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Drug-target residence time : a case for the adenosine A1 and A2A receptors

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Chapter 6

*Molecular basis of allosteric modulation
Insight from a binding kinetics study at
the human adenosine A₁ receptor*

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About this chapter

Many G protein-coupled receptors (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-) labeling of the orthosteric ligand, which is impractical for studying a range of ligands. In this chapter we describe the application of a competition association assay at the adenosine A_1 receptor (A_1R) for this purpose. We used the competition association assay to examine the binding kinetics of several unlabeled A_1R orthosteric agonists in the absence or presence of two allosteric modulators. We also tested two bitopic ligands, in which an orthosteric and an allosteric pharmacophore were covalently linked with different spacer lengths in between. The relevance of the competition association assay for the bitopic ligands' binding kinetics was also explored by analyzing simulated data. The binding kinetics of an unlabeled 'cold' orthosteric ligand was influenced upon the addition of an allosteric modulator and such an effect was 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. The competition association assay is a useful tool for exploring the allosteric modulation of the human adenosine A_1 receptor. This assay may have general applicability to study allosteric modulation at other GPCRs as well.

6.1 Introduction

Adenosine receptors are a subfamily within the class A of G protein-coupled receptors (GPCRs), which includes four subtypes, A_1 , A_{2A} , A_{2B} and A_3 .¹ These receptors are ubiquitously expressed in many tissues and have been shown to play important roles in numerous physiological processes.² Adenosine receptors can be activated by their endogenous ligand adenosine or by a range of synthetic small molecule agonists that share the same binding site as the natural ligand, which is referred to as the orthosteric site.³ Adenosine receptors also possess an allosteric site, which is a binding pocket topographically distinct from the orthosteric site.⁴ 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.⁵⁻¹⁰ 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.^{11,12} Moreover, the extent of allosteric modulation also depends on the nature of the orthosteric ligand, so-called probe-dependency.^{8,13} These complexities add difficulties in understanding 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 (MOA).^{14,15} 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.¹⁶ However, current kinetic studies of GPCR allosterism mostly rely on the 'probe' dissociation experiment, i.e., determining the change in a radiolabeled orthosteric ligand's dissociation rate.^{12,17,18} This radiolabeling process is both labor 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 hA_1R in our lab,¹⁹ based on the mathematical model described by Motulsky and Mahan.²⁰ In the current study we examined the association and dissociation rates of several A_1R agonists in the absence or presence of different allosteric modulators (Figure

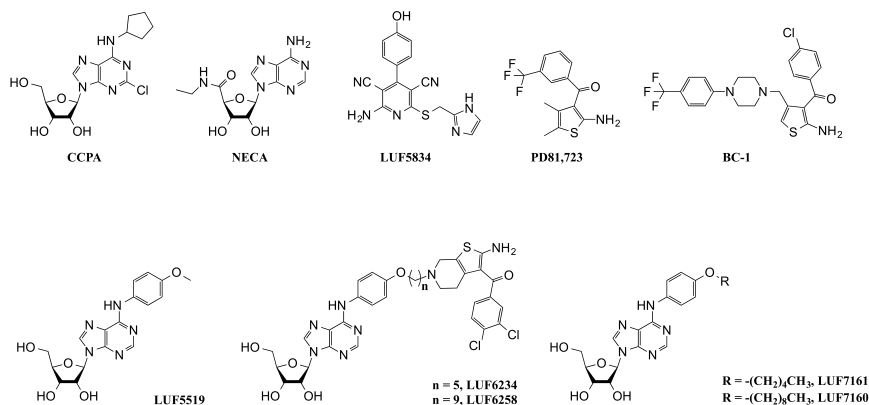


Figure 6.1 | Chemical structures of compounds used in this chapter. CCPA, NECA and LUF5519 are ribose-containing adenosine A₁ receptor agonists, while LUF5834 is a non-ribose agonist;^{21–23} PD81,723 and BC-1 are allosteric enhancers for adenosine A₁ receptor agonists.²⁴ LUF6234 and LUF6258 are in-house synthesized bitopic ligands for the adenosine A₁ receptor with five-carbon linker and nine-carbon linker, respectively.²⁶ LUF7161 and LUF7160 are newly synthesized monovalent ligands with five-atom linker and nine-atom linker, respectively.

6.1). Amongst these compounds, CCPA (2-chloro-N⁶-cyclopentyladenosine) and NECA (N⁵-ethylcarboxamidoadenosine) are ribose-containing A₁R receptor agonists,^{21,22} while LUF5834 (2-amino-4-(4-hydroxyphenyl)-6-(1H-imidazol-2-ylmethyl-sulfanyl)-pyridine-3,5-dicarbonitrile) is a A₁R agonist without the ribose moiety.²³ 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.*²⁴, 2-amino-4-((4-(4-(trifluoromethyl)phenyl)piperazin-1-yl)methyl)thiophen-3-yl)(4-chlorophenyl)methanone] are allosteric enhancers of the A₁R^{24,25}. The binding kinetics of two in-house synthesized ‘bitopic’ ligands, namely LUF6234 and LUF6258 (Figure 6.1),²⁶ were also investigated, as they are useful tools for the exploration of A₁R allosteric modulation.²⁷ These bitopic ligands share the same orthosteric and allosteric pharmacophores, mimicking LUF5519 [A₁R agonist, N⁶-(4-methoxyphenyl)adenosine] and PD81,723 respectively, which are covalently linked via a spacer with different lengths, i.e., five- and nine-carbon atoms, respectively.

6.2 Methods

Chemicals and reagents

[³H]-1,3-dipropyl-8-cyclopentylxanthine ([³H]-DPCPX, specific activity 103 Ci/mmol) was purchased from ARC, Inc. (St. Louis, MO, USA). Unlabeled DPCPX and CCPA were from Sigma (St. Louis, MO, USA). NECA was purchased from Sigma-Aldrich (Steinheim, Germany). *N*⁶-cyclopentyladenosine (CPA) was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). LUF5834, PD81,723 and BC-1 were prepared in-house following synthesis routes as reported previously.²³⁻²⁵ LUF5519, LUF6234 and LUF6258 were also synthesized in-house as described by Narlawar *et al.*²⁶ The synthesis of LUF7160 [*N*⁶-(4-nonyloxyphenyl) adenosine] and LUF7161 [*N*⁶-(4-pentyloxyphenyl)adenosine] was performed following the same method in Narlawar *et al.*²⁶. CHAPS [3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate)] was obtained from Carl Roth GmbH (Karlsruhe, Germany). Chinese hamster ovary (CHO) cells stably expressing the human A₁R (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.

