Kinetic aspects of the interaction between ligand and G protein-coupled receptor. The case of the adenosine receptors.

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Abstract

Ligand-receptor binding kinetics is an emerging topic in the drug research community. Over the past years medicinal chemistry approaches from a kinetic perspective have been increasingly applied to G protein-coupled receptors including the adenosine receptors (AR), which are involved in a plethora of physiological and pathological conditions. The study of ligand-AR binding kinetics offers room for detailed structure-kinetics relationships next to more traditional structure-activity relationships. Their combination may facilitate the triage of candidate compounds in hit-to-lead campaigns. Furthermore, kinetic studies also help in understanding AR allosterism. Allosteric modulation may yield a change in the activity and conformation of a receptor resulting from the binding of a compound at a site distinct from where the endogenous agonist adenosine binds. Hence, in this review we summarize available data and evidence for the binding kinetics of orthosteric and allosteric AR ligands. We hope this review will raise awareness to consider the kinetic aspects of drug-target interactions on both ARs and other drug targets.

Contents

1. INTRODUCTION 5
1.1. Adenosine receptors 5
1.2. Ligand-receptor binding kinetics as an emerging concept 7
1.3. Current methods to measure binding kinetics on ARs 8
1.3.1. Kinetic association assay 9
1.3.2. Kinetic dissociation assay 9
1.3.3. Competition association assay 9
1.3.4. Kinetic assay in the presence of an allosteric modulator 10
1.3.5. Emerging approaches 11
2. ORTHOSTERIC LIGANDS' BINDING KINETICS 12
2.1. A_1AR 12
2.1.1. A_1AR receptor agonists 14
2.1.2. A_1AR receptor antagonists 17
2.2. A_{2A}AR 19
2.2.1. A_{2A}AR receptor agonists 19
2.2.2. A_{2A}AR receptor antagonists 22
2.3. A_{2B}AR 25
2.4. A_3AR 27
2.4.1. A_3AR receptor agonists 27
2.4.2. A_3AR receptor antagonists 28
2.5. Summary 29
3. THE INFLUENCE OF AN ALLOSTERIC MODULATOR ON THE ORTHOSTERIC LIGAND'S BINDING KINETICS 30
3.1. A_1AR 30
3.1.1. 2-amino-3-substituted thiophenes 33
3.1.2. 2-aminothiazoles 37
3.1.3 Thiadiazoles as putative allosteric modulators. 39
3.1.4. Non-selective allosteric modulators. 39
3.1.5. Binding sites for A_1AR allosteric modulators 40
3.2. A_{2A}AR 42

3.2.1. Non-selective allosteric modulators. 42
3.2.2. Emerging selective allosteric modulators 42
3.2.3. Binding sites for A_{2A}AR allosteric modulators 44
3.3. A_{2B}AR 45
3.4. A_3AR 45
3.4.1. Isoquinoline and quinoline derivatives 45
3.4.2. Imidazoquinoline derivatives 47
3.4.3. Non-selective allosteric modulators 48
3.4.4. Binding site for A_3AR allosteric modulators 49
3.5. Summary 50
4. BINDING KINETICS OF BITOPIC LIGANDS 50
5. FUTURE PERSPECTIVES AND CHALLENGES FOR KINETIC CHARACTERIZATIONS 51
5.1. Structure-kinetics relationship (SKR) and structure-activity relationship (SAR) studies 51
5.2. Signaling bias and ligand-receptor binding kinetics 52
5.3. Equilibrium selectivity and kinetic selectivity profiling 53
5.4. Measuring the binding kinetics of allosteric modulators 54
5.5. Molecular mapping of the binding site for allosteric modulators 54
5.6. Characterization of ligand-receptor binding kinetics in living cells 55
6. CONCLUDING REMARKS 57
ACKNOWLEDGMENTS 58
REFERENCES 59
BIOGRAPHIES 74

1. INTRODUCTION

1.1. Adenosine receptors

Adenosine receptors (ARs) belong to the superfamily of G protein-coupled receptors (GPCRs), the largest class of drug targets so far.[1] ARs have four subtypes: A_1AR, A_{2A}AR, A_{2B}AR and A_3AR. Their activation by the endogenous agonist adenosine and subsequent signaling has been extensively studied.[2,3] The A_1 and A_3 subtypes are mainly coupled to the enzyme adenylate cyclase in an inhibitory fashion via $G_{i/o}$ proteins, whereas the A_{2A} and A_{2B} subtypes stimulate this enzyme via a $G\alpha_s$ protein.[2,3] ARs are ubiquitously expressed throughout the human body and involved in a wide range of physiological and pathological conditions. For instance, the A_1 and A_{2A} ARs are present in the cardiovascular system and play critical roles in regulating myocardial oxygen consumption and coronary blood flow.[4,5] These two receptors are also highly expressed in the brain, regulating the release of other neurotransmitters such as dopamine and glutamate.[6] The A_{2B} and A_3 ARs are located mainly peripherally and are involved in many processes such as inflammation and immune responses.[7,8]

Many selective orthosteric agonists and antagonists for ARs have been developed.[9] Several clinical trials are currently in progress (see reviews[10,11] and references therein for detailed information). One agonist targeting the A_{2A} receptor, namely regadenoson, has been approved by the US Food and Drug Administration (FDA) for myocardial perfusion imaging in patients with suspected coronary artery disease.[12] Istradefylline, a selective A_{2A}AR antagonist, has received market approval in Japan for adjunctive treatment of Parkinson's disease.[13]

In addition to the extensive repertoire of orthosteric AR ligands—compounds that share the same binding site as adenosine —many allosteric modulators have been developed over the past decades.[14] An allosteric modulator binds to a site on the target protein distinct from the orthosteric primary ligand binding site to which endogenous ligands such as adenosine and

synthetic derivatives bind.[15] The binding of an allosteric modulator is supposed to cause a conformational change of the receptor, which, in turn, may enhance the action (affinity and/or efficacy) of a given orthosteric ligand (positive allosteric modulator, PAM) or inhibit it (negative allosteric modulator, NAM). In this light a ligand that occupies an allosteric site on a receptor but does not alter the action of the orthosteric ligand is termed a neutral allosteric ligand (NAL) or silent allosteric modulator (SAM).[16-18] Furthermore, 'bitopic' or 'dualsteric' (orthosteric/allosteric) ligands bind to both orthosteric and allosteric sites, creating self-allosterism.[19-21] In principle, a strict allosteric modulator does not have any activity alone. It requires the presence of an orthosteric ligand (for instance, the endogenous hormone or neurotransmitter) to show its action. Therefore, an allosteric modulator of ARs could possibly tune the effect of endogenous adenosine in an event-responsive and temporally specific manner, which might have therapeutic advantages compared to an orthosteric AR ligand.[22]

New insights into the molecular aspects of ligand binding stem from the X-ray crystallographic structure of GPCRs[23], and the A_{2A}AR is at the forefront in this regard.[24] To date, several A_{2A}AR structures in complex with an agonist[25-27] or antagonist[28-32] are available, which provide atomic details of ligand-receptor interactions and enable structure-based drug design and discovery for the AR and other GPCRs as well. The binding pocket of the A_{2A}AR in complex with 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) in an inactive state is depicted in Figure 1A, or with 6-(2,2-diphenylethylamino)-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-*N*-(2-(3-(1-(pyridin-2-yl)piperidin-4-yl)ureido)ethyl)-9*H*-purine-2-carboxamide (UK432,097) in an active conformation (Figure 1B). In the inactive conformation (Figure 1A), the bicyclic triazolotriazine core of ZM241385 is anchored by an aromatic stacking interaction with Phe168$^{5.29}$ (residue superscripts refer to the Ballesteros-Weinstein numbering[33]), an aliphatic hydrophobic interaction with

Ile274$^{7.39}$ and a hydrogen bonding interaction with Asn253$^{6.55}$. Adjacent to Phe168$^{5.29}$ the polar residue Glu169$^{5.30}$ interacts with the exocyclic amino group attached to the bicyclic core of ZM241385.[28,32,34] Comparison of the active and inactive conformations of the A_{2A}AR highlighted structural changes for receptor activation, particularly within the ligand binding region (Figure 1B). These changes include a tightening of hydrophilic residues in TM3, TM5 and TM7 around the ribose group of UK432,097 (Thr88$^{3.36}$, Val84$^{3.32}$, Leu85$^{3.33}$, Ser277$^{7.42}$, His278$^{7.43}$).[25] Unstable water molecules that appear in the inactive state of the A_{2A}AR are removed from deep in the binding pocket.[28,29,32] The 1.8-angstrom high-resolution crystal structure also identified the presence of a sodium ion, a general allosteric modulator for GPCRs, and several clusters of structural water molecules with potential roles in receptor stabilization and function.[32] Crystal structures for A_1, A_{2B} and A_3 ARs are not available yet. Although probing the binding sites of orthosteric and allosteric ligands for these subtypes largely relies on homology modeling and docking[35,36] or on site-directed mutagenesis studies[37-39], the predictability has been satisfactory, as evidenced by the identification, design and modification of both known and novel AR ligands.[24,40-42]

1.2. Ligand-receptor binding kinetics as an emerging concept

Ligand-receptor binding kinetics is an emerging pharmacological concept, which is receiving increasing attention in the drug research community. Several recent reviews have highlighted the importance of kinetic profiling in the pipeline of the drug design and discovery process. It allows resolving ligand-receptor interactions into both molecular recognition (k_{on}) and complex stability (k_{off}) and thus provides additional parameters for triage and advancement of drug candidates in the hit-to-lead campaign.[43-45] For instance, several seminal studies in the field showed, in retrospect, that drugs with desirable receptor residence time (RT = 1 /k_{off})[46] might provide the

following advantages: for therapies aiming for prolonged target occupancy, a long-RT compound might exert sustained pharmacological effects even if the compound has a rapid pharmacokinetic profile;[47] on the other hand, when a drug's target-related toxicity outweighs its therapeutic benefit, a rapidly dissociating compound that displays short-lived intervention may reduce the occurrence of on-target toxicity.[48] Although conceptually tempting, ligand-receptor binding kinetics is still in its infancy and has been largely neglected in the traditional drug discovery process. The prevailing strategy in current practice is largely based on steady-state metrics, e.g. affinity or potency values, as predictors for *in vivo* efficacy. However, the current high attrition rate, often due to insufficient clinical efficacy, suggests that the equilibrium-based strategy might be too simplistic.[49] Introducing analysis of binding kinetics, and in particular residence time in the earlier phase of the drug design and discovery process might provide a better predictor for *in vivo* efficacy, and thereby contribute to the development of a new generation of improved medicinal products.[50-53]

Kinetic characterization of AR ligands is emerging but has not been thoroughly reviewed before. Here, without claiming to be exhaustive we summarize orthosteric ligands on all ARs with known kinetic information. We also review and discuss the kinetics of orthosteric ligands in the presence of an allosteric modulator, given that the modulatory effect is best understood in terms of the kinetics of the ligand-receptor interaction.

