.4
+10 μM PD81,723	$2.6 \pm 0.2 \times 10^8$	0.78 ± 0.08	1.3 ± 0.1	3.0 ± 0.2
+10 μM BC-1	$4.2 \pm 0.4 \times 10^7^{**}$	$0.034 \pm 0.007^{***}$	$29 \pm 3^{***}$	$0.81 \pm 0.11^{***}$

Data are shown as mean \pm SEM of three separate experiments each performed in duplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the values in the absence of an allosteric modulator; Student's *t*-test. ^a k_{on} (k_3) and k_{off} (k_4) of unlabelled A₁ receptor agonists in the absence or presence of an allosteric modulator (PD81,723 or BC-1) were determined in [³H]-DPCPX (2.6 nM) competition association assays. ^bRT (residence time) = $1/k_{off}$. ^cKinetic $K_D = k_{off}/k_{on}$; k_{on} (k_3) and k_{off} (k_4) values of unlabeled antagonists were generated from [³H]-DPCPX (2.6 nM) competition association assays at 25°C.

association assay. These three ligands, LUF6232, LUF6234 and LUF6258, with four-, five- and nine-carbon spacer length, respectively (Figure 1), were previously designed and synthesized as tools for understanding A₁ receptor allosterism (Narlawar *et al.*, 2010). Here, we further examined the binding kinetics of the compounds and compared their values to the orthosteric ligand LUF5519 in combination with PD81,723 (each representing the orthosteric and allosteric pharmacophore of the bitopic ligands, respectively; see Figure 1 for chemical structures). In the absence of an allosteric modulator, LUF5519 displayed an on-rate of $2.0 \pm 0.4 \times 10^6 M^{-1}\cdot min^{-1}$ and an off-rate of $1.19 \pm 0.19 min^{-1}$, which led to an affinity of $595 \pm 92 nM$ (Figure 6A; Table 7). Upon the addition of 10 μM PD81,723, the association rate of LUF5519 ($k_{on} = 2.4 \pm 1.4 \times 10^6 M^{-1}\cdot min^{-1}$) did not significantly change, whereas the dissociation rate decreased by approximately threefold from 1.19 ± 0.19 to $0.38 \pm 0.05 min^{-1}$ (Figure 6A; Table 7). Further raising the concentration of PD81,723 to 33 μM increased LUF5519's on-rate to $9.1 \pm 1.1 \times 10^6 M^{-1}\cdot min^{-1}$, while a smaller effect on its off-rate was observed ($0.46 \pm 0.07 min^{-1}$). Taken together, this resulted in an enhanced kinetic K_D value of $51 \pm 16 nM$. However, after covalently linking the allosteric and orthosteric pharmacophores, all three bitopic ligands demonstrated a significantly decreased association rate of over 33-fold in comparison to LUF5519 (LUF6232, $6.0 \pm 0.3 \times 10^4 M^{-1}\cdot min^{-1}$, LUF6234, $5.3 \pm 0.7 \times 10^4 M^{-1}\cdot min^{-1}$ and LUF6258, $0.8 \pm 0.3 \times 10^4 M^{-1}\cdot min^{-1}$), indicative of an extra hurdle for the receptor to accommodate large flexible molecules. With respect to their off-rates, all three compounds displayed significantly longer receptor RTs

(LUF6232, RT = $4.5 \pm 1.3 min$, LUF6234, RT = $19 \pm 3 min$ and LUF6258, RT = $48 \pm 11 min$) than LUF5519 (RT = $0.8 \pm 0.1 min$, $P < 0.05$). Interestingly, the presence of 'external' 10 μM PD81,723 significantly increased the association rates of these bitopic ligands (LUF6232, $k_{on} = 2.8 \pm 0.5 \times 10^6 M^{-1}\cdot min^{-1}$; LUF6234, $k_{on} = 3.1 \pm 0.1 \times 10^6 M^{-1}\cdot min^{-1}$; LUF6258, $k_{on} = 3.0 \pm 1.4 \times 10^4 M^{-1}\cdot min^{-1}$), although LUF6258 was less affected than its four- and five-carbon linker analogues. Such effects were more pronounced when 33 μM PD81,723 was added. As for the dissociation rate, the bitopic ligands demonstrated different profiles upon the addition of 10 μM PD81,723: LUF6234's RT was significantly reduced to $2.5 \pm 0.3 min$, whereas the RTs of LUF6232 and LUF6258 were not affected (5.3 ± 0.6 and $48 \pm 15 min$), even after further raising the concentration of PD81,723 to 33 μM (Table 7). In addition, we examined the binding kinetics of a newly synthesized monovalent ligand LUF7161 (Figure 1), which served as a control, as it contains the orthosteric part and the five-atom linker. It follows from Table 7 that LUF7161 had a similar dissociation rate ($k_{off} = 1.21 \pm 0.32 min^{-1}$) as LUF5519, yet its association rate to the A₁ receptor was approximately 10-fold lower ($k_{on} = 2.7 \pm 0.7 \times 10^5 M^{-1}\cdot min^{-1}$) than LUF5519. Upon the addition of 10 and 33 μM PD81,723, the association rate of LUF7161 was 14- and 17-fold higher, respectively, whereas its dissociation rates were less affected in comparison to its kinetics in the absence of the allosteric modulator. The other monovalent ligand with nine-atom linker (LUF7160) did not display significant radioligand displacement up to concentrations of 33 μM (data not shown); thus, its binding kinetics were not determined.