Cell culture and membrane preparation

Cell culture and membrane preparation were performed as reported previously.¹⁹

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 one hour. The

displacement experiments were performed using eleven concentrations of competing ligands with 2.6 nM [^3H]-DPCPX in the absence or presence of 10 μM of PD81,723 or BC-1. For the determination of the allosteric potency of PD81,723 and BC-1, 100 nM (IC_{80} value) CCPA was used in the presence of increasing concentrations of these two allosteric modulators to examine the potentiated [^3H]-DPCPX displacement. Nonspecific binding was determined in the presence of 100 μM CPA and represented less than 10% of the total binding. Incubations were terminated and samples were obtained as described previously.¹⁹

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 10 μM PD81,723. For the dissociation assay, membrane aliquots containing 5 μg of protein were allowed to reach equilibrium for one hour 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 10 μM PD81,723. The amount of radioligand still bound to the receptor was measured at various time intervals for a total of one hour. Incubations were terminated and samples were obtained as described previously.¹⁹

Radioligand competition association assay

The binding kinetics of unlabeled ligands was quantified at 25 °C using the competition association assay based on the theoretical framework by Motulsky and Mahan.²⁰ This method has been recently adopted and validated on the adenosine A_1R in our lab,¹⁹ which enables accurate determination of unlabeled ligands' binding kinetics. In the present chapter we followed the same method. In brief, we used a concentration of approximately 3- to 10-fold K_i of the unlabeled 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 one hour, except for LUF6234 and LUF6258, which were incubated for 2 hours given their slow kinetic profiles. Incubations were terminated and samples were obtained as described previously.¹⁹

Data analysis

All experimental data was analyzed by using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). 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 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 unlabeled ligands were calculated by fitting the data in the competition association model using ‘kinetics of competitive binding’:²⁰

$$\begin{aligned} K_A &= k_1 \cdot [L] \cdot 10^{-9} + k_2 \\ K_B &= k_3 \cdot [I] \cdot 10^{-9} + k_4 \\ S &= \sqrt{(K_A - K_B)^2 + 4 \cdot k_1 \cdot k_3 \cdot L \cdot I \cdot 10^{-18}} \\ K_F &= 0.5 \cdot (K_A + K_B + S) \\ K_S &= 0.5 \cdot (K_A + K_B - S) \\ Q &= \frac{B_{\text{max}} \cdot k_1 \cdot L \cdot 10^{-9}}{K_F - K_S} \\ Y &= Q \cdot \left(\frac{k_4 \cdot (K_F - K_S)}{K_F \cdot K_S} + \frac{k_4 - K_F}{K_F} (-K_F \cdot X) - \frac{k_4 - K_S}{K_S} e^{(-K_S \cdot X)} \right) \end{aligned} \quad (2)$$

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, L the concentration of [^3H]-DPCPX used (nM), $B_{\max}$ the total binding (DPM) and I the concentration unlabeled ligand (nM). 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 unlabeled ligand and k_4 , which is the k_{off} value (min^{-1}) of the unlabeled ligand. Ligand-receptor residence times (RT) were calculated using the following equation:²⁸

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

The derived association and dissociation rates of the unlabeled 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 5-11) were used to follow the time (t)-wise changes in target ‘AB’ occupancy by a bitopic ligand, ‘ab’, or a monovalent ligand, ‘c’, based on the thermodynamic model in Figure 6.2.²⁹ The abbreviation notions for different molecular complexes are in parentheses in Figure 6.2.

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

$$\frac{d[aAB]}{d(t)} = k_{+a} \cdot [AB] \cdot [ab] - k_{-a} \cdot [aAB] + k_{-b} \cdot [aABb] - k_{+b} \cdot [aAB] \cdot [L] \quad (6)$$

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

$$\frac{d[aABb]}{d(t)} = k_{+a} \cdot [ABb] \cdot [L] - k_{-a} \cdot [aABb] + k_{+b} \cdot [aAB] \cdot [L] - k_{-b} \cdot [aABb] \quad (8)$$

$$\frac{d[cAB]}{d(t)} = k_{+c} \cdot [AB] \cdot [c] - k_{-c} \cdot [cAB] + k_{-b} \cdot [cABb] - k_{+b} \cdot [cAB] \cdot [ab] \quad (9)$$

$$\frac{d[cABb]}{d(t)} = k_{+b} \cdot [cAB] \cdot [ab] - k_{-c} \cdot [cABb] + k_{+c} \cdot [ABb] \cdot [c] - k_{-c} \cdot [cABb] \quad (10)$$

$$[AB]_{\text{occ}} = 100 \cdot \frac{[aAB] + [aABb] + [ABb] + [cABb]}{[AB] + [aAB] + [aABb] + [ABb] + [cABb] + [cAB]} \quad (11)$$

Where k_{+a} and k_{+b} ($M^{-1} \cdot \text{min}^{-1}$) 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, L the total concentration of the bitopic ligand (M), c the concentration of the monovalent ligand (M), k_{+c} and k_{-c} are the association rate and dissociation rate for c-A binding/unbinding. α_{ab} and α'_{ab} are the penalty factors to correct the kinetics of binding/unbinding of the second pharmacophore of a bitopic ligand. The macroscopic k_{on} and k_{off} value of a bitopic ligand can be theoretically calculated using the following equations:²⁹

$$k_{on} = k_{+a} + k_{+b} \quad (12)$$

$$k_{off} = k_{-a} \cdot \frac{k_{-b}}{[L] \cdot k_{+a}} + k_{-b} \cdot \frac{k_{-a}}{[L] \cdot k_{+b}} \quad (13)$$

Data simulations were performed to mimic the binding of a radioligand (represented by the target occupancy of 'c') by solving Equations 5-11 during a period of 5 min, either alone or in the presence of increasing concentrations of 'ab' with different combinations of microscopic kinetics defined as follows:

- 1) $k_{+a} = 1 \times 10^5 M^{-1} \cdot \text{min}^{-1}$, $k_{-a} = 1 \text{ min}^{-1}$, $k_{+b} = 1 \times 10^5 M^{-1} \cdot \text{min}^{-1}$, $k_{-b} = 1 \text{ min}^{-1}$;
- 2) $k_{+a} = 1 \times 10^5 M^{-1} \cdot \text{min}^{-1}$, $k_{-a} = 1 \text{ min}^{-1}$, $k_{+b} = 1 \times 10^6 M^{-1} \cdot \text{min}^{-1}$, $k_{-b} = 1 \text{ min}^{-1}$;
- 3) $k_{+a} = 1 \times 10^6 M^{-1} \cdot \text{min}^{-1}$, $k_{-a} = 1 \text{ min}^{-1}$, $k_{+b} = 1 \times 10^5 M^{-1} \cdot \text{min}^{-1}$, $k_{-b} = 1 \text{ min}^{-1}$.

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 simulated data were collected at 0.5 min intervals for a total of 5 min and subsequently subjected to the competition association model using '*kinetics of competitive binding*'.²⁰ The kinetics data obtained thereof were compared to the theoretically calculated values (by subjecting the defined microscopic rate constants mentioned above into Equation 12 and 13) to explore the relevance of using competition association assay for bitopic ligands' binding kinetics.

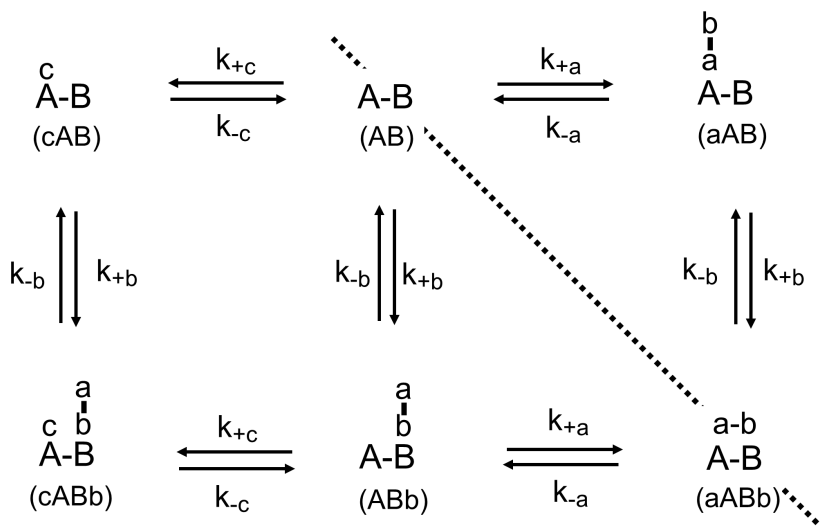


Figure 6.2 | Schematic representation of the monovalent, 'c', and bitopic, 'ab', ligand-target site interactions. The bitopic ligand bears two distinct pharmacophores: 'a' and 'b'. 'AB' is the target with distinct binding sites 'A' and 'B'. 'a' and 'c' only bind to 'A' in a competitive manner and 'b' only binds to 'B'. The abbreviated notation for the free target and target complexes is in parentheses. Formation of a trimeric species in which two bitopic ligands are bound to a single target (i.e. one via 'a' and the other via 'b') is not included in the model because, with the present microscopic rate constants, this species only marginally contributes to the total binding (data not shown). k_{+a} , k_{+b} and k_{+c} (in $\text{M}^{-1}\cdot\text{min}^{-1}$) 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. The broken line divides the thermodynamic cycle model of bivalent ligand binding into two pathways/lanes that 'ab' can adopt to form the fully bound aABb state as outlined elsewhere.²⁹

6.3 Results

Quantification of the affinity (K_i) of A_1R ligands in the absence or presence of PD81,723 or BC-1 in displacement experiments

To determine the affinities of several agonists at the A_1R in the absence or presence of allosteric modulators, radioligand displacement experiments were performed. All compounds tested produced concentration-dependent inhibition of specific [3H]-DPCPX binding (Figure 6.3) and their affinities are detailed in Table 6.1. Among the agonists tested, the binding of CCPA and NECA were preferably fitted by a two-site/state competition model, whereas the non-ribose agonist LUF5834 was best described by a one-site/state competition model. In the presence of 10 μM 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. Similarly to CCPA, NECA had 3.7-fold and 21-fold increased affinities (110 ± 50 nM and 20 ± 2 nM) in the presence of PD81,723 or BC-1, respectively. Both were mainly from a gain in affinity at the low-affinity binding site. In contrast to the ribose-containing agonists, i.e., CCPA and NECA, the binding affinity of LUF5834 to the A_1R 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 [3H]-DPCPX displacement from the A_1R by 100 nM CCPA (IC_{80}) in the presence of increasing concentrations of these two allosteric modulators. It follows from Figure 6.3D that BC-1 had a 13-fold higher potency ($EC_{50} = 1.5 \pm 0.2$ μM) than PD81,723 ($EC_{50} = 19 \pm 2$ μM) for modulating CCPA binding to the A_1R .