1.3. Current methods to measure binding kinetics on ARs

Over the years several approaches to measure binding kinetics have been developed, and new techniques are emerging as well. These methods have been well documented in several recent reviews.[45,54,55] Here we focus on the commonly used kinetic assays on the ARs (Figure 2).

1.3.1. Kinetic association assay

As shown in Figure 2A, the association experiment is usually initiated by incubating the receptor-bearing material with a ligand either radiolabeled or fluorescently labeled for a different period. k_{obs}, the observed rate constant to approach equilibrium, can be obtained by Equation (1) and then fitted into Equation (2) to obtain the ligand's association rate (k_{on}).

$$Y = Y_{max} \cdot (1 - e^{-k_{obs} \cdot t}) \quad (1)$$

$$k_{on} = (k_{obs} - k_{off}) / [\text{labeled ligand}] \quad (2)$$

Where t is a given time, Y is the amount of specific labeled ligand binding, Y_{max} the specific labeled ligand binding at equilibrium, k_{off} the dissociation rate constant being determined in independent dissociation experiments as follows.

1.3.2. Kinetic dissociation assay

Firstly, the receptor-bearing material is pre-incubated with the labeled ligand to reach equilibrium. Secondly, an excess amount of assay buffer (i.e., infinite dilution) or a receptor-saturating concentration of unlabeled ligand is added to prevent ligand (re)association (Figure 2B). In this way labeled ligand dissociation is initiated, which usually follows a monophasic or biphasic exponential decay depending on different mechanisms.

1.3.3. Competition association assay

An alternative that avoids labeling every ligand of interest is the competition association assay (Figure 2C), in which a labeled ligand is co-incubated with the receptor-bearing material in the absence (i) or presence of an unlabeled ligand of interest (ii and iii). With this method the association and dissociation rate constants for unlabeled ligands are calculated by fitting the data into Equation (3) developed by Motulsky and Mahan[56]:

$$K_A = k_1[L] + k_2$$
$$K_B = k_3[I] + k_4$$
$$S = \sqrt{(K_A - K_B)^2 + 4 \cdot k_1 \cdot k_3 \cdot L \cdot I}$$
$$K_F = 0.5(K_A + K_B + S)$$
$$K_S = 0.5(K_A + K_B - S)$$
$$Q = \frac{B_{\max} \cdot k_1 \cdot L}{K_F - K_S}$$
$$Y = Q \cdot \left(\frac{k_4 \cdot (K_F - K_S)}{K_F \cdot K_S} + \frac{k_4 - K_F}{K_F} e^{(-K_F \cdot X)} - \frac{k_4 - K_S}{K_S} e^{(-K_S \cdot X)}\right) \quad (3)$$

Where X is the time, Y is the specific binding of the labeled ligand, k_1 the k_{on} of the labeled ligand predetermined in association experiments, k_2 the k_{off} predetermined in dissociation experiments, L the concentration of the labeled ligand used, B_{max} the total binding and I the concentration of unlabeled ligand. Combining these parameters into Equation (3) allows the following parameters to be calculated: k_3, the k_{on} of the unlabeled ligand and k_4, the k_{off} of the unlabeled ligand.

Different kinetic profiles of an unlabeled ligand are also reflected in the kinetic association curves (Figure 2C). According to the theory of Motulsky and Mahan[56], if the competitor dissociates faster from its target than the labeled ligand, the specific binding of the radioligand will slowly and monotonically approach its equilibrium in time (ii). However, when the competitor dissociates more slowly, the association curve of the labeled ligand will consist of two phases starting with a typical 'overshoot' and then a decline until a new equilibrium is reached (iii). This assay also allows further adaptation into a high-throughput format, which will be discussed in Section 2.

1.3.4. Kinetic assay in the presence of an allosteric modulator

The influence of an allosteric modulator on the binding kinetics of a given orthosteric ligand (the probe) is usually quantified in a dissociation experiment by determining the change in the probe's

dissociation rate constant (Figure 2D). In brief, the assay contains the following phases: (1) formation of the probe-receptor binary complex by preincubating the receptor-bearing material with a probe; (2) addition of an allosteric modulator either before or together with the initiation of the probe's dissociation—methods vary in different labs—to stabilize the allosteric ternary complex; (3) dissociation of the probe by adding a displacer in an excess amount or by an infinite dilution step. After a period of incubation, the allosteric activity is scored based on a comparison of the residual binding of the probe in the presence (i) or absence (ii) of the allosteric modulator. A PAM will retard the probe's dissociation from the receptor (as in Figure 2D), whereas a NAM will fasten the dissociation process.

The on- and off-rate of a given orthosteric ligand in the presence of an allosteric ligand can also be quantified by the competition association assay, which was further adapted in our lab for the evaluation of allosteric activity using an unlabeled orthosteric ligand.[57] The result obtained is very informative, which provides (1) a measure of the kinetic characteristics of a probe, including its association and dissociation rate constants and kinetic K_D (k_{off}/k_{on}), and (2) an examination of a specific feature of GPCR allosteric modulation, the so-called "probe-dependency"[16,58].

1.3.5. Emerging approaches

Alternative to the abovementioned filtration assays, the scintillation proximity assay (SPA) enable one to monitor dynamic ligand-AR interactions in a "mix and measure" format without a filtration step to separate bound from free ligand as is the case in a traditional receptor-binding assay. An application of the SPA assay is recently reported on the A_1AR, demonstrating its practical convenience in kinetic measurements.[59]

Kinetics of ligand-receptor interaction can be obtained by monitoring fluorescent ligand binding in combination with fluorescence correlation spectroscopy (FCS). This technique measures fluctuations in the fluorescence intensity of fluorescently labeled particles diffusing

through a small illuminated detection volume. This allows free ligands to be distinguished from slowly diffusing receptor-bound ligands without their physical separation.[60] One major advantage of this method is that the actual ligand amounts can be measured. It can be used at the single cell level and even at the level of single molecules.[54] Several cases were reported by Hill and coworkers, who characterized kinetic ligand binding to different ARs.[61-64]

Furthermore, advances in label-free technology enable kinetic determinations of AR ligands as well, as represented by the surface plasmon resonance (SPR) technology[65,66] or the more recently application of mass spectrometry[67].

2. ORTHOSTERIC LIGANDS' BINDING KINETICS

As mentioned above, the most straightforward and common way of measuring ligand-receptor binding kinetics is using a kinetic radioligand binding assay.[45,54] Radiolabeled agonists or antagonists selective for each AR subtype are available. They are tool/reference compounds that can be applied to investigate the kinetics of known or novel unlabeled AR ligands using the mathematical model developed by Motulsky and Mahan.[56] In the following section we will discuss the kinetic profile of orthosteric ligands for each AR subtype.

2.1. A_1AR

Chemical structures of representative A_1AR ligands are in **Figure 3** and their binding kinetics is summarized in **Table 1**.

Table 1. Binding kinetics of A_1AR ligands.

Cmpd	**Target**	**T (°C)**	**k_{on} ($M^{-1}\cdot min^{-1}$)**	**k_{off} (min^{-1})**	**Kinetic K_D** (nM)	**Ref.**
CPA	Rat brain tissue	23	5.5×10^7	0.023	0.42	[68]
(*S*)-ENBA	Rat brain membranes	25	1.2×10^8	0.056 (fast phase); 0.0032 (slow phase)	n.a.	[69]
(*R*)-PIA	Rat brain membranes	37	6.6×10^6	0.078	12	[70]
(*R*)-IHPIA	Rat brain membranes	30	3.1×10^7	1.3 (fast phase); 0.0093 (slow phase)	n.a.	[71]
LUF5834	$CHOhA_1AR$ membranes	25	2.0×10^8	0.92	4.6	[57]
Capadenoson	$CHOhA_1AR$ membranes	25	2.4×10^7	0.036	1.5	[72]
LUF6976	$CHOhA_1AR$ membranes	25	3.9×10^8	0.87	2.2	[73]
LUF7050	$CHOhA_1AR$ membranes	25	4.3×10^5	0.016	37	[73]
LUF6941	$CHOhA_1AR$ membranes	25	2.6×10^6	0.0076	2.9	[72]
ABA-X-BY630	$CHOhA_1AR$ whole cells	37	2.6×10^7	2.0	77	[74]
XAC	Rat adipocyte membranes	25	3.0×10^7	0.12	4	[75]
DPCPX	Rat brain membranes	25	9.0×10^7	0.045	0.50	[76]
DPCPX	$CHOhA_1AR$ membranes	25	1.4×10^8	0.21	1.5	[77]
I-BW-A844U	Bovine brain membranes	19-22	6.7×10^8	0.093	0.14	[78]
LUF5962	$CHOhA_1AR$ membranes	25	6×10^7	0.021	0.35	[77]
LUF6057	$CHOhA_1AR$ membranes	25	4.8×10^8	3.0	6.3	[77]

Kinetic $K_D = k_{off}/k_{on}$. n.a., not applicable, K_D values cannot be calculated due to the biphasic nature of the dissociation processes.