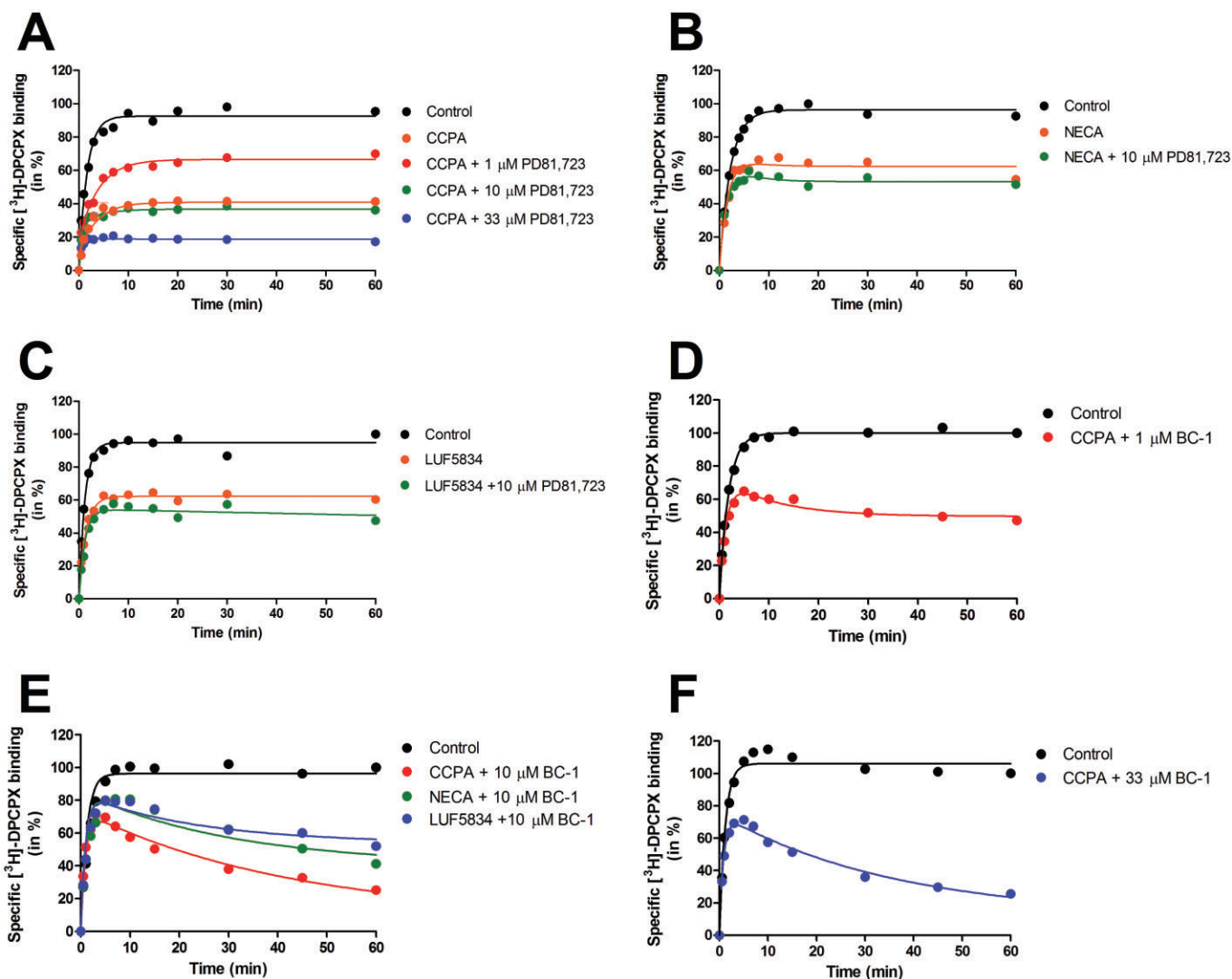


Figure 5

(A) [³H]-DPCPX competition association assay in the absence or presence of unlabelled CCPA with or without different concentrations of PD81,723. (B) [³H]-DPCPX competition association assay in the absence or presence of unlabelled NECA with or without 10 μM PD81,723. (C) [³H]-DPCPX competition association assay in the absence or presence of unlabelled LUF5834 with or without 10 μM PD81,723. (D) [³H]-DPCPX competition association assay in the absence or presence of unlabelled CCPA with or without 1 μM BC-1. (E) [³H]-DPCPX competition association assay in the absence or presence of unlabelled CCPA, NECA or LUF5834 with or without 10 μM BC-1. (F) [³H]-DPCPX competition association assay in the absence or presence of unlabelled CCPA with or without 33 μM BC-1. Data were fitted to Equation 2 described in the Methods section to calculate the k_{on} (k_3) and k_{off} (k_4) values of unlabelled ligands by using the respective k_1 and k_2 values of [³H]-DPCPX under different conditions. Representative graphs from one experiment performed in duplicate (see Table 5 for kinetic values).