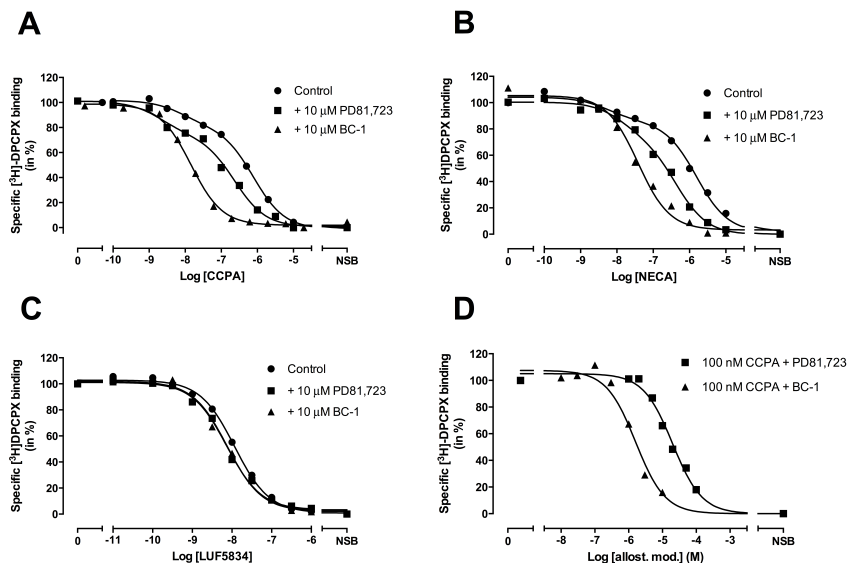


Figure 6.3 | Displacement of specific $[^3\text{H}]$ -DPCPX binding from the adenosine A_1 receptor at 25 °C by CCPA (Panel A), NECA (Panel B) or LUF5834 (Panel C) in the absence or presence of 10 μM PD81,723 or BC-1 or by 100 nM CCPA (normalized as 100%) in the presence of increased concentrations of PD81,723 or BC-1 (Panel D). Representative graphs from one experiment performed in duplicate.

Quantification of the association (k_{on} (k_1)) and dissociation rates (k_{off} (k_2)) of $[^3\text{H}]$ -DPCPX in the absence or presence of PD81,723

Since knowledge of the association [k_{on} (k_1)] and dissociation rates [k_{off} (k_2)] of $[^3\text{H}]$ -DPCPX was necessary for subsequent competition association assays in the presence of an allosteric modulator, we performed a direct $[^3\text{H}]$ -DPCPX association and dissociation assay to determine DPCPX's binding kinetics in the absence or presence of 10 μM PD81,723. As detailed in Table 6.2, $[^3\text{H}]$ -DPCPX displayed a fast association ($1.7 \pm 0.3 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$) and dissociation rate ($0.23 \pm 0.02 \text{ min}^{-1}$) in the absence of PD81,723, which values were not significantly affected by the presence of PD81,723 ($k_{\text{on}} =$

Table 6.1 | [³H]-DPCPX displacement experiments by CCPA, NECA and LUF5834 on CHO-hA₁R membranes in the absence or presence of 10 μM PD81,723 or BC-1

<i>Cmpd</i>	<i>CCPA</i>			<i>NECA</i>			<i>LUF5834</i>		
	K _H (nM)	K _L (nM)	R _H (%)	K _L (nM)	K _H (nM)	K _L (nM)	R _H (%)	K _L (nM)	K _H (nM)
Control	16±1	338±27	20±4	184±18	4±1	731±94	18±3	411±52	7.8±1.5
+ 10 μM PD81,723	2±1	77±5	27±7	43±8	6±4	200±78	26±9	110±50	9.6±2.6
+ 10 μM BC-1	n.a.	n.a.	n.a.	4.5±0.3	8±2	128±27	10±3	20±2	6.7±1.5

Data are shown as mean ± s.e.m of three separate experiments each performed in duplicate. K_H and K_L, K_i values for the high and low-affinity state, respectively and are shown for data that could be fitted to a two-state model; a one-state K_i analysis was also performed to effectively compare all affinities. R_H, the fraction of receptors in the high affinity state and is shown in %. n.a., not available.

1.8 ± 0.1 × 10⁸ M⁻¹·min⁻¹; k_{off} = 0.22 ± 0.01 min⁻¹). Similarly, BC-1, sharing the same chemical scaffold as PD81,723 (Figure 6.1), was reported to have a negligible effect on the binding of [³H]-DPCPX.²⁴ Hence, it allowed us to further explore the binding kinetics of unlabeled agonists in the absence or presence of an allosteric modulator using the rate constants (k₁ = 1.7 ± 0.3 × 10⁸ M⁻¹·min⁻¹ and k₂ = 0.22 ± 0.01 min⁻¹) of the antagonist [³H]-DPCPX.

Quantification of the binding kinetics of unlabeled agonists on the A₁R in the absence or presence of an allosteric modulator by using the competition association assay

In our previous work at the adenosine A₁R, we have validated the competition association assay to test a ligand's binding kinetics.¹⁹ Here we adopted the method to examine the binding kinetics of three A₁R 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 orthosteric ligand's affinity by influencing their binding kinetics (Table 6.3, Figure 6.4). Taking CCPA as an example, its dissociation rate decreased almost four-fold in the presence of 10 μM PD81,723 (k_{off} = 0.29 ± 0.02 min⁻¹), while its association rate increased 2.5-fold. As a result, the affinity (K_D = 131 ± 21 nM) of CCPA was increased

Table 6.2 | The association and dissociation rates of [³H]-DPCPX binding to CHO-hA₁R membranes in the absence or presence of 10 μM PD81,723.

Cmpd	<i>k</i> _{on} (M ⁻¹ .min ⁻¹) ^a	<i>k</i> ^{off} (min ⁻¹) ^b	<i>K</i> _D (nM) ^c
DPCPX	1.7 ± 0.3 × 10 ⁸	0.23 ± 0.02	1.5 ± 0.3
+ PD81,723	1.8 ± 0.1 × 10 ⁸	0.22 ± 0.01	1.2 ± 0.1

Values are means ± s.e.m of three separate experiments each performed in duplicate.
^a Association [*k*_{on} (*k*₁)] of [³H]-DPCPX to CHO-hA₁R membrane at 25°C; *k*_{on} = (*k*_{obs} - *k*_{off}) / [radioligand]. ^b Dissociation [*k*_{off} (*k*₂)] of [³H]-DPCPX from CHO-hA₁R membrane at 25°C.
^ckinetic *K*_D = *k*_{off} / *k*_{on}.