2.1.1. A_1AR receptor agonists

In general, most A_1AR agonists are derivatives of the endogenous ligand adenosine (**1**), containing a ribose group attached to the N^9 position of the adenine moiety. Substitution at the N^6 position with a wide range of alkyl, cycloalkyl or arylalkyl groups generally causes A_1AR selectivity.[9] Among these synthetic efforts, N^6-cycloalkyl substitution yielded N^6-cyclopentyladenosine (**2**, CPA), which has become a reference compound to examine the pharmacology of the A_1AR. Kinetic characterization of tritium-labeled CPA binding to the A_1AR in rat brain tissue revealed its association rate of 5.5×10^7 $M^{-1}\cdot min^{-1}$ and dissociation rate of 0.023 min^{-1}, equaling a "kinetic K_D" (k_{off}/k_{on}) of 0.42 nM.[68] Its 2-chloro substituted analogue, 2-chloro-N^6-cyclopentyladenosine (**3**, CCPA), has a high selectivity for the A_1AR too, combined with slow kinetics with equilibrium reached after three hours for both its association to and dissociation from the receptor.[79,80] Replacing the cyclopentyl group in CPA with cyclohexyl resulted in N^6-cyclohexyladenosine (**4,** CHA), which was reported to have a slow dissociation half-life ($t_{1/2}$ = 60 min) from the A_1AR in both guinea pig and bovine brain.[81]

Substitution at the N^6 position with bicycloalkyl groups can further improve the ligand's selectivity compared to that with the monocycloalkyl moiety. One example is the development of 1*R*,2*S*,4*S*-N^6-2-endo-norbornyladenosine, [**5**, (*S*)-ENBA].[69] It displayed a higher selectivity ratio (A_{2A}/A_1 K_i ratio = 4700) in rat brain membranes than CPA (A_{2A}/A_1 K_i ratio = 780). Kinetic characterization of the tritiated form of (*S*)-ENBA revealed that the compound had a fast association to the A_1AR (k_{on} 1.2×10^8 $M^{-1}\cdot min^{-1}$). Its dissociation from the receptor was best fitted to a biphasic model (k_{off_1} = 0.056 min^{-1} for the fast dissociation phase and k_{off_2} = 0.0032 min^{-1} for the slow dissociation phase), probably representing the kinetics at both low- and high-affinity binding states.

Other lines of research, such as arylalkyl substitution at the N^6 position of adenosine analogues, also led to the discovery of selective A_1AR agonists (**6-8**), for instance, N^6-[(*R*)-phenylisopropyl]adenosine [**6**, (*R*)-PIA] and derivatives (**7, 8**). The binding kinetics of (*R*)-PIA was determined in rat brain membranes at 37 °C (k_{on} = 6.58 × 10^6 $M^{-1}{\cdot}min^{-1}$, k_{off} = 0.078 min^{-1}, kinetic K_D = 12 nM).[70] After this, Schwabe and colleagues reported a radioiodinated *para*-hydroxy substituted analogue, namely [^{125}I]-N^6-(3-iodo-4-hydroxyphenylisopropyl)adenosine ([^{125}I]-IHPIA), and used it in its racemic form to characterize the A_1AR in rat cerebral cortex membranes.[82] In a follow-up study, Munshi *et al.* specifically synthesized and examined the respective kinetics of the iodinated stereoisomers of N^6-(4-hydroxylphenyl)isopropyladenosine (HPIA), i.e., N^6-[(*R*)-(3-iodo-4-hydroxyphenyl)isopropyl]adenosine [**7**, (*R*)-IHPIA] and N^6-[(*S*)-(3-iodo-4-hydroxyphenyl)isopropyl]adenosine [**8**, (*S*)-IHPIA] in rat brain membranes.[71] In contrast to the fast binding of the (*S*)-isomer (less than 10 min for complete ligand association), the (*R*)-isomer interacted slowly with the receptor and required almost 2 h to reach equilibrium, when a concentration close to the K_d was used. The association rate was estimated with a k_{on} value of 3.1 × 10^7 $M^{-1}{\cdot}min^{-1}$. The dissociation of specifically bound [^{125}I]-(*R*)-IHPIA was biphasic, displaying an initial fast phase (1.3 min^{-1}) followed by a slower phase (0.0093 min^{-1}) comparable to that of (*S*)-ENBA mentioned above.

5'-*N*-ethylcarboxamidoadenosine (**9**, NECA) is another radiolabeled agonist with well-characterized binding kinetics. It displayed very fast kinetics on pig aorta smooth muscle membranes at 0 °C.[83] Within 2 min more than half of the maximal binding of [^{3}H]-NECA was attained. The dissociation of this radioligand was very rapid as well. Almost 80% of the specifically bound [^{3}H]-NECA was displaced by 100 μM NECA within 1 min.

Next to the classical derivatives of purine nucleosides as A_1AR agonists, non-adenosine agonists have been unveiled by research from our group and Bayer Co. (Germany), such as 2-

amino-4-(4-hydroxyphenyl)-6-[(1*H*-imidazol-2-ylmethyl)thio]-3,5-pyridinecarbonitrile (**10,** LUF5834)[84,85] and 2-amino-6-[[2-(4-chlorophenyl)-1,3-thiazol-4-yl]methylsulfanyl]-4-[4-(2-hydroxyethoxy)phenyl]pyridine-3,5-dicarbonitril (**13,** capadenoson)[86,87]. Both compounds had (relatively) fast binding kinetics at a CHO cell line expressing the recombinant human A_1AR (CHOhA_1AR). In a follow-up study, the synthesis and evaluation of 4-amino-aryl-5cyano-2-thiopyrimidines was reported by our group.[73] These compounds displayed divergent kinetic profiles with ligand-receptor RTs ranging from 1 min (4-amino-6-(benzo[*d*][1,3]dioxol-5-yl)-2-((2-morpholinothiazol-4-yl)methylthio)pyrimidine-5-carbonitrile, **11**, LUF6976) to 1 h (4-amino-6-(benzo[*d*][1,3]dioxol-5-yl)-2-((2-(4-iodophenyl)thiazol-4-yl)methylthio)pyrimidine-5-carbonitrile, **12**, LUF7050), and extensive structure-affinity (SAR) and structure-kinetics relationships (SKR) were established. A similar analysis of SAR and SKR for novel A_1AR agonists was performed in another study, in which Louvel *et al*. reported the synthesis and biological evaluation of new derivatives of capadenoson.[72] This allowed the discovery of 2-amino-6-{[2-(4-chlorophenyl)thiazol-4-yl]methylthio}-4-(4-methoxyphenyl)pyridine-3,5-dicarbonitrile (**14,** LUF6941) with an increased RT of 132 min, which is nearly 26-fold longer than that of 2-amino-4-(4-methoxyphenyl)-6-(thiazol-4-ylmethylthio)pyridine-3,5-dicarbonitrile (**15**, LUF7064)—an analogue without a phenyl group attached to the thiazole. These studies demonstrated that minor chemical modifications can have a dramatic effect on ligand-receptor residence time, while the affinity remains more or less the same (LUF6941, K_i = 5.0 nM; LUF7064, K_i = 1.3 nM). Such a drug development strategy of SAR in combination with SKR could be very useful in future kinetics-directed medicinal chemistry efforts.[88,89]

In addition to the application of radiolabeled A_1AR agonists for kinetic investigations new fluorescent agonists are available.[60,62,90] In combination with scanning confocal microscopy and fluorescence correlation spectroscopy, these tool compounds allow noninvasive imaging and

quantification of the kinetics of ligand-receptor interactions in living single cells.[91] One exemplary case is the fluorescent adenosine derivative ABA-X-BY630 (**16**), an N^6-aminobutyladenosine congener conjugated with the commercially available amine-reactive fluorophore BODIPY630/650-X.[74] The fluorescent ligand had a relatively slow on-rate (2.6×10^7 $M^{-1}\cdot min^{-1}$) and a fast off-rate (2.0 min^{-1}) at CHOhA_1AR cells. It can be argued that the attached large fluorophore and the long aliphatic linker to N^6-aminobutyladenosine may strongly influence the overall kinetics of the fluorescent agonist.[92] Considering this, it might be interesting to compare the kinetics of the parental agonist (N^6-aminobutyladenoine) in another assay format under the same conditions (e.g. cell line, temperature, etc.) for better understanding of the fluorescent ligand's binding kinetics.