Data simulations and assay validation for determining the bitopic ligands' binding kinetics in the competition association assay

Simulated data were collected by consecutively solving the differential equations in the Methods section (Equations 5–12), which mimic the binding of a radioligand in the absence or presence of a bitopic ligand (Supporting Information Tables S1–S4). Next, the binding kinetics of the bitopic ligand were analysed using two methods: (i) by subjecting the defined microscopic kinetic constants (Table 4, 'input parameters') into Equations 13 and 14 to obtain the theoretical macroscopic binding parameters (Table 4, 'calculated param-

eters') and (ii) by subjecting the simulated data into the model of competition association assay to obtain the 'collected parameters' in Table 6 (Supporting Information Fig. S1). In general, the binding kinetics analysed by using both methods correlate well with each other. The association rates of the bitopic ligands from the competition association assay are similar to the theoretical values in all four examples. For instance, the collected k_{on} value in Example 1 is $2.3 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$, which correlates well with its corresponding theoretical k_{on} value of $2.0 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$. Likewise, the dissociation rate can be reliably determined by using the competition association assay. All collected/experimental k_{off}

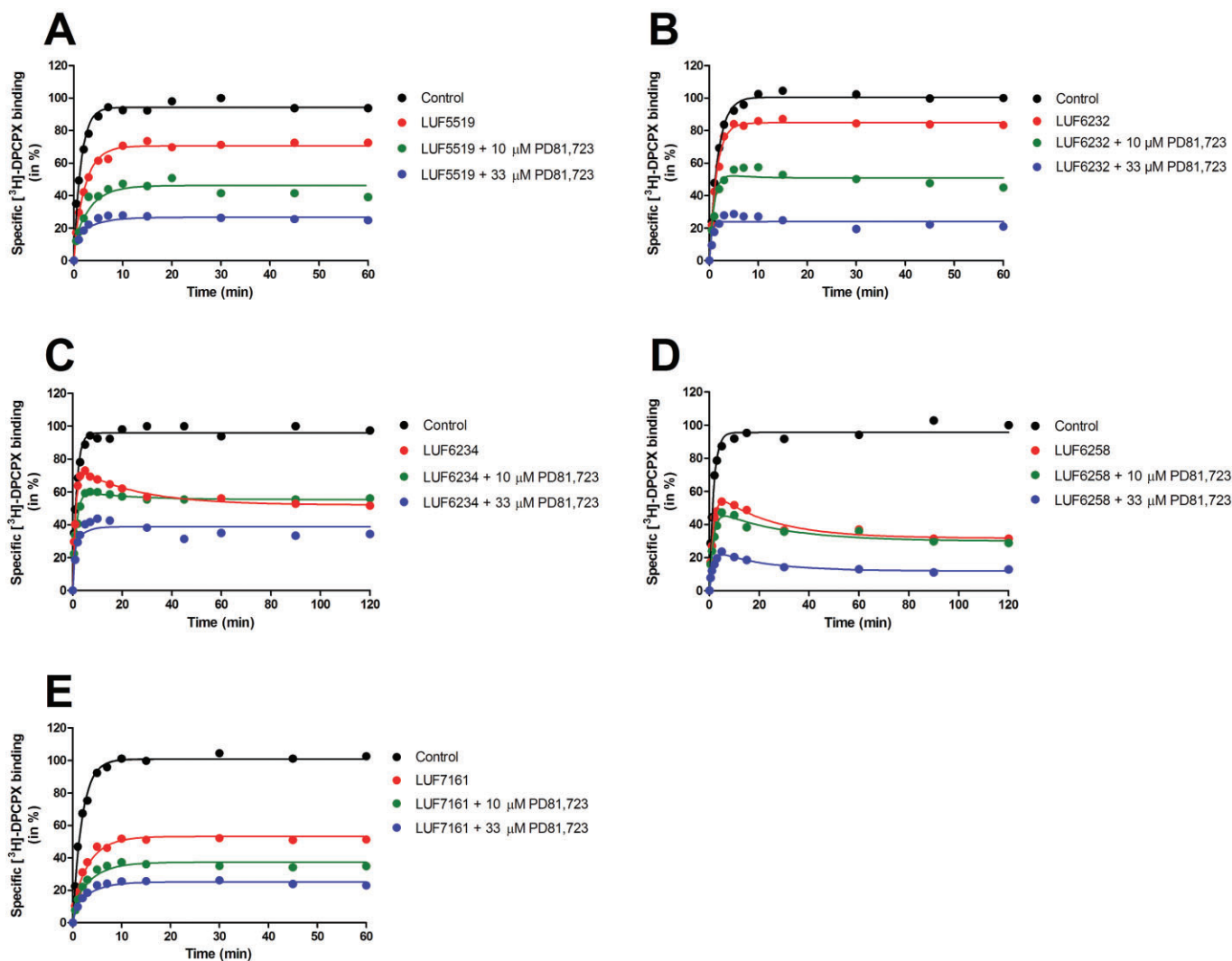


Figure 6

(A) [^3H]-DPCPX competition association assay in the absence or presence of unlabelled LUF5519 with or without 10 or 33 μM PD81,723. (B) [^3H]-DPCPX competition association assay in the absence or presence of unlabelled LUF6232 with or without 10 or 33 μM PD81,723. (C) [^3H]-DPCPX competition association assay in the absence or presence of unlabelled LUF6234 with or without 10 or 33 μM PD81,723. (D) [^3H]-DPCPX competition association assay in the absence or presence of unlabelled LUF6258 with or without 10 or 33 μM PD81,723. (E) [^3H]-DPCPX competition association assay in the absence or presence of unlabelled LUF7161 with or without 10 or 33 μM PD81,723. Data were fitted to the equations described in the methods to calculate the k_{on} (k_3) and k_{off} (k_4) values of unlabelled ligands by using the k_1 and k_2 values of [^3H]-DPCPX. Representative graphs from one experiment performed in duplicate (see Table 6 for kinetic values).

values turned out to be within twofold difference from the corresponding calculated values. Thus, the competition association assay enables the determination of the binding kinetics of bitopic ligands.