over 10-fold in the presence of PD81,723 (11 ± 1 nM). In comparison, 10 μM BC-1 lowered CCPA's off-rate by ten-fold (*k*_{off} = 0.11 ± 0.02 min⁻¹) and raised its on-rate by three-fold (*k*_{on} = 2.7 ± 0.4 × 10⁷ M⁻¹.min⁻¹). Overall, this resulted in an increased A₁R affinity of 4.1 ± 0.6 nM. This finding correlated to our aforementioned statement that BC-1 is a more potent A₁R allosteric modulator than PD81,723, yet with more detailed kinetic information. Secondly, the allosteric effect was concentration-dependent. It follows from Table 6.3 and Figure 6.4B that BC-1 slowed the dissociation rate of CCPA by eight- to ten-fold at low concentrations (i.e., 1 μM or 10 μM), while at a relatively high concentration (i.e., 33 μM), it further decreased CCPA's off-rate by another three-fold. A similar trend was observed for PD81,723, though the effect was smaller in comparison to that of BC-1 (Figure 6.4A). Thirdly, A₁R allosteric modulation was 'probe-dependent', i.e., 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 two- to five-fold, while the dissociation rate of the non-ribose agonist (LUF5834) was not significantly affected (0.92 ± 0.09 min⁻¹ versus 0.78 ± 0.08 min⁻¹, *P* = 0.31). Similarly, in the presence of the more potent allosteric modulator BC-1, all three orthosteric ligands underwent varying changes in their dissociation rates. A significant difference was observed for the residence times of LUF5834 and CCPA, i.e., three- or ten-fold increased to 3.1 ± 0.2 min⁻¹ and 9.1 ± 0.9 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 to the ribose-containing ligands in the presence of 10 μM BC-1. CCPA and NECA had an almost three-fold increase in their on-rates, while for LUF5834 a slightly decreased *k*_{on} value was observed (1.1 ± 0.1 ×

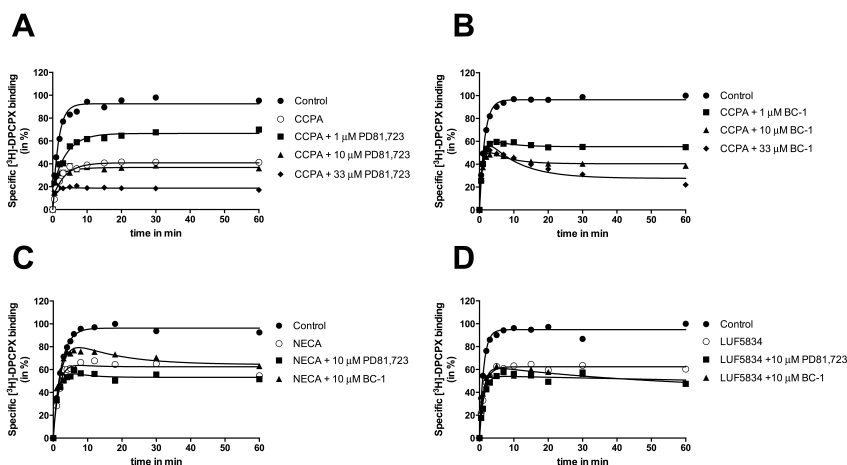


Figure 6.4 | Panel A, [^3H]-DPCPX competition association assay in the absence or presence of unlabeled CCPA with or without different concentrations of PD81,723. Panel B, [^3H]-DPCPX competition association assay in the absence or presence of unlabeled CCPA with different concentrations of BC-1. Panel C, [^3H]-DPCPX competition association assay in the absence or presence of unlabeled NECA with or without 10 μM PD81,723 or BC-1. Panel D, [^3H]-DPCPX competition association assay in the absence or presence of unlabeled LUF5834 with or without 10 μM PD81,723 or BC-1. Concentrations of 3- to 10-fold K_i of the unlabeled orthosteric ligands were used to obtain the binding data, which were fitted to Equation 2 described in the methods to calculate the k_{on} and k_{off} values of unlabeled ligands. Representative graphs from one experiment performed in duplicate. (See Table 6.3 for kinetic values).

$10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$). Lastly, a good correlation ($r^2 = 0.90$, $p < 0.0001$) between the pK_i (K_i values from one-state K_i analysis) obtained from equilibrium displacement assays and the kinetically derived pK_D values was found for all tested ligands. This proved the accuracy of the competition association assay for determination of an orthosteric agonist's binding kinetics in the absence or presence of an allosteric modulator.

Table 6.3 | Binding kinetic 'inventory' (association rate, dissociation rate, residence time 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 derived from competition association assays.

<i>Cmpd</i>	$k_{on} (M^{-1}.min^{-1})^a$	$k_{off} (min^{-1})^a$	<i>RT (min)</i> ^b	$K_D (nM)^c$
CCPA	$9.6 \pm 1.8 \times 10^6$	1.17 ± 0.24	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 ± 7
+10 μM PD81,723	$2.4 \pm 0.5 \times 10^7$	0.29 ± 0.02	3.4 ± 0.2	11 ± 1
+33 μM PD81,723	$3.4 \pm 0.8 \times 10^7$	0.20 ± 0.02	5.0 ± 0.4	5.9 ± 1.9
+1 μM BC-1	$3.5 \pm 0.4 \times 10^7$	0.15 ± 0.01	6.6 ± 0.4	4.4 ± 0.3
+10 μM BC-1	$2.7 \pm 0.4 \times 10^7$	0.11 ± 0.02	9.1 ± 0.9	4.1 ± 0.6
+33 μM BC-1	$3.2 \pm 0.3 \times 10^7$	0.038 ± 0.004	26 ± 3	1.2 ± 0.1
NECA	$0.9 \pm 0.4 \times 10^6$	0.47 ± 0.19	2.1 ± 0.5	482 ± 167
+10 μM PD81,723	$3.0 \pm 0.3 \times 10^6$	0.21 ± 0.02	4.8 ± 0.3	70 ± 6
+10 μM BC-1	$3.2 \pm 0.5 \times 10^6$	0.09 ± 0.03	11 ± 2	28 ± 6
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	$1.1 \pm 0.1 \times 10^8$	0.32 ± 0.02	3.1 ± 0.2	2.9 ± 0.2

Data are shown as mean $\pm$ s.e.m of three separate experiments each performed in duplicate.

^a k_{on} (k_3) and k_{off} (k_4) of unlabeled A₁R 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. ^b RT (Residence Time) = $1 / k_{off}$. ^c Kinetic 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 assay association assays at 25 °C.