2.1.2. A_1AR receptor antagonists

Xanthine and xanthine derivatives, including the natural products caffeine (**17**) and theophylline, are prototypical antagonists at all AR subtypes.[93] Chemical modifications of the xanthine core structure at the 8-position with aryl or cycloalkyl groups have led to antagonists with high affinity and selectivity for the A_1AR.[9] One of the first cases available with kinetic profiling dates back to 1980, when Goodman *et al.* reported the dissociation rate of the tritiated 1,3-diethylphenylxanthine (**18**, DPX).[81] In bovine brain the antagonist exhibits high-affinity binding (K_i = 5 nM) and rapid dissociation kinetics ($t_{1/2}$ = 1 min) measured at 0 °C. Following this study, Jacobson *et al*. prepared an amine-functionalized analogue of 1,3-DPX in tritiated form coined xanthine amine congener (**19**, XAC) for use as another antagonist radioligand.[94] The kinetics for [^{3}H]-XAC were later measured on rat adipocyte membranes and specifically examined for the effect of guanine nucleotides on ligand binding to the A_1AR. It was shown that Gpp(NH)p (Guanosine 5'-[β,γ-imido]triphosphate), a nonhydrolyzable form of GTP, enhanced the association rate ($k_{on} = 3 \times 10^7$ $M^{-1}\cdot min^{-1}$) of the radioligand to the receptor by 4-fold (k_{on} = 1.3 ×

10^8 $M^{-1}\cdot min^{-1}$) but had no effect on the dissociation rate (k_{off} = 0.12 min^{-1}) of the radioligand-receptor complex.[75] A similar study was performed by Prater *et al.*, in which the authors evaluated the effects of GTPγS [guanosine 5'-(3-O-thio)triphosphate], another nonhydrolyzable form of GTP, on the binding of radioiodinated 3-(4-amino-3-I-phenethyl)-1-propyl-8-cyclopentylxanthine (**20**, I-BW-A844U)[95] to the A_1AR in bovine brain membranes.[78] Contrary to the result for [^{3}H]-XAC, the kinetics of ^{125}I-BW-A844U were not significantly changed in the absence (k_{on} = 6.0 × 10^8 $M^{-1}\cdot min^{-1}$, k_{off} = 0.098 min^{-1}) or presence (k_{on} = 6.7 × 10^8 $M^{-1}\cdot min^{-1}$, k_{off} = 0.093 min^{-1}) of GTPγS, although the B_{max} value was increased by 28%. Combining with other lines of evidence, Prater *et al.* confirmed that the effects of GTPγS could be attributed to the decreased affinity of receptors for a pool of endogenous adenosine that cannot be readily removed from membranes. Such a finding is important, especially for accurate kinetic measurements on tissue preparations.

Further structural modifications on the xanthine core by means of combining 1,3-dipropyl- and 8-cyclopentyl substitutions led to additive effects for 8-cyclopentyl-1,3-dipropylxanthine (**21**, DPCPX) in terms of A_1AR affinity and selectivity over other ARs.[76] Additionally, the nonspecific binding of radiolabeled DPCPX was very low. Its properties are superior to those of other radioligands. Therefore, [^{3}H]-DPCPX has been benchmarked for subsequent pharmacological and kinetic characterizations on the A_1AR. Using rat brain membranes, Lohse *et al*. determined [^{3}H]-DPCPX's association and dissociation rate constants (k_{on} = 9 × 10^7 $M^{-1}\cdot min^{-1}$, k_{off} = 0.045 min^{-1}) for the first time, yielding a 'kinetic K_D' of 0.5 nM. Notably, DPCPX displayed different binding kinetics at the recombinant CHOhA$_1$AR membranes (k_{on} = 1.4 × 10^8 $M^{-1}\cdot min^{-1}$, k_{off} = 0.21 min^{-1}) at the same assay temperature[57,77], suggesting species differences.[88]

To allow kinetic characterizations in a high-throughput format, our group developed a so-called dual-point competition association assay based on the mathematical model developed by

Motulsky and Mahan.[56] The method measures radioligand binding at two different time points (t_1 and t_2) and their ratio (binding at t_2/binding at t_1) in the presence of an unlabeled competitor coined the kinetic rate index (KRI).[77] This approach enables direct comparison of ligand-receptor RTs, i.e. a KRI above 1.0 indicates a relatively slow dissociation from the target; a KRI below 1.0 or equal to 1.0 predicts relatively fast or similar dissociation kinetics as the radioligand. With this method we were able to screen a large number of in-house A_1AR antagonists and then rank their KRI values. We discovered the non-xanthine antagonists 8-cyclopentyl-2,6-diphenyl-7H-purine (**22,** LUF5962) and *N*-(6-(benzo[*d*][1,3]dioxol-5-yl)-5-cyano-2-phenylpyrimidin-4-yl)butyramide (**23**, LUF6057) have the highest and lowest KRI values, respectively. Consistently, these two compounds showed the slowest and fastest dissociation rates of 0.021 min^{-1} and 0.33 min^{-1}, respectively, in a follow-up competition association assay.

2.2. A_{2A}AR

Figure 4 has the chemical structures of representative A_{2A}AR ligands. Their binding kinetics are summarized in **Table 2**.

2.2.1. A_{2A}AR receptor agonists

One of the first kinetic investigations at the A_{2A}AR was performed with the radiolabeled 2-[*p*-(2-carboxyethyl)-phenethylaminol]-5'-*N*-ethylcarboxmido adenosine, (**24**, CGS21680). The ligand binds to the A_{2A}AR in rat striatal membranes with an on-rate of 2.1×10^7 $M^{-1}\cdot min^{-1}$ and an off-rate of 0.033 min^{-1}, equaling to a RT of 31 min.[96] Notably, two adenosine receptors (A_1AR and A_{2A}AR) are expressed in brain tissue at high levels.[97,98] CGS21680 displayed a high affinity to the A_{2A}AR (15 nM) while a 140-fold lower affinity was reported at the rat brain A_lAR.[99] This provides the advantage of directly labeling the A_{2A}AR receptor for binding and kinetic determinations, without the need to block the A_1AR. In contrast, when using nonselective

radioligands such as [^{3}H]-NECA[100] or [^{3}H]-XAC[101] in brain tissue/membrane preparations, the influence from the A_1AR needs to be blocked.

Table 2. Binding kinetics of A_{2A}AR ligands.

Cmpd	**Target**	**T (°C)**	**k_{on} ($M^{-1}\cdot min^{-1}$)**	**k_{off} (min^{-1})**	**Kinetic K_D** (nM)	**Ref.**
CGS21680	Rat striatal membranes	23	2.1×10^7	0.033	1.6	[96]
NECA	HEK293hA_{2A}AR membranes	4	1.9×10^6	0.053	28	[102]
UK432,097	HEK293hA_{2A}AR membranes	5	5.0×10^5	0.004	8.0	[103]
LUF5835	HEK293hA_{2A}AR membranes	5	1.6×10^7	0.29	18	[103]
PD115,199	Rat striatal membranes	25	1.1×10^9	1.24	1.1	[104]
XAC	Rabbit striatal membranes	24	1.3×10^8	0.36	2.8	[101]
SCH58261	CHOhA_{2A}AR membranes	25	6.4×10^8	1.5	2.3	[105]
ZM241385	HEK293hA_{2A}AR membranes	4	1.3×10^8	0.014	0.11	[106]
LUF6805	HEK293hA_{2A}AR membranes	4	2.0×10^8	0.35	1.8	[106]
LUF6632	HEK293hA_{2A}AR membranes	4	3.4×10^7	0.0031	0.091	[106]
34	Purified A_{2A}-StaR	25	2.4×10^8	0.061	0.25	[107]

Kinetic $K_D = k_{off}/k_{on}$.

The availability of a cell line stably expressing recombinant hA_{2A}ARs further provides convenience for kinetic investigations at one receptor subtype. This allows one to even use nonselective ligands for kinetic measurements. For instance, in our lab we determined the binding kinetics of [^{3}H]-NECA on HEK293hA_{2A}AR cell membranes by plotting its k_{obs} values as a function of the concentrations.[102] As a result, a significant linear correlation was observed, suggesting that the association of [^{3}H]-NECA to the A_{2A}AR follows a simple one-step process.

The k_{on} and k_{off} values derived from this method were 1.9×10^6 $nM^{-1} \cdot min^{-1}$ and 0.053 min^{-1}, respectively, equaling to a kinetic K_D of 27.9 nM.

In another study from our lab, we used the Motulsky and Mahan competition association assay[56] to analyze the binding kinetics for ten A_{2A}AR full and partial agonists, including both adenosine-like and non-adenosine derivatives.[103] A strong correlation was observed between the compounds' receptor RTs and their intrinsic efficacies gathered from a cAMP assay and a cell-based label-free impedance assay. On the contrary, the affinity of the A_{2A}AR agonists was not correlated with efficacy. Of all ten agonists tested, UK432,097 (**25**) displayed the longest RT at the receptor. The structural basis for its long receptor occupancy was elucidated by Stevens and colleagues from the Scripps institute, when they determined the crystal structure of the A_{2A}AR.[25] It was observed that the co-crystalized UK432,097 was accommodated in an extensive interaction network, including 11 hydrogen bonds, one strong aromatic stacking and a number of van der Waals interactions in the A_{2A}AR/UK432,097 complex. In comparison, the non-adenosine agonist, 2-amino-4-(3-hydroxyphenyl)-6-(1*H*-imidazol-2-ylmethylsulfanyl)pyridine-3,5-dicarbonitrile, (**26**, LUF5835), displayed the shortest receptor RT of 3.4 min. Such differences in kinetics highlight the structural importance of the ribose group, as demonstrated in several other adenosine-like agonist-bound crystal structures as well, in which the sugar moiety inserts deeply into the binding cavity and is stabilized by key residues such as $S277^{7.42}$ and $H278^{7.43}$.[25,27,108]

The elucidation of the agonist-bound A_{2A}AR structure also enabled molecular dynamics simulations of ligand-receptor interactions. Recently, Lee and Lyman reported microsecond-time scale simulations of the A_{2A}AR bound to either adenosine or UK432,097.[109] The binding of adenosine to the A_{2A}AR was highly dynamic, in stark contrast to the case with UK432,097, which was stabilized in a much tighter neighborhood in the binding cavity. The two aromatic rings of UK432,097 blocked the entrance to the binding pocket, gating the access of water to the

interior of the protein, consistent with its long receptor RT. On the contrary, the binding pocket appears much more hydrated in the case of adenosine and thus the ligand has to constantly compete with water molecules to form transient hydrogen bonds. Furthermore, the authors also suggested the possibility of an additional binding site located closer to the extracellular entrance to the binding pocket. It is likely that such a binding site is vital along the trajectories of ligand association to and egress from the receptor, resembling the so-called “extracellular vestibule” on other GPCRs.[110,111]

Based on the results mentioned above, several features that affect the kinetics of agonists at the A_{2A}AR can be hypothesized. Firstly, upon binding agonists that contain a polar ribose group – which is true for the majority of adenosine receptor agonists - can form a number of hydrogen bonds with the receptor. This anchoring accommodates the ligand in the binding pocket, otherwise occupied by three water molecules. Secondly, the ligand’s dissociation process is influenced by the hydration state of the binding pocket after binding of the agonist. In a highly hydrated binding cavity, the bound ligand competes with water molecules to form polar interactions with the receptor. This possibly reduces the ligand’s residence time in the receptor, while ligands can stay longer in a less hydrated binding site. Thirdly, ligands that form transient interactions with the receptor along their dissociation trajectories might display longer residence times on the target, while not so for those with fewer interactions.