Discussion

In the present study, we examined the allosteric modulation of the adenosine A_1 receptor from a kinetic perspective, focusing on the orthosteric ligand. It follows from Table 2 that the competition association assay (Motulsky and Mahan, 1984) can be applied to determine the binding kinetics of unlabelled orthosteric ligands in the presence of an allosteric

modulator that could either modulate the binding of the radioligand (e.g. BC-1) or not (e.g. PD81,723). This confirms the general applicability of the assay to study GPCR allostereism. Two additional validation experiments were performed though to further prove the general applicability, especially in the case of examining orthosteric agonists, while using an antagonistic radioligand. Firstly, it was necessary to examine the effect of different agonist's concentrations on its occupancy of the two different receptor states (coupled to or uncoupled from the G protein) and the corresponding binding kinetics. Hence, we examined the GTP effect on the equilibrium affinity and binding kinetics of CCPA and NECA, while we used a radiolabelled antagonist, [^3H]-DPCPX. We observed that the kinetics of CCPA was insensitive to the

Table 6

Binding of the monovalent ligand 'c' ($k_{+c} = 1 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$, $k_{-c} = 0.3 \text{ min}^{-1}$ and $[c] = 100 \text{ nM}$) was simulated during a time lapse of 50 min in the absence or presence of different concentrations of the bitopic ligands 'ab' ($k_{+a} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$, $k_{-a} = 1 \text{ min}^{-1}$, $k_{+b} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$, $k_{-b} = 1 \text{ min}^{-1}$ and $[L] = 0.29 \text{ mM}$) with different cooperativity factors

Example	Cooperativity factor	Calculated parameters ^a		Collected parameters ^b	
		$k_{\text{on}} (\text{M}^{-1} \cdot \text{min}^{-1})$	$k_{\text{off}} (\text{min}^{-1})$	$k_{\text{on}} (\text{M}^{-1} \cdot \text{min}^{-1})$	$k_{\text{off}} (\text{min}^{-1})$
1	$\alpha_{ab} = 10$	2.0×10^5	0.0069	2.3×10^5	0.0046
2	$\alpha_{ab} = 0.1$	1.6×10^5	0.41	1.1×10^5	0.72
3	$\alpha'_{ab} = 10$	1.9×10^5	0.0066	2.1×10^5	0.0039
4	$\alpha'_{ab} = 0.1$	2.0×10^5	0.50	1.3×10^5	0.90

The 'macroscopic' kinetic binding parameters of the bitopic ligands were calculated using Equations 13 and 14 or by subjecting the simulated data (Supporting Information Tables S1–S4) into the competition association assay based on the theoretical framework by Motulsky and Mahan (1984). ^aThe 'macroscopic' kinetic binding parameters of the bitopic ligands were calculated using Equations 13 and 14. ^b k_{on} and k_{off} values were determined by analysing the simulated data (Supporting Information Tables S1–S4) in the competition association model 'kinetics of competitive binding' (Motulsky and Mahan, 1984).

presence of 1 mM GTP (Table 4) in our system and its kinetic K_D values correlated to the equilibrium affinity in the presence of GTP (141 nM). This convinced us that we could continue without applying GTP in our further kinetic assays. Secondly, we analysed the binding kinetics of CCPA at high or low concentrations, as the compound was shown to have two affinity sites in the equilibrium binding assay. One could reason that a different set of kinetic parameters might be obtained at high or low agonist concentrations, as the high and low affinity states will be occupied to different degrees. However, we did not observe significant variations when the agonist was tested at three different, single concentrations or by globally analysing them (Table 4). Of note, similar findings were observed for an agonist at the adenosine A_{2A} receptor (Guo *et al.*, 2012). Taken together, this suggested that the agonist's binding kinetics obtained from the competition association assay using the antagonist [³H]-DPCPX was predominantly from the G protein-uncoupled sites/states, at least on the adenosine receptors.

The competition association assay offers several, not yet fully realised advantages for the investigation of allosteric modulation. Firstly, it enables the measurement of the kinetic characteristics of a probe in an unlabelled form, expanding the availability of orthosteric ligands for probe-dependent investigations. Secondly, the method allows the determination of the complete range of binding kinetics. This includes the association rate, dissociation rate, residence time and the 'kinetic K_D ' for the ligand studied. These abundant details are very useful to interpret the dynamic interactions between the ligand and receptor, especially given that many allosteric modulators have the propensity to alter the dissociation rates of orthosteric ligands. For instance, upon the addition of PD81,723 or BC-1, the receptor-residence times (RT) of CCPA or NECA were prolonged. Such a change can be immediately captured in the competition association assay from the typical 'overshoot' pattern as in Figure 5, indicating a compound's slower dissociation process than the radioligand (Motulsky and Mahan, 1984). PD81,723 and other GPCR allosteric modulators are known to influence a probe's affinity by changing its dissociation rate (Bruns and