Quantification of the binding kinetics of bitopic ligands for the A₁R in the absence or presence of an allosteric modulator by using the competition association assay

The binding kinetics of two in-house synthesized A₁R bitopic ligands were investigated in the competition association assay. These two ligands, i.e., LUF6234 with five-carbon spacer length and LUF6258 with nine-carbon spacer length (Figure 6.1), were previously designed and synthesized as tools for understanding A₁ receptor allostereism.²⁶ Here we further examined

Table 6.4 | Binding parameters for bitopic ligands and their constituent parts in the absence or presence of PD81,723 at CHO A_1 R derived from competition association assays.

Cmpd	k_{on} ($M^{-1}.min^{-1}$)^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 μM PD81,723	$2.4 \pm 1.4 \times 10^6$	0.38 ± 0.05	2.6 ± 0.4	158 ± 41
LUF6234 (linker n=5)	$5.3 \pm 0.7 \times 10^4$	0.052 ± 0.007	19 ± 3	981 ± 65
+ 10 μM PD81,723	$3.1 \pm 0.1 \times 10^6$	0.40 ± 0.05	2.5 ± 0.3	129 ± 13
LUF6258 (linker n=9)	$0.8 \pm 0.3 \times 10^4$	0.021 ± 0.008	48 ± 11	2625 ± 810
+ 10 μM PD81,723	$3.0 \pm 1.4 \times 10^4$	0.021 ± 0.011	48 ± 15	700 ± 284
+ 33 μM 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 μM PD81,723	$3.8 \pm 0.9 \times 10^6$	0.87 ± 0.21	1.1 ± 0.1	229 ± 45

Data are shown as mean $\pm$ s.e.m of three separate experiments each performed in duplicate.

^a k_{on} (k_3) and k_{off} (k_4) of unlabeled A_1 R agonists in the absence or presence of an allosteric modulator (PD81,723 or BC-1) were determined in [3 H]-DPCPX (2.6 nM) competition association assays. ^b RT (Residence Time) = $1 / k_{off}$. ^c Kinetic K_D = k_{off} / k_{on} ; k_{on} (k_3) and k_{off} (k_4) values of unlabeled antagonists were generated from [3 H]-DPCPX (2.6 nM) competition association assay association assays at 25°C.

the binding kinetics of both compounds and compared their values to the orthosteric ligand LUF5519 in combination with PD81,723 (each representing the orthosteric and allosteric pharmacophore of LUF6234 and LUF6258, respectively; see Figure 6.1 for chemical structures). In the absence of an allosteric modulator, LUF5519 displayed an on-rate of $2.0 \pm 0.4 \times 10^6 M^{-1}.min^{-1}$ and off-rate of $1.19 \pm 0.19 min^{-1}$, which led to an affinity of $595 \pm 92 nM$ (Figure 6.5, Table 6.4). Upon the addition of 10 μM PD81,723, the association rate of LUF5519 ($k_{on} = 2.4 \pm 1.4 \times 10^6 M^{-1}.min^{-1}$) did not significantly change while the dissociation rate decreased by approximately three-fold from $1.19 \pm 0.19 min^{-1}$ to $0.38 \pm 0.05 min^{-1}$ (Figure 6.5A and Table 6.4). However, after covalently linking the allosteric and orthosteric pharmacophores, both bitopic ligands demonstrated a significantly decreased association rate in comparison to LUF5519 (LUF6234, $5.3 \pm 0.7 \times 10^4 M^{-1}.min^{-1}$ and LUF6258, $0.8 \pm 0.3 \times 10^4 M^{-1}.min^{-1}$), indicative of an extra hurdle for the receptor to accommodate large flexible molecules. With respect to their off-rates, both compounds displayed significantly longer receptor residence times (LUF6234, RT = $19 \pm 3 min$ and LUF6258, RT = $48 \pm$

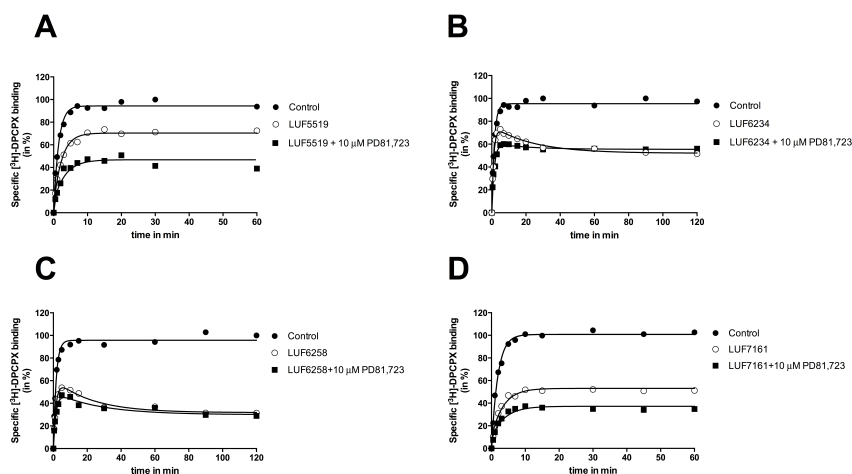


Figure 6.5 | Panel A, [^3H]-DPCPX competition association assay in the absence or presence of unlabeled LUF5519 with or without 10 μM PD81,723. Panel B, [^3H]-DPCPX competition association assay in the absence or presence of unlabeled LUF6234 with or without 10 μM PD81,723. Panel C, [^3H]-DPCPX competition association assay in the absence or presence of unlabeled LUF6258 with or without 10 μM PD81,723. Panel D, [^3H]-DPCPX competition association assay in the absence or presence of unlabeled LUF7161 with or without 10 μM PD81,723. Concentrations of 3- to 10-fold K_i of the unlabeled orthosteric ligands were used to obtain the binding data, which were fitted to the equations described in the methods to calculate the k_{on} and k_{off} values of unlabeled ligands. Representative graphs from one experiment performed in duplicate. (See Table 6.4 for kinetic values).