2.2.2. A_{2A}AR receptor antagonists

Binding kinetics studies for A_{2A}AR antagonists are emerging. This started from the application of tritium-labeled non-selective adenosine receptor antagonists, such as *N*-[2-(dimethylamino)ethyl]-*N*-methyl-4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)benzenesulfonamide (**27**, PD115,199)[104] and XAC (**19**)[101]. The kinetics of both compounds were measured in the presence of an A_1AR-selective antagonist to eliminate the binding to the

A_1AR in rat/rabbit brain striatal membranes. Such an approach allowed a reliable determination of the A_{2A}AR-related kinetics. XAC displayed rapid binding kinetics (k_{on} = 1.3 × 10^8 M^{-1}·min^{-1}, k_{off} = 0.36 min^{-1}) at 24 °C. The association and dissociation of PD115,199 was fast as well at a similar temperature (25 °C) with an on-rate of 1. 1 × 10^9 M^{-1}·min^{-1} and an off-rate of 0.36 min^{-1}.[101,104]

In 1994, Nonaka *et al*. examined the potential of the radiolabeled 8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxanthine (**28**, KF17837S) as the first selective antagonist radioligand for the A_{2A}AR.[112] This compound was characterized with kinetics similar to that of [^{3}H]-XAC, yet slower than [^{3}H]-PD115,119. Another xanthine derivative with high A_{2A}AR affinity and selectivity is 3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine (**29**, MSX-2), developed by Müller and colleagues.[113] This xanthine derivative also displayed binding kinetics to the A_{2A}AR in a similar range as the other aforementioned analogues.

Non-xanthine antagonists have also been developed. Two examples are 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (**30**, SCH58261) and ZM241385 (**31**). Both SCH58261 and ZM241385 have been prepared in a tritiated form[114,115], the latter of which has also been available in a radioiodinated form[116], enabling direct measurement of their on- and off-rates in kinetic radioligand binding assays. [^{3}H]-SCH58261 showed fast association and dissociation at both rat striatal membranes[114] and membranes from CHO cells stably transfected with the human A_{2A}AR[105] at 25 °C (rat: k_{on} = 1.15 × 10^9 M^{-1}·min^{-1}, k_{off} = 1.12 min^{-1}; human: k_{on} = 6.4 × 10^8 M^{-1}·min^{-1}, k_{off} = 1.5 min^{-1}). Kinetics for [^{3}H]-ZM241385 have been extensively studied in several studies, including characterizations at different temperatures[103,115] or at A_{2A}AR membranes from different species[117]. Such abundant information, together with its ideal binding properties (i.e., low non-specific binding and high receptor affinity), benchmarked

ZM241385 as a proper reference compound for follow-up studies.[103,106] One example is a combined SKR and SAR study from our group.[106] An ensemble of 24 A_{2A}AR antagonists, all ZM241385 derivatives with variations at the C_2-position, displayed only minor differences in affinity, while they varied substantially in their dissociation rates from the receptor. Among these derivatives, N^5-(2-(4-(3,4-difluorophenyl)piperazin-1-yl)ethyl)-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazine-5,7-diamine (**32**, LUF6632), a high-affinity A_{2A}AR antagonist previously synthesized and reported by Vu *et al.*[118], showed the slowest dissociation rate (k_{off} = 0.0031 min^{-1}), which was approximately five-fold slower than that of ZM241385. In comparison, 2-(furan-2-yl)-N^5-(2-(4-phenylpiperidin-1-yl)ethyl)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazine-5,7-diamine (**33**, LUF 6805) displayed the fastest dissociation rate of 0.35 min^{-1}, equaling to a RT of 3 min. LUF6805 and LUF6632 was also compared in a cAMP assay using different formats (co-application vs. pre-incubation). It appeared that LUF6632, rather than LUF6805, was a competitive, insurmountable antagonist at the hA_{2A}AR—a phenomenon caused by so-called hemi-equilibrium due to its long A_{2A}AR residence time profile.[119]

Next to the application of radioligand binding assays, several studies used surface plasmon resonance (SPR) measurement for binding and kinetic investigations. One of the examples is the discovery of 1,2,4-triazine derivatives as A_{2A}AR antagonists, in which 4-(3-amino-5-phenyl-1,2,4-triazin-6-yl)-2-chlorophenol (**34**) displayed the slowest dissociation rate (0.061 min^{-1}) while its analogue 5,6-diphenyl-1,2,4- triazin-3-amine (**35**) showed a dissociation rate more than 1000-fold faster.[107] The crystal structure of the A_{2A}AR in complex with **34** was elucidated, illustrating that the compound bound deeply inside the orthosteric binding cavity having significant interactions with Ile66$^{2.64}$, Leu85$^{3.33}$, Asn181$^{5.42}$ and Asn253$^{6.55}$. An analysis of the binding site in the apo A_{2A}AR structure (with the co-crystalized ligand removed) demonstrated that the compound occupied exactly the same region where a cluster of “unhappy”

water molecules (> 2.2 kcal/mol vs bulk solvent) existed.[120] Displacing these water molecules is entropically favorable, which perhaps accounts for the slow dissociation profile of **34**.[121]

It is important to mention that the required immobilization of the (purified) protein on the SPR chip can potentially block the accessibility of the intra- or extracellular side of the receptor, and may consequently affect ligand-receptor binding kinetics. The influence of lipid composition upon assay performance was recently investigated in a comparative study of the adenosine A_{2A} receptor employing four different reconstitution approaches.[66] When the receptor was reconstituted in lipid nanodiscs, protein stability was enhanced and the kinetic data obtained were more similar to native receptors compared with those solubilized in detergents. More specifically, the difference was mainly in their association rates ($k_{on/nanodisc}$ = 3.67 $\times 10^6$ $M^{-1} \cdot s^{-1}$, $k_{on/detergent}$ = 7.34 $\times 10^5$ $M^{-1} \cdot s^{-1}$), while less so in their dissociation rates ($k_{off/nanodisc}$ = 0.76 $\times 10^{-2}$ s^{-1}, $k_{off/detergent}$ = 1.60 $\times 10^{-2}$ s^{-1}). This resulted in different K_D values, where nanodisc-solubilized A_{2A}AR receptors displayed a K_D value (1.98 nM) quite similar to those embedded in native membranes (K_D = 0.93 nM) as determined in radioligand binding assays. In comparison, the K_D value for detergent solubilized receptors was 21.7 nM. Similar findings were shared by Cooke and colleagues from Heptares Therapeutics as well.[65] In combination these studies demonstrate the influence of native membrane composition upon ligand-receptor interactions.[54]

2.3. A_{2B}AR

Ligand-receptor binding kinetics at the A_{2B}AR have been less investigated compared to the other AR subtypes. To date, most of the kinetic data for the A_{2B}AR were obtained by using kinetic radioligand binding assays. **Figure 5** has the chemical structures of representative A_{2B}AR ligands and their binding kinetics are in **Table 3**.

Table 3. Binding kinetics of A_{2B}AR ligands.

Cmpd	Target	T (°C)	k_{on} ($M^{-1}\cdot min^{-1}$)	k_{off} (min^{-1})	Kinetic K_D (nM)	Ref.
MRS1754	HEK293hA_{2B}AR membranes	25	2.2×10^7	0.027	1.2	[122]
MRE2029-F20	CHOhA_{2B}AR membranes	4	1.7×10^7	0.031	1.8	[123]
OSIP339391	HEK293hA_{2B}AR membranes	22	9.5×10^7	0.039	0.41	[124]

Kinetic $K_D = k_{off}/k_{on}$.

N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl) -phenoxy]acetamide (**36**, MRS1754) was the first selective A_{2B}AR antagonist reported with binding kinetics (k_{on} = 2.2 × 10^7 $M^{-1}\cdot min^{-1}$, k_{off} = 0.027 min^{-1}).[122] Following this study, Baraldi and workers reported the synthesis and evaluation of a series of 8-heterocyclic substituted xanthine derivatives.[125] This led to the development of *N*-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide (**37**, MRE2029-F20) in a tritiated form. Its kinetics were determined on recombinant CHOhA_{2B}AR cell membranes with an association rate of 1.7 × 10^7 $M^{-1}\cdot min^{-1}$ and a dissociation rate of 0.031 min^{-1}.[123] Müller and colleagues also developed a xanthine derivative, namely 1-alkyl-8-(piperazine-1-sulfonyl)phenylxanthine (**38**, PSB603), with kinetics similar to that of MRS1754.[126] Other than the xanthine-like analogues, non-xanthine antagonists have been developed as well. One representative example is *N*-(2-{2-phenyl-6-[4-(3-phenylpropyl)-piperazine-1-carbonyl]-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-ylamino}-ethyl)-acetamide (**39**, OSIP339391).[124] Its binding kinetics on the A_{2B}AR were in the same range of other A_{2B}AR ligands, although OSIP339391 displayed a higher affinity (K_D = 0.17 nM) than others (MRS1754, K_D = 1.1 nM; MRE2029-F20, K_D = 1.7 nM; PSB603, K_D = 0.40 nM).

2.4. A_3AR

Kinetic data for the A_3AR are emerging. In **Figure 6** the chemical structures of representative A_3AR ligands are depicted. Their binding kinetics are in **Table 4**.