Fergus, 1990; Bhattacharya and Linden, 1995; Lazareno and Birdsall, 1995; Gao *et al.*, 2001; Bauer *et al.*, 2012). Their effect on the association rates of orthosteric ligands, however, has been less investigated. Here, by using the competition association assay, we found that an allosteric modulator could also change an orthosteric ligand affinity by influencing its association rate. For NECA, the association rate increased by 3.3-fold upon the addition of 10 μM PD81,723, which, together with the 2.2-fold decrease in the dissociation rate, resulted in an overall 6.9-fold change in affinity. We also reaffirmed that the allosteric effect of both A₁ receptor modulators was concentration-dependent, as evidenced by the concentration-dependent increase in the RT for CCPA (Table 5). Moreover, the kinetics of different orthosteric ligands were not equally altered, which reflects the specific feature of GPCR allosteric modulation, the so-called 'probe-dependency' (Christopoulos, 2002). For orthosteric ligands, such a phenomenon is not only limited to their affinity or potency, the parameters most often investigated for GPCR allosterism, but is also reflected in the less examined dissociation and association rate. As evidence, the association and/or dissociation rates for CCPA and NECA were significantly changed upon the addition of 10 μM PD81,723, but not by LUF5834 (Table 5). Apparently, all these details of ligand–receptor interaction can be simultaneously obtained by using the competition association approach. It should be pointed out that the current assay has limited advantages when it comes to the quantification of parameters for the modulators, for instance, the cooperativity factor, α . To fully characterize the latter, the approach developed by Lazareno and Birdsall (1995) may be more suitable.

In the present study, we furthermore performed a competition association assay with bitopic ligands to study their kinetic behaviour. A bitopic ligand is a useful tool to study the molecular mechanism of allosteric modulation (Lane *et al.*, 2013). However, interpretation of its binding can be very complex. Different bitopic ligands may display three divergent binding modes: the ligand can be bound to the allosteric site only, to the orthosteric site only or to both sites

Table 7

Binding parameters for bitopic ligands and their constituent parts in the absence or presence of PD81,723 at CHO A_1 R membranes, derived from competition association assays

Compound	k_{on} ($M^{-1}\cdot min^{-1}$) ^a	k_{off} (min^{-1}) ^a	RT (min) ^b	K_D (nM) ^c
LUF5519	$2.0 \pm 0.4 \times 10^6$	1.19 ± 0.19	0.8 ± 0.1	595 ± 92
+10 μ M PD81,723	$2.4 \pm 1.4 \times 10^6$	$0.38 \pm 0.05^*$	$2.6 \pm 0.4^*$	$158 \pm 41^*$
+33 μ M PD81,723	$9.1 \pm 1.1 \times 10^{6**}$	$0.46 \pm 0.07^*$	$2.2 \pm 0.2^{**}$	$51 \pm 6^{**}$
LUF6232 (linker $N = 4$)	$6.0 \pm 0.3 \times 10^5$	0.22 ± 0.11	4.5 ± 1.3	367 ± 106
+10 μ M PD81,723	$2.8 \pm 0.5 \times 10^{6**}$	0.19 ± 0.04	5.3 ± 0.6	$68 \pm 11^*$
+33 μ M PD81,723	$1.0 \pm 0.2 \times 10^{7**}$	0.21 ± 0.04	4.8 ± 0.5	$21 \pm 3^*$
LUF6234 (linker $N = 5$)	$5.3 \pm 0.7 \times 10^4$	0.052 ± 0.007	19 ± 3	981 ± 65
+10 μ M PD81,723	$3.1 \pm 0.1 \times 10^{6***}$	$0.40 \pm 0.05^{**}$	$2.5 \pm 0.3^{**}$	$129 \pm 13^{***}$
+33 μ M PD81,723	$7.8 \pm 0.9 \times 10^{6***}$	$0.35 \pm 0.05^{**}$	$2.9 \pm 0.2^{**}$	$45 \pm 5^{***}$
LUF6258 (linker $N = 9$)	$0.8 \pm 0.3 \times 10^4$	0.021 ± 0.008	48 ± 11	2625 ± 810
+10 μ M PD81,723	$3.0 \pm 1.4 \times 10^4$	0.021 ± 0.011	48 ± 15	700 ± 284
+33 μ M PD81,723	$5.8 \pm 3.4 \times 10^4$	0.023 ± 0.014	43 ± 15	397 ± 194
LUF7161	$2.7 \pm 0.7 \times 10^5$	1.21 ± 0.32	0.8 ± 0.2	4481 ± 985
+10 μ M PD81,723	$3.8 \pm 0.9 \times 10^{6*}$	0.87 ± 0.21	1.1 ± 0.1	$229 \pm 45^*$
+33 μ M PD81,723	$4.7 \pm 0.9 \times 10^{6**}$	0.74 ± 0.15	1.4 ± 0.2	$157 \pm 25^*$

Data are shown as mean \pm SEM of three separate experiments each performed in duplicate. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the values in the absence of an allosteric modulator; Student's *t*-test. ^a k_{on} (k_3) and k_{off} (k_4) of unlabelled A_1 receptor agonists in the absence or presence of an allosteric modulator (PD81,723 or BC-1) were determined in [³H]-DPCPX (2.6 nM) competition association assays. ^bRT (residence time) = $1/k_{off}$. ^cKinetic $K_D = k_{off}/k_{on}$; k_{on} (k_3) and k_{off} (k_4) values of unlabelled antagonists were generated from [³H]-DPCPX (2.6 nM) competition association assays at 25°C.