11 min) than LUF5519 ($RT = 0.8 \pm 0.1$ min). Interestingly, the presence of ‘external’ PD81,723 significantly increased the association rates of both bitopic ligands (LUF6234, $k_{\text{on}} = 3.1 \pm 0.1 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$; LUF6258, $k_{\text{on}} = 3.0 \pm 1.4 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$), though LUF6258 was less affected than its five-carbon linker analogue. As for the dissociation rate, both bitopic ligands demonstrated different profiles upon the addition of PD81,723: LUF6234’s residence time was significantly reduced to 2.5 ± 0.3 min, while LUF6258’s residence time was not affected (48 ± 15 min), even after further raising the concentration of PD81,723 to 33 μM (Table 6.4). In addition, we examined the binding kinetics of a newly synthesized monovalent ligand LUF7161 (Figure 6.1), which served as a control, since it contains the orthosteric part and the five-atom linker. It follows from Table 6.4 that LUF7161 had a similar dissociation rate ($k_{\text{off}} = 1.21 \pm 0.32 \text{ min}^{-1}$) as LUF5519, yet its association rate

Table 6.5 | 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 5 min in the absence or presence of different concentrations of three bitopic ligands 'ab' (their 'microscopic' association and dissociation kinetic constants of both pharmacophores were defined as Examples 1 to 3, input parameters). The 'macroscopic' kinetic binding parameters of the bitopic ligands were calculated by using Equation 12 and 13 or by subjecting the simulated data into the competition association assay.²⁰

Example	Input parameters ^a				Calculated parameters ^b		Collected parameters ^c	
	k_{+a} ($\text{M}^{-1} \cdot \text{min}^{-1}$)	k_{-a} (min^{-1})	k_{+b} ($\text{M}^{-1} \cdot \text{min}^{-1}$)	k_{-b} (min^{-1})	k_{on} ($\text{M}^{-1} \cdot \text{min}^{-1}$)	k_{off} (min^{-1})	k_{on} ($\text{M}^{-1} \cdot \text{min}^{-1}$)	k_{off} (min^{-1})
1	1×10^5	1	1×10^5	1	2.0×10^5	0.069	1.90×10^5	0.11
2	1×10^5	1	1×10^6	1	1.1×10^6	0.035	1.04×10^6	0.083
3	1×10^6	1	1×10^5	1	1.1×10^6	0.035	1.07×10^6	0.036

^a k_{+a} and k_{+b} are the microscopic association rate constants and k_{-a} and k_{-b} are the microscopic dissociation rate constants of the bitopic ligand, 'ab', for a-A, and b-B binding to the target, 'AB', respectively. ^b The 'macroscopic' kinetic binding parameters of the bitopic ligands were calculated by using Equation 12 and 13. ^c k_{on} and k_{off} values were determined by analyzing the simulated data in the competition association model 'kinetics of competitive binding'.²⁰

to the A₁R was approximately ten-fold lower ($k_{\text{on}} = 2.7 \pm 0.7 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$) than LUF5519. Upon the addition of 10 μM PD81,723, the association rate of LUF7161 was 14-fold higher, while its dissociation rate was less affected in comparison to its kinetics in the absence of the allosteric modulator. The other monovalent ligand, i.e., 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.

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 *Methods* (Equations 5-11), which mimic the binding of a radioligand in the absence or presence of a bitopic ligand. Next, the

binding kinetics of the bitopic ligand were analyzed using two methods: 1) by subjecting the defined microscopic kinetic constants (Table 6.5, ‘input parameters’) into Equation 12 and 13 to obtain the theoretical macroscopic binding parameters (Table 6.5, ‘calculated parameters’); 2) by subjecting the simulated data into the model of competition association assay to obtain the ‘collected parameters’ in Table 6.5. In general, the binding kinetics analyzed 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 three examples. For instance, the collected k_{on} value in Example 1 is $1.90 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$, which correlates to 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, although Example 2 demonstrates an approximately two-fold difference between the theoretically calculated k_{off} (0.035 min^{-1}) and the corresponding value from the competition association assay (0.085 min^{-1}), which is robust enough for practical applications. Thus, the competition association assay enables the determination of a bitopic ligand’s binding kinetics.

6.4 Discussion

In the present chapter we examined the allosteric modulation of the adenosine A_1 receptor from a kinetic perspective. We used a competition association assay to determine the binding kinetics of several unlabeled orthosteric agonists upon the addition of two allosteric modulators, i.e., PD81,723 and BC-1. The competition association assay offers several, not yet ‘tapped’ advantages for the investigation of allosteric modulation. Firstly, it enables the measurement of the kinetic characteristics of a probe in an unlabeled form, expanding the availability of orthosteric ligands for probe-dependent investigations. Secondly, the method allows the determination of a complete ‘inventory’ of binding kinetics. This includes a ligand’s association rate, dissociation rate, residence time and the ‘kinetic K_D ’. 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 orthosteric ligand’s dissociation rates. For instance, upon the addition of PD81,723 or BC-1 the receptor-residence times 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 6.4B or 6.4C, indicating a compound’s slower dissociation process than the radioligand.²⁰ PD81,723 and other GPCR allosteric modulators are known to influence a probe’s affinity by changing its dissociation rate.^{25,30-33} Their effect on the orthosteric ligand’s association rate, however, has been less investigated. Here, by using the competition association assay we found that an allosteric modulator could also change an orthosteric ligands’ affinity by influencing its association rate. For instance, CCPA’s association rate increased 2.5-fold upon the addition of 10 μ M PD81,723, which, together with the four-fold decrease in the dissociation rate, resulted in the overall ten-fold change in CCPA’s affinity. We also reaffirmed that the allosteric effect of both A₁R modulators was concentration-dependent, evidenced by the concentration-dependent increase in CCPA’s RT (Table 6.3). 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’.¹³ Such a phenomenon is not only limited to an orthosteric ligand’s affinity or potency, the parameters often investigated for GPCR allosterism, but is also reflected in the less examined orthosteric ligand’s dissociation and association rate. As evidence, CCPA and NECA’s association and dissociation rates were significantly changed upon the addition of 10 μ M PD81,723, while not for LUF5834 (Table 6.3). Apparently, all these details of ligand-receptor interaction can be simultaneously obtained by using the competition association approach.