Table 4. Binding kinetics of A_3AR ligands.

Cmpd	Target	T (°C)	k_{on} ($M^{-1}\cdot min^{-1}$)	k_{off} (min^{-1})	Kinetic K_D (nM)	Ref.
I-AB-MECA	HEK293hA_3AR membranes	37	6.1×10^7	0.042	0.69	[127]
MRS5127	HEK293hA_3AR membranes	25	2.4×10^8	0.51	2.1	[128]
MRE3008F20	CHOhA_3AR membranes	4	7.6×10^7	0.042	0.55	[129]

Kinetic $K_D = k_{off}/k_{on}$.

2.4.1. A_3AR receptor agonists

The most widely used radioligand on the A_3AR is the high-affinity agonist, 3-iodo-4-aminobenzyl-5'-*N*-methylcarboxamidoadenosine (**39**, I-AB-MECA),[130] first prepared in its radioiodinated form by Olah *et al*.[131] The kinetics of [^{125}I]-I-AB-MECA are relatively slow (k_{on} = 6.1×10^7 $M^{-1}\cdot min^{-1}$; k_{off} = 0.042 min^{-1}, 37 °C) compared to aforementioned agonistic radioligands on other AR subtypes.[127] This is most likely due to its bulky aromatic substitution pattern at the N^6 position of the adenine moiety, resembling the role of the phenyl moieties in UK432,097 on the A_{2A}AR.

Placing an aliphatic substitution on the C-2 position of the adenine core seems to yield compounds with slow kinetics too, as exemplified by the 2-substituted adenosine receptor agonist, 2-hexyn-1-yl-N^6-methyladenosine (**41**, HEMADO). Its association at a concentration of

1 nM reached equilibrium between 30 min and 60 min, while its dissociation from the A_3AR was complete in 60 min, all at room temperature.[132]

In comparison, the radioiodinated (1′*R*,2′*R*,3′*S*,4′*R*,5′*S*)-4′-[2-chloro-6-(3-iodobenzylamino)-purine]-2′,3′-O-dihydroxy-bicyclo-[3.1.0]hexane (**42**, MRS5127), reported by the Auchampach group, displayed much faster kinetics with association and dissociation both completed in less than 10 min (k_{on} = 2.4 × 10^8 M^{-1}·min^{-1}, k_{off} = 0.51 min^{-1}).[128] Notably, the 5'-hydroxylmethylene moiety in the ribose group is absent in MRS5127 and was replaced with a rigid *N*-methanocarba(bicyclo[3.1.0]hexane) ring system as a ribose substituent. This might be the structural basis for MRS5127's shorter residence time, since the truncation at the 5'-position probably causes the loss of H-bond interactions with residues in the binding pocket—a key feature for long agonist-AR residence time, as demonstrated in the aforementioned A_{2A}AR crystal structures[25,27,108] and in the molecular dynamics study.[109]

2.4.2. A_3AR receptor antagonists

A series of tricyclic imidazo[2,1-*i*]purinones derived from xanthine derivatives has been prepared with the aim of improving A_3AR affinity.[133] Among this series, 8-ethyl-4-methyl-2-phenyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purin-5-one (**43**, PSB-11) possesses high affinity for the hA_3AR (K_i = 2.3 nM) and selectivity over other ARs. Kinetic characterization revealed this ligand quickly associates to and dissociates from CHOhA_3AR cell membranes. Both processes were completed within 10 min.[134] In another study, the Baraldi group reported a series of substituted pyrazolotriazolo pyrimidines as selective antagonists for the A_3AR.[135] *N*-[2-(2-Furanyl)-8-propyl-8*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-yl]-*N*'-(4-methoxyphenyl)urea (**44**, MRE3008F20) was one of a number of compounds showing high A_3AR affinity (K_i = 0.29 nM). The kinetics of MRE3008F20 were determined in a kinetic radioligand binding assay with k_{on} = 7.6 × 10^7 M^{-1}·min^{-1} and k_{off} = 0.042 min^{-1}.[129]

2.5. Summary

The association rate constants of AR ligands vary substantially, across several orders-of-magnitude. It had been assumed that target engagement is a diffusion-limited process by definition, with a corresponding rate constant for a ligand and a receptor encountering each other in the right stoichiometry and to form a binary complex.[136,137] The data in **Tables 1-4** demonstrate that this general assumption is not necessarily true. Most of the association rate constants are in the range well below the diffusion-limited value (about $6 \times 10^9 \sim 6 \times 10^{10}$ $M^{-1} \cdot min^{-1}$).

Of note, the binding kinetics of orthosteric AR ligands summarized in Table 1-4 were obtained on varies species. Additionally, earlier kinetic studies were largely performed on tissue preparations rather than on membranes prepared from recombinant cell lines. These experimental variations could result in a different kinetic readout, which needs to be taken into account when comparing these data.

The residence times of AR ligands also vary significantly, ranging from a few seconds to several minutes or hours, which might correspond to divergent biological effects. It is tempting to speculate whether long or short RT AR ligands are needed to direct future medicinal chemistry efforts. A ligand's potential clinical indication as well as its on-target side effects should, therefore, be carefully considered. For instance, on the A_3AR, acute or chronic treatment with the agonist IB-MECA induced paradoxical effects: the acute administration of the agonist to an *in vivo* gerbil model of global ischemia exacerbated histologic and functional damage, while chronic treatment of the same agonist over several weeks led to a highly neuroprotective, postischemic effect.[138,139] Given these primary observations it might be useful to design long RT agonists for the A_3AR. A_{2A}R antagonists have been developed for the treatment of Parkinson's disease (PD) in combination with dopaminergic therapies.[140,141] A compound with a long receptor RT might be

preferred, since a sustained receptor occupancy can potentially reduce the wear-off effect of dopaminergic medications (e.g. levodopa) in between doses.[141] Such information obtained from a kinetic perspective may provide additional rationales for the design of new AR ligands.

3. THE INFLUENCE OF AN ALLOSTERIC MODULATOR ON THE ORTHOSTERIC LIGAND'S BINDING KINETICS

ARs, as many GPCRs, can be allosterically modulated.[14,15] However, a 'pure' modulating effect is rare for ARs (and other GPCRs), which is often "contaminated" with inhibition of the binding at the orthosteric site. Particularly in rhodopsin-like (class A) GPCRs the binding site for an allosteric modulator may be quite close to the orthosteric binding pocket, leading to the modulator also displaying activities as a competitor agonist/antagonist and decreasing the binding of the orthosteric ligand. Furthermore, an allosteric action to increase the binding of any adenosine remaining in e.g., membrane preparations may also contribute to a decrease in equilibrium radioligand binding. Given these complications, determining the binding kinetics, in particular the dissociation rate of an orthosteric ligand (or so-called "probe") in the presence of an allosteric modulator has been used as an unambiguous measure of allosteric activity.[142] In the following section, chemical classes of allosteric ligands and their structure-activity/kinetic relationships will be discussed. We will mainly focus on the allosteric effects on binding and kinetics of the orthosteric ligands unless mentioned otherwise.

3.1. A_1AR

Chemical structures of representative A_1AR allosteric modulators are represented in **Figure 7**. The allosteric activity of these compounds is summarized in **Table 5**.

Table 5. Allosteric and antagonistic activities of A_1AR allosteric modulators.

Cmpd	Allosteric effect	EC_{50} (μM) [a]	% antagonism [b]	Ref.
PD81,723	k_{off} of [^{3}H]-CCPA decreased 30%; AE score = 28%	15	40	143,144
46	122% enhancement [c]	6.0	32	145
T-62	123% enhancement [c]	6.8	40	145
LUF5484	151% enhancement [c]	6.2	35	145
49	k_{off} of [^{3}H]-CCPA decreased 46%	nd	4.8	143
50 [d]	AE score = 99%	nd	13	146
51 [d]	AE score = 91%	6.4	28	147
54 [e]	k_{off} of [^{3}H]-NECA decreased 52%	nd	nd	148
55 [e]	k_{off} of [^{3}H]-NECA decreased 59%	nd	nd	149
56	AE score = 57%	6.6	58	150
57	AE score = 18%	2.1	82	150
58	AE score = 77%	16	61	150
59	AE score = 80%	1.4	9.2	144
60 [f]	AE score = 60%	14	23	151
61	AE score = 74%	0.3	nd	152
SCH202676	k_{off} of [^{3}H]-DPCPX decreased 69%	nd	-49	153
LUF5794	k_{off} of [^{3}H]-DPCPX decreased 63%	nd	-32	153
Amiloride [g]	k_{off} of [^{3}H]-DPCPX decreased 46%	nd	nd	154
HMA [h]	k_{off} of [^{3}H]-DPCPX decreased 69%	nd	nd	154

Data were obtained at 10 μM of test compounds on membranes of CHO cells expressing recombinant A_1AR at 25 °C unless mentioned otherwise. AE scores were measured 10 min after the initiation of dissociation, and ranges from 0 to 100%. A score of 100% means no dissociation and a score of zero means complete dissociation. nd, not determined. [a] EC_{50} for enhancing activity. [b] Orthosteric antagonistic activity, % inhibition of an orthosteric radioligand by 10 μM test compounds. Negative values indicated enhancement of radioligand binding. [c] Enhancing activity by test compounds expressed as percentage decrease in [^{3}H]-CCPA dissociation over control (0%) and that of

10 μM PD81,723 (100%). Data were obtained on rat brain cortex membranes. [d] Data were obtained at 100 μM of the test compound. [e] Data were obtained at 1 μM of the test compound at 37 °C. [f] Data were obtained at 50 μM of the test compound. [g] Data were obtained at 1 mM of the test compound on rat forebrain membranes at 25 °C. [h] Data were obtained at 0.1 mM of the test compound on rat forebrain membranes at 25 °C