simultaneously if the length of the linker is optimal. Moreover, one receptor can be occupied by two bitopic ligands, that is, one to the allosteric site and the other to the orthosteric site (Steinfeld *et al.*, 2007; Narlawar *et al.*, 2010; Lane *et al.*, 2013). A kinetic investigation of a bitopic ligand could add information to an affinity- or potency-based evaluation. Therefore, we selected three previously reported bitopic ligands (Narlawar *et al.*, 2010), namely LUF6232, LUF6234 and LUF6258, and examined their binding kinetics in the present study. We found that the theoretically calculated macroscopic kinetics correlated well with the values that were determined by analysing the simulated data in the competition association assay (Table 6). Additionally, our kinetic K_D values were generally in the same range as the K_i values reported by Narlawar *et al.* (2010), although their K_i of LUF6234 was slightly higher than we determined. Thus, the competition association assay enables sufficient quantification of the net changes of the macroscopic binding kinetics of bitopic ligands. Next, we observed that, because of the covalent link between the orthosteric and allosteric pharmacophores, the dissociation process of LUF6234 and LUF6258 was much slower than their orthosteric constituent, LUF5519, in the presence of PD81,723 (Table 7). Such phenomenon can be explained by the fact that a freshly dissociated pharmacophore is obliged to remain in 'forced proximity' to its cognate binding site as long as the tethered, companion pharmacophore is still bound. This favours its rebinding to that site, thereby significantly slowing down the net dissociation process (Vauquelin, 2013; Vauquelin and Charlton, 2013). To

our knowledge, our present observation of the gained target RT of the bitopic ligand provides the first experimental evidence for such rebinding mechanism of low MW ligands. One could reason that such slow dissociation rates of LUF6234 and LUF6258 could occur via another mechanism where the bitopic ligand is bound to the orthosteric site and the allosteric portion may be close to, but not interacting with the allosteric site. If this would be the case, the bitopic ligand could flip between orthosteric and allosteric binding modes, while remaining in close proximity to or even occluding the orthosteric site and therefore extending its apparent RT. To distinguish between these two mechanisms, we also included the binding kinetics of another bitopic ligand, LUF6232, which has a four-carbon linker, that is, shorter than LUF6234. In comparison, the RT of LUF6232 was increased to a lesser extent than its analogues with a five- or nine-carbon linker. Specifically, its value (4.5 ± 1.3 min) was more close to the RT of the monovalent ligand LUF5519 or LUF7161 in the presence of 10 or 33 μ M PD81,723. This result further confirmed that both the allosteric and orthosteric sites need to be occupied by, rather than being close to, the corresponding portion of the bitopic ligand to exert the synergistic effect of long receptor occupancy, and excluded the mechanism of increased RT by a bitopic ligand through 'flipping' between the two binding modes.

To further clarify mode of binding of bitopic ligands to the A_1 receptor, we examined the effect of the addition of 'external' PD81,723 in studies with the bitopic ligand. In this way, the monovalent modulator should be able to occupy the