In the present chapter, we furthermore performed a competition association assay with bitopic ligands to study their kinetic behavior. A bitopic ligand is a useful tool to study the molecular mechanism of allosteric modulation.²⁷ 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 simultaneously if the length of the linker is optimal. Moreover, one receptor can be occupied by two bitopic ligands, i.e., one to the allosteric site and the other to the orthosteric site.^{26,27,34} A kinetics investigation of the bitopic ligand could add information to an affinity- or potency-based evaluation. Therefore, we selected two previously reported bitopic ligands,²⁶ namely LUF6234 and LUF6258, and examined their binding kinetics in the present chapter. We found that the theoretically calculated macroscopic kinetics correlated well with the values that were determined by analyzing the simulated data in the competition association assay (Table 6.5). Thus,

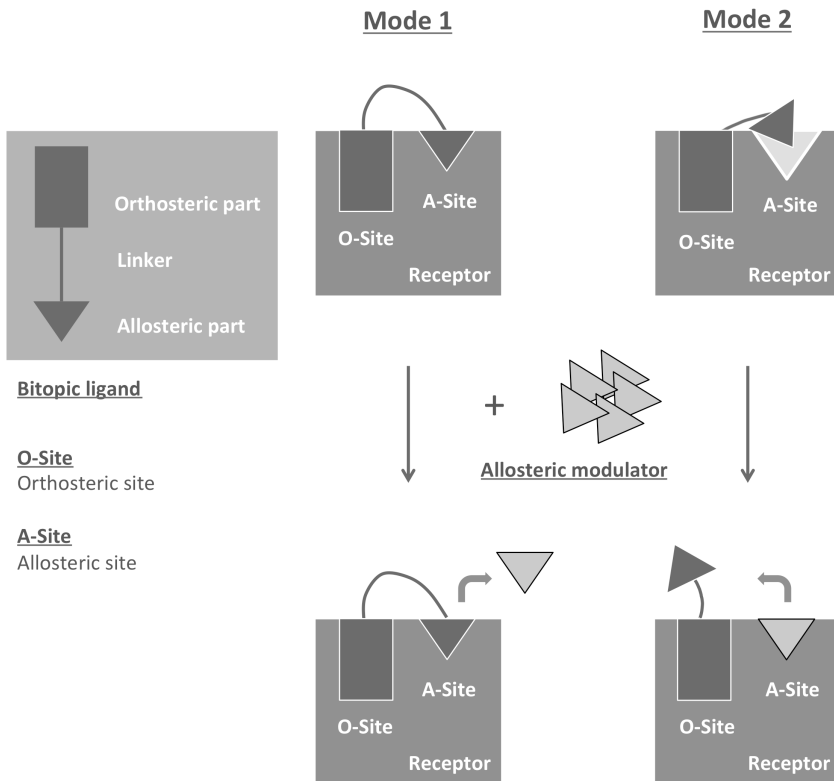


Figure 6.6 | Proposed diagram of two distinct binding modes for the bitopic ligands in the absence or presence of further added allosteric modulator. Mode 1: the linker length of a bitopic is optimal to occupy both orthosteric and allosteric sites of the receptor. Mode 2: the linker length of a bitopic is sufficient to occupy both sites on the receptor, yet in a non-optimal manner. Upon the addition of excess allosteric modulator, the monovalent ligand can occupy the allosteric site on the receptor and prevent the rebinding of the freshly dissociated allosteric pharmacophore in Mode 2 due to the bitopic ligand's less optimal binding pose. In contrast, the binding of the bitopic ligand with optimal linker length (Mode 1) is less affected in the presence of the monovalent ligand.

the competition association assay enables sufficient quantification of the net changes of the bitopic ligands' macroscopic binding kinetics. Next, we observed that due to covalently linking the orthosteric and allosteric pharmacophores the dissociation rates of both compounds were much slower than their orthosteric constituent, LUF5519, in the presence of PD81,723 (Table 6.4). 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 favors its rebinding to that site, thereby significantly slowing down the net dissociation process.^{29,35} To our knowledge, our present observation of the gained target-residence time of the bitopic ligand provides the first experimental evidence for such rebinding mechanism of low molecular weight ligands.

To further understand the two bitopic ligands' mode of binding to the A₁R, 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 allosteric site on the receptor and thus prevent the rebinding of the freshly dissociated allosteric pharmacophore. Interestingly, both association and dissociation of LUF6234 became faster compared to its kinetics in the absence of PD81,723, indicative of competition between the allosteric part of LUF6234 and the 'free' allosteric modulator at the A₁R allosteric binding site. In contrast, the dissociation rate of LUF6258 was unaffected upon the addition of 10 μ M PD81,723, which was also the case when we raised the concentration of PD81,723 to 33 μ M. Further increasing the concentration of the allosteric modulator may allow it to displace the allosteric pharmacophore of LUF6258 from the A₁R allosteric site.³⁵ However, we did not attempt to do so, given that PD81,723 also significantly displaces the binding of an orthosteric ligand at concentrations of 100 μ M and higher,³⁶ while solubility is an issue too. Apparently, the monovalent ligand PD81,723 is less efficient in preventing the rebinding of the nine-linker compound than its five-linker analogue at the same concentration. Such a discrepancy is most likely due to the flexibility of their linkers, where the nine-carbon linker has more freedom of rotation and thus increased the likelihood that both pharmacophores can tightly interact with the receptor (Figure 6.6, Mode 1). 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 6.6, Mode 2).

Notably, our proposed binding modes of the two bitopic ligands expand on the conclusions previously drawn by Narlawar *et al.*²⁶ where the authors suggested that the bitopic ligand with linker length of five (LUF6234) failed to completely occupy both allosteric site and orthosteric site while the one with linker length of nine (LUF6258) was able to occupy both sites. This conclusion was based on 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.²⁶ Following this hypothesis, we would have reached the same conclusion if merely affinity was taken into consideration, since we also observed that LUF6234 displayed a strongly increased affinity upon the addition of PD81,723, while less so for LUF6258. However, it is also noteworthy from our study that both LUF6234 and LUF6258 displayed significantly increased receptor residence times compared to that of the monovalent ligand in the presence of PD81,723 (Table 6.4). Such a result strongly indicates that both allosteric and orthosteric sites on the A₁R 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 residence time comparable to the monovalent LUF7161, 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 validated and showed the use of a competition association assay at the hA₁R to determine the allosteric modulation of the binding kinetics of different A₁R agonists. Our data suggests that the influence of an A₁R allosteric enhancer (positive allosteric modulator) on the binding kinetics of the orthosteric agonist is dependent on 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 bitopic ligands' binding kinetics was also explored by analyzing 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 radiolabeled or no, 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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