The first AR allosteric ligand for the A_1AR was reported by Bruns and Fergus in 1990.[155] Several compounds from a series of 2-amino-3-benzoylthiophenes, originally prepared as intermediates in the synthesis of benzodiazepine-like compounds[156], were serendipitously discovered to possess an allosteric enhancing (AE) effect on the A_1AR. These compounds were reported to increase the binding of the agonist radioligand [^{3}H]-CHA to the A_1AR in rat brain membranes and retard the dissociation of the radioligand, implying an allosteric mechanism of action. The compounds also behaved as competitive antagonists at the same receptor, since concentration-effect curves were bell-shaped with up to 45% stimulation of binding at 10 μM followed by inhibition at higher concentrations. Among these first-generation AR enhancers, 2-amino-4,5-dimethyl-3-thienyl)-[3-(triflurомethyl)-phenyl]methanone (**45**, PD81,723) was found to be the most potent compound, displaying the best ratio of enhancement and antagonism in a following-up study from the same research group.[142] Subsequently, PD81,723 has been investigated pharmacologically in greater detail by various research groups. It was convincingly demonstrated that PD81,723 also enhanced the preconditioning effect of endogenous adenosine in the heart, which proves the aforementioned concept of allosteric modulation.[157] Furthermore, long-term exposure of cells expressing the human A_1AR to PD81,723 induced only minor desensitization and down-regulation, which is encouraging in terms of therapeutic potential.[158] For these reasons, PD81,723 became the reference allosteric modulator of A_1AR and many follow-up structure-activity relationship studies were performed.

3.1.1. 2-amino-3-substituted thiophenes

Initial structure-activity relationships for enhancement of A_1AR agonist binding (i.e. slowing the probe's dissociation rate) by 2-amino-3-benzoylthiophenes were reported by Burns and coworkers.[142] They concluded that the 2-amino group was essential as well as the carbonyl of the benzoyl moiety. Alkyl substitution at the 4-position of the thiophene ring augmented activity, as did substitutions on the phenyl ring, with the 3-trifluomethyl substituent showing optimal activity.

Based on the initial SAR, van der Klein *et al*. modified the 4,5-dimethyl group and the substitution at the benzoyl moiety of PD81,723, yielding a series of compounds bearing 4,5-dialkyl group superior to PD81,723 in terms of enhancing/antagonistic ratio.[145] More specifically, (2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)(3-(trifluoromethyl)phenyl)methanone (**46**), (2-amino-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)(4-chlorophenyl)methanone (**47**, T-62) and (2-amino-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)(3,4-dichlorophenyl)methanone (**48**, LUF5484) showed significant enhancement (122%, 123% and 151% respectively) in [^{3}H]-CPA dissociation experiments compared to PD81,723 (100%). Their antagonistic activity on the A_1AR was comparable to that of PD81,723. From this study it was concluded that lipophilic benzoyl substitutions, such as 3-CF_3 and 3,4-diCl, and thiophene 4-alkyl substitution favored AE activity, whereas bulky substitution at the 5-position favored antagonist properties. T-62 was later prepared in a tritiated form as the first radiolabeled A_1AR allosteric enhancer by Baraldi group.[159] The biological evaluation of [^{3}H]-T-62, although not very conclusive, suggested the presence of an allosteric binding site on the A_1AR different from the binding site for orthosteric agonists and antagonists.

The enhancing action of cycloalkyl-substituted thiophene derivatives was subsequently confirmed by Baraldi and coworkers.[143] It was demonstrated that the cycloalkylthiophenes tended

to be more potent than their 4,5-dimethyl analogues. In addition to the chemical modification at the 4- and 5-positions of the thiophene moiety, the authors also varied the structures by replacing the original benzoyl moiety with a naphthoyl ring. This led to the discovery of (2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)(4-chloronaphthalen-1-yl)methanone (**49**), more potent than PD81,723 in slowing down the dissociation of [^{3}H]-CCPA from CHOhA_1AR cell membranes (i.e. k_{off} decrease = 46% compared to 30% for PD81,723 at 10 μM). The inhibition activity of **49** at 10 μM on the A_1AR was modest as that of PD81,723. Furthermore, the allosteric effect of **49** was corroborated in a cAMP assay, in which forskolin-stimulated cAMP accumulation, challenged by CPA, was further decreased to 45%, similar to that of PD81,723 (50%).

The initial SAR proposed by Bruns *et al*. was further extended and updated by Tranberg *et al*., who performed a systematic survey of the 2-amino-3-aroyl-4,5-alkylthiophenes derivatives.[146] The authors specifically introduced polymethylene bridges of various length linking the 4 and 5-positions, in combination with divergent aromatic substitutions at the 3-position. Compounds from aforementioned studies[142,145] were also included to have a direct comparison of the AE activity. In this study, the AE score of different compounds was assayed as a decrease of the dissociation rate of ^{125}I-ABA from the A_1AR. GTPγS was specially introduced in the assay to speed up the dissociation of the radioligand, since in the absence of the nucleotide the dissociation of ^{125}I-ABA was very slow ($t_{1/2} \approx 60$ min). Briefly, compounds without 4- and 5-substitutions had little or no AE activity. The same holds for the replacement of the 2-amino group with 2-carboxyl. Increasing the size of the thiophene C-4 and C-5 substituents significantly enhanced AE activity, which follows an ascending order of 4,5-diH, 4,5-diMe, cPent, cHex and cHep. With respect to C-3 position substitution, bulky (or hydrophobic) groups at the *meta* and *para* positions of the benzoyl group augmented AE activity. Replacing the 3-benzoyl group with a 3-naphthoyl group also had a favorable effect on the AE. Taken together, it is reasonable to

speculate that the allosteric binding site of the A_1AR has ample space to accommodate 3-aroyl substituents that are bulky and/or hydrophobic but not necessarily planar; a second region of the binding pocket could interact with alkyl substituents at thiophene's C-4 and/or C-5 positions. Of the tested compounds (2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophen-3-yl)(3-methoxyphenyl)methanone (**50**) was discovered with the highest AE score (99%) while its inhibition of the antagonist radioligand [^{3}H]-DPCPX was 13 %, much lower than that of PD81,723 (42%) at 100 μM.

In a follow-up study, the same research group also tried 3,4- and 5-modifications at the 2-amino-3-benzoylthiophene derivatives, including large space-filling groups, such as phenyl or substituted phenyl at the C-4 and/or C-5 positions.[147] This generated various compounds with superior AE activity to that of PD81,723. The compound with the best AE score (91%) was [2-amino-5-bromo-4-(3-trifluoromethylphenyl)thiophen-3-yl]phenylmethanone (**51**) and at 100 μM it displaced less [^{3}H]-DPCPX (29%) compared to PD81,723 (85%).

Substitutions at both C-4 and C-5 positions of thiophenes appear to have synergistic effects in enhancing the AE activity on the A_1AR. To further study the role of the C4-position alone, a series of 2-amino-3-(*p*-chlorobenzoyl)thiophenes with different phenyl-substituted piperazine moieties attached to the C-4 part of the thiophene ring was prepared and biologically evaluated by the Baraldi group.[160] The authors found that the nature of substituents on the phenyl ring tethered to the piperazine seem to exert a significant influence on the allosteric enhancer activity, with the 4-chlorophenyl analogue, (2-amino-4-((4-(4-chlorophenyl)piperazin-1-yl)methyl)-5-phenylthiophen-3-yl)(4-chlorophenyl)methanone (**52**), and the 4-trifluoromethyl analogue, (2-amino-5-phenyl-4-((4-(4-(trifluoromethyl)phenyl)piperazin-1-yl)methyl)thiophen-3-yl)(4-chlorophenyl)methanone (**53**), being the most active compounds in binding and functional cAMP studies.

Encouraging results were found for 4-neopentyl substituted analogues as well.[161] As a follow-up study, Baraldi and coworkers further examined the role of the C5-position on the AE activity in a second series of 4-neopentyl derivatives.[148] The presence of an phenylacetylene at the 5-position of the thiophene proved optimal for activity. As evidence, even at a low concentration (1μM), (2-amino-4-neopentyl-5-(2-phenylethynyl)thiophen-3-yl)(4-chlorophenyl)methanone (**54**) significantly retarded the dissociation of the agonist [^{3}H]-NECA from the A_1AR (control: k_{off} = 0.016 min^{-1}; + **54**, k_{off} = 0.0076 min^{-1}), superior to that in the presence of PD81,723 at the same concentration (k_{off} = 0.0143 min^{-1}).

Romagnoli *et al*. continued and extended the series of 2-amino-3-(*p*-chlorobenzyl)thiophene to elaborate on the combined C-4 and C-5 positions of the scaffold.[149] They found that the allosteric contributions of phenyl and benzyl groups at these sites were clearly position dependent. Analogues with the phenyl group at the C-5 position and the benzyl group at the C-4 position were shown to be more efficacious than their corresponding regioisomeric 4-aryl-5-benzylthiophenes. In an SAR study, [2-amino-4-(4-chlorobenzyl)-5-(4-chlorophenyl)-3-thienyl](4-chlorophenyl)methanone (**55**) showed the most prominent AE activity, significantly retarding the dissociation of [^{3}H]-NECA from the A_1AR (control: k_{off} = 0.019 min^{-1}; + **55**: k_{off} = 0.0079 min^{-1}). Compound **55** also showed a good anti-nociceptive effect in a formalin assay using a mouse model, confirming that allosteric enhancers at the A_1AR could be useful in pain modulation.

The structure-activity relationships at the C-5 position have also been extensively examined by Scammells and colleagues.[150] The authors prepared two series of 5-substituted 2-amino-4-(3-trifluoromethylphenyl)thiophenes. In the 3-ethoxycarbonyl series, ethyl 2-amino-5-(4-chlorophenyl)-4-(3-trifluoromethylphenyl)thiophene-3-carboxylate (**56**) was the most potent and efficacious (EC_{50} = 6.6 μM, AE score = 57.0 %) in slowing down [^{125}I]-ABA's dissociation rate.