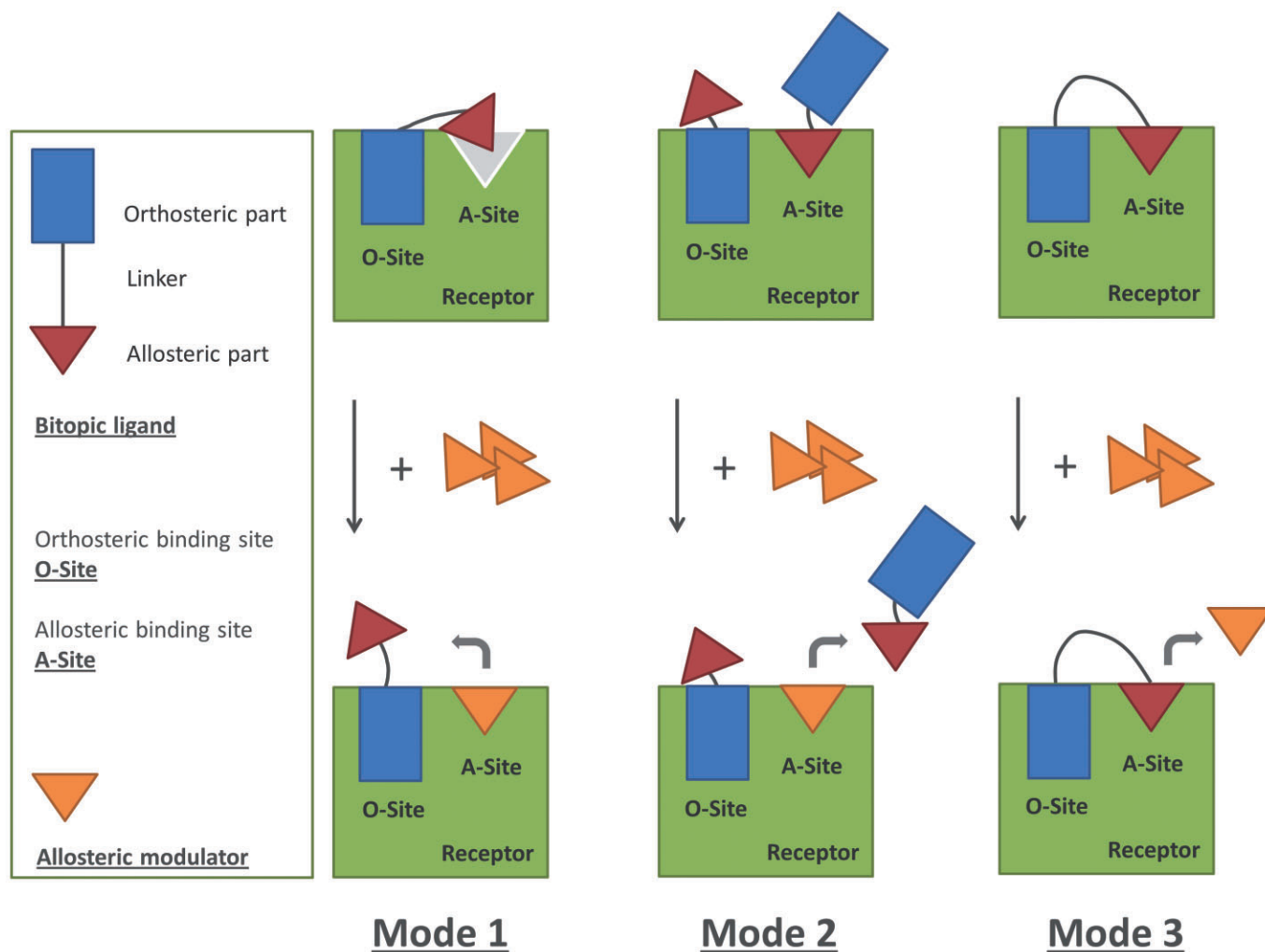


Figure 7

Proposed diagram of three distinct binding modes for the bitopic ligands in the absence or presence of further added allosteric modulator. Mode 1: the linker length of a bitopic ligand is sufficient to occupy both sites on the receptor, yet in a non-optimal manner. Mode 2: the linker length of a bitopic ligand is not sufficient to occupy both sites on the receptor. Two ligands could possibly bind to one receptor with either the orthosteric part to the 'O-site' or with the allosteric part to the 'A-site' on the receptor. Mode 3: the linker length of a bitopic is optimal to occupy both orthosteric and allosteric sites of the receptor. Upon the addition of excess allosteric modulator, the allosteric modulator can prevent the rebinding of the freshly dissociated allosteric pharmacophore in Mode 1 due to the bitopic ligand's less optimal binding pose or occupy the 'A-site' on the receptor and thus 'displace' the bitopic ligand binding to the receptor via its allosteric pharmacophore in Mode 2. In contrast, the binding of the bitopic ligand with optimal linker length (Mode 3) is less affected in the presence of the allosteric modulator.

allosteric site on the receptor preventing the rebinding of the freshly dissociated allosteric pharmacophore. In this sense, both association and dissociation of LUF6234 became faster compared with its kinetics in the absence of PD81,723, indicative of competition between the allosteric part of LUF6234 and the 'free' allosteric modulator at the A₁ receptor allosteric binding site. Such binding mechanism can be illustrated in Mode 1 (Figure 7). On the contrary, the RT of a ligand that cannot simultaneously occupy both the allosteric and orthosteric binding site should be less affected upon the addition of the external monovalent modulator, like LUF6232 (Mode 2, Figure 7). Indeed, the dissociation rate of LUF6232 was unaffected upon the addition of 10 or 33 μM PD81,723. Interestingly, the dissociation rate of LUF6258 was

also unaffected upon the addition of 10 or 33 μM PD81,723. Based upon this result alone, one could hypothesize that LUF6258's binding mode may fall into the same category as its four-carbon linker analogue, LUF6232 (i.e. Mode 2). However, this mode fails to explain LUF6258's long RT. Therefore, we reasoned that this unique phenomenon of LUF6258 binding was most likely due to the flexibility of its linker: it has more freedom of rotation and thus increased the likelihood that both pharmacophores could interact tightly with the receptor (Figure 7, Mode 3). In contrast, the shorter five-carbon linker might be able to bridge both allosteric and orthosteric sites simultaneously, yet in a non-optimal manner due to its short length and thus higher energy constraint (Figure 7, Mode 1).

Notably, our proposed binding modes of LUF6234 and LUF6258 expand on the conclusions previously drawn by Narlawar *et al.* (2010), where the authors suggested that the bitopic ligand with linker length of five (LUF6234) failed to completely occupy both allosteric site and orthosteric site, whereas the one with linker length of nine (LUF6258) was able to occupy both sites. This conclusion was based upon the hypothesis that if the linker is of sufficient length to allow simultaneous occupancy of the allosteric and orthosteric binding sites, the addition of further PD81,723 will have a minimal effect on the potency of the bitopic ligand, while for the bitopic ligand with insufficient linker length affinity will be significantly increased (Narlawar *et al.*, 2010). Following this hypothesis, we would have reached the same conclusion if merely affinity was taken into consideration, as we also observed that LUF6234 displayed a strongly increased kinetic K_D upon the addition of 10 μ M PD81,723 (7.6-fold), while less so for LUF6258 (3.8-fold). However, it is also noteworthy from our study that both LUF6234 and LUF6258 displayed significantly increased receptor RTs compared to that of the monovalent ligand, LUF5519 or LUF7161, in the presence of PD81,723 (Table 7). Such a result strongly indicates that both allosteric and orthosteric sites on the A_1 receptor were simultaneously occupied for both compounds, which appeared to induce a synergistic effect – greater than simply combining two monovalent ligands – on the global receptor conformation. If LUF6234's linker length had been insufficient to occupy both sites, it should have had a RT comparable to the monovalent LUF7161 or the bitopic ligand LUF6232 that has a shorter linker, which, in fact, is not the case for this bitopic ligand. Apparently, investigation of binding kinetics adds significantly to understanding the molecular mechanism of the binding of a bitopic ligand.