In the 3-benzoyl series, a series of 5-phenyl moieties with both electron-donating and -withdrawing activity were prepared as well as an analogue without a C-5 substituent. It was found that [2-amino-5-phenyl-4-(3-trifluoromethylphenyl)thiophen-3-yl]phenylmethanone (**57**) conferred the greatest AE potency (ED_{50} = 2.1 μM, AE score = 18%) but its counterpart with no 5-substitution, i.e. [2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl]phenylmethanone (**58**), proved to be most efficacious (ED_{50} = 16 μM, AE score = 77%). However, all compounds showed higher antagonist activity than PD81,723 in a [^{3}H]-DPCPX competitive binding assay, evidence for their additional antagonist profile.

Nikolakopoulos *et al*. studied three series of 2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene and 2-amino-5,6,7,8-tetrahydrocyclohepta[*b*]thiophenes with 3-carboxylates and carboxamides as potential A_1AR allosteric enhancers.[144] A structure-activity relationship study of these compounds led to the discovery of 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylic acid (**59**) with 80% AE activity, higher than 28% of PD81,723.

Further optimization attempts were performed by introducing a fused ring to the thiophene core connecting the C-4 and the C-3 positions. Accordingly, a series of dihydrothieno[3,4-*d*]pyridazines was synthesized and pharmacologically explored as new A_1AR allosteric modulators.[151] However, these compounds displayed potent orthosteric displacement, although all compounds from this series recognized the allosteric binding site on the A_1AR, as revealed by an agonist radioligand kinetic dissociation assay. Ethyl 5-Amino-4-oxo-3-phenyl-3,4-dihydrothieno[3,4-*d*]pyridazine-1-carboxylate (**60**) is an illustrative compound showing a good AE score (60%) and modest inhibition of [^{3}H]-DPCPX binding (23%)

3.1.2. 2-aminothiazoles

2-Aminothiazoles represent another chemical class of allosteric modulators for the A_1AR, firstly reported by Chordia *et al*.[152] A SAR analysis of ligands from this series indicates that (1) tricyclic

2-aminothiazole derivatives are generally more AE active than their bicyclic counterparts, and among the tricyclic derivatives, compounds with the 6+5+5 ring system are more potent; (2) exchanging the positions of nitrogen and sulfur has a deleterious effect on the AE activity, suggesting an important role of the nitrogen for molecular recognition; (3) an electron-donating moiety on the aromatic ring improves AE activity. 2-Amino-3a,8a-dihydro-8*H*-indeno[1,2-*d*]thiazol-7-yl acetate hydroiodide (**61**) was the most potent and efficacious compound in this series.

A number of 2-aminothiazoles and their amide derivatives was also prepared in our lab. However, these compounds exerted little AE activity, in stark contrast to the abovementioned findings from Chordia *et al.*[152] Interestingly, in a subsequent study Chordia *et al.* investigated these contrasting results and reconfirmed the AE activity of the 2-aminothiazoles. The authors suggested that one possible reason for the contradictory observations might be the different purification methods from two labs. Chordia *et al.* used 2-aminothiazole hydroiodide salts, whereas our lab prepared the free base form by base treatment of hydroiodide salts and purification by silica gel column chromatography. It was found that the free base variants of this type of compounds in freshly made solutions were only weak allosteric enhancers. However, the AE activity in solutions of these compounds in DMF, DMSO or acetonitrile increased after 4 h and finally led to a constant activity equivalent to that of the hydroiodide salts after 12 h.[162] Another putative factor complicating the interpretation of the different results might be the so-called probe-dependency, that is, the extent of allosteric action depending on the nature of the orthosteric ligand.[163,164] Notably, the assessment of AE activity of the 2-aminothiazoles was undertaken with different orthosteric radioligands (i.e. [^{3}H]-CCPA and ^{125}I-ABA) in different labs. Given this, it might be interesting to further examine the same compound prepared in the

same form (free base or hydroiodide salt) using the same radioligand to confirm the AE profile of the 2-aminothiazoles.

3.1.3 Thiadiazoles as putative allosteric modulators.

Several ligands have been reported as promiscuous modulators for GPCRs. One exemplary case is *N*-(2,3-diphenyl-1,2,4-thiadiazol-5(*2H*)-ylidene)methanamine (**62**, SCH202676). It has been shown to interact with many GPCRs, including opioid, muscarinic, adrenergic and dopaminergic receptors.[165] The allosteric modulating effect of SCH-2022676 on the ARs was examined in our lab, along with a series of newly prepared thiadiazoles with varied substitutions at the 2-, 3- and 5-positions. SCH-2022676 and 5-*N*-methylimino-2-(4-methoxyphenyl)-3-phenyl-*2H*-[1,2,4]thiadiazole (**63**, LUF5794) strongly decreased the dissociation rate of [^{3}H]-DPCPX (from 0.35 min^{-1} to 0.11 min^{-1} and 0.13 min^{-1}, respectively), thereby resulting in a prolonged $t_{1/2}$ value for the dissociation process. The apparent K_D of [^{3}H]-DPCPX in the presence of LUF5794 was slightly increased, indicating its role as an enhancer of antagonist binding to the A_1AR.[153] However, a follow-up study from our lab showed that thiadiazoles are sulfhydryl modifying agents rather than allosteric modulators as they appear to reversibly modify the sulfhydryl groups of cysteine residues in cell membrane preparations.[166] This general feature explains their non-selective modulating effect on GPCR binding and functioning. A similar mechanism was suggested for 1,2,4-thiadiazoles acting as cathepsin B inhibitors[167] or melanocortin MC4 receptor agonists[168].

3.1.4. Non-selective allosteric modulators.

An allosteric regulation of ligand binding by sodium ions was observed on the A_1AR as well as for GPCRs from other subfamilies such as dopamine D_2,[169] opioid,[170] or α-adrenergic receptors,[171] among others.[38] At high-salt concentrations, agonist affinity for the receptor decreased one order of magnitude. The effect occurred through the binding of Na^+ at the

Asp55$^{2.50}$ site. As evidence receptor agonist affinity did not change over a range of sodium ion concentrations from 0-400 mM when the residue was mutated to Asp55Ala$^{2.50}$.[38]

Amiloride (**64**), a potassium-sparing diuretic drug, is also acting as a promiscuous agent for many GPCRs,[171-173] including the AR. The action of the compound was initially postulated to be linked to its known action of inhibiting Na^+ transporter systems.[174,175] However, on the A_1AR the addition of NaCl (145 mM) enhanced the maximal binding capacity of [^{3}H]-DPCPX, which was against the inhibitory effect of [^{3}H]-DPCPX binding in the presence of amiloride. Additionally, the relationship between the structure of amiloride analogues and their affinity for the A_1AR differed from the known SAR for the Na^+/H^+ exchange/transporter system. These results thus excluded the involvement of the Na^+/H^+ exchange system.[176] Amiloride increased the dissociation rate constant of [^{3}H]-DPCPX significantly (control, k_{off} = 0.13 min^{-1}; + 1 mM amiloride, k_{off} = 0.19 min^{-1}). However, at the same concentration amiloride displayed no effect on the dissociation rate constant of the agonist radioligand [^{3}H]-*R*-PIA. Similarly, 5-(*N*,*N*-hexamethylene)amiloride (**65**, HMA), an amiloride analogue, showed a negative allosteric effect on the dissociation of [^{3}H]-DPCPX from the A_1AR (control, k_{off} = 0.13 min^{-1}; + 0.1 mM HMA, k_{off} = 0.22 min^{-1}) while not so on [^{3}H]-*R*-PIA.[154]

3.1.5. Binding sites for A_1AR allosteric modulators

Probing the binding site for an allosteric modulator is difficult, since residues at the allosteric site in GPCRs are supposed to be less conserved among family subtypes. Although the crystallographic structure of the M_2 muscarinic receptor simultaneously bound to the orthosteric agonist iperoxo and the positive allosteric modulator LY2119620 has been recently been elucidated[177], still little is known about the possible molecular mechanism of allosteric modulation at other GPCRs.[178] To date, in the absence of structural information, the exact location of the allosteric site on the A_1AR remains unknown.

Since most of the allosteric modulators on the A_1AR showed mixed profiles of orthosteric antagonist as well as allosteric modulator, several lines of research suggest that the allosteric site might be in the proximity of the orthosteric site. Such speculation was corroborated by probing the allosteric site using a series of bitopic ligands with an increasing linker length between the orthosteric and allosteric pharmacophores. Both functional and kinetic investigations of these compounds, further discussed below in Section 4, suggested that the compound with a nine carbon atom spacer can simultaneously bridge both binding sites.[57,179] This narrows down the putative region of the allosteric site within the boundary of the second extracellular loop (ECL2) of the receptor. In another study, Peeters *et al*. performed an alanine scan on both ECL2 and ECL3 to investigate the influence on the allosteric effect of PD81,723. The result highlighted the role of $Try156^{ECL2}$ and $Glu164^{ECL2}$, as demonstrated by the significantly affected modulating effect of PD81,723 on the binding and function of CPA at the Ala mutant of both residues.[180] Taken together, these studies provided useful information increasing our understanding of the molecular aspects of allosteric modulation at the A_1AR.

Table 6. Allosteric activities of A_{2A}AR allosteric modulators.

Cmpd	**Allosteric effect** [a]	**Ref.**
Sodium ions [b]	-38%	[181]
HMA [c]	12-fold	[181]
Amiloride [c]	1.2-fold	[181]
66 [d]	-37%	[182]
ZB1854 [e]	-31%	[183]

[a] Expressed as the influence on dissociation rate of [^{3}H]-ZM241385 from the A_{2A}AR. Positive and negative values indicate increasing or