In summary, we have validated and shown the use of a competition association assay at the human adenosine A_1 receptor to determine the allosteric modulation of the binding kinetics of different A_1 receptor agonists. Our data suggest that the influence of an A_1 receptor allosteric enhancer (positive allosteric modulator) on the binding kinetics of the orthosteric agonist is dependent upon the concentration of the allosteric modulator and the nature of the orthosteric ligand and that both the association and dissociation rates can be affected. Furthermore, we examined the binding kinetics of two bitopic ligands and investigated their binding mode. The relevance of the competition association assay for the binding kinetics of bitopic ligands was also explored by analysing a series of simulations and comparing the result to the theoretically determined kinetics. Taken together, the competition association assay enables accurate determination of the binding kinetics of an orthosteric ligand, whether radiolabelled or not, in the absence or presence of an allosteric modulator. We believe that this approach will have general applicability for the study of allosteric modulation at other GPCRs as well.

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Author contributions

D. G. designed and conducted the experiments, analysed the data and wrote the manuscript. S. N. V. conducted the experiments. A. M. conducted the experiments and analysed the data. J. P. D. V. synthesized the compounds and contributed to the writing of the manuscript. G. V. conducted the simulations, analysed the data and wrote the manuscript. A. P. I. designed the experiments, assessed the results and wrote the manuscript. L. H. H. designed the experiments, assessed the results and wrote the manuscript.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.12836>

Figure S1 Competition association assay of a radioligand, 'c' (with $k_{on} = 1 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$, $k_{off} = 0.3 \text{ min}^{-1}$ and $[c] = 100 \text{ nM}$), during a period of 50 min, either alone or in the presence of

increasing concentrations of 'ab' ($k_{+a} = 1 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-a} = 1 \text{ min}^{-1}$, $k_{+b} = 1 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-b} = 1 \text{ min}^{-1}$ and $[L] = 0.29 \text{ mM}$) with different cooperativity factors: (1) $\alpha_{ab} = 10$; (2) $\alpha_{ab} = 0.1$; (3) $\alpha'_{ab} = 10$; (4) $\alpha'_{ab} = 0.1$. Data input into this analysis were from Table S1 to S4 respectively.

Table S1 Data simulation of the binding of radioligand, 'c', during a period of 50 min, either alone or in the presence of increasing concentrations of the bitopic ligand, 'ab'. The microscopic binding kinetics of the bitopic ligand 'ab' was defined as $k_{+a} = 1 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-a} = 1 \text{ min}^{-1}$, $k_{+b} = 1 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-b} = 1 \text{ min}^{-1}$ and $[L] = 0.29 \text{ mM}$. The binding kinetics of the monovalent ligand 'c' was defined as $k_{+c} = 1 \times 10^7 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-c} = 0.3 \text{ min}^{-1}$ and $[c] = 100 \text{ nM}$. The cooperativity factors of 'ab' were set as $\alpha_{ab} = 10$.

Table S2 Data simulation of the binding of radioligand, 'c', during a period of 50 min, either alone or in the presence of increasing concentrations of the bitopic ligand, 'ab'. The microscopic binding kinetics of the bitopic ligand 'ab' was defined as $k_{+a} = 1 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-a} = 1 \text{ min}^{-1}$, $k_{+b} = 1 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-b} = 1 \text{ min}^{-1}$ and $[L] = 0.29 \text{ mM}$. The binding kinetics of the monovalent ligand 'c' was defined as $k_{+c} = 1 \times$

$10^7 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-c} = 0.3 \text{ min}^{-1}$ and $[c] = 100 \text{ nM}$. The cooperativity factors of 'ab' were set as $\alpha_{ab} = 0.1$.

Table S3 Data simulation of the binding of radioligand, 'c', during a period of 50 min, either alone or in the presence of increasing concentrations of the bitopic ligand, 'ab'. The microscopic binding kinetics of the bitopic ligand 'ab' was defined as $k_{+a} = 1 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-a} = 1 \text{ min}^{-1}$, $k_{+b} = 1 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-b} = 1 \text{ min}^{-1}$ and $[L] = 0.29 \text{ mM}$. The binding kinetics of the monovalent ligand 'c' was defined as $k_{+c} = 1 \times 10^7 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-c} = 0.3 \text{ min}^{-1}$ and $[c] = 100 \text{ nM}$. The cooperativity factors of 'ab' were set as $\alpha'_{ab} = 10$.

Table S4 Data simulation of the binding of radioligand, 'c', during a period of 50 min, either alone or in the presence of increasing concentrations of the bitopic ligand, 'ab'. The microscopic binding kinetics of the bitopic ligand 'ab' was defined as $k_{+a} = 1 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-a} = 1 \text{ min}^{-1}$, $k_{+b} = 1 \times 10^5 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-b} = 1 \text{ min}^{-1}$ and $[L] = 0.29 \text{ mM}$. The binding kinetics of the monovalent ligand 'c' was defined as $k_{+c} = 1 \times 10^7 \text{ M}^{-1}\cdot\text{min}^{-1}$, $k_{-c} = 0.3 \text{ min}^{-1}$ and $[c] = 100 \text{ nM}$. The cooperativity factors of 'ab' were set as $\alpha'_{ab} = 0.1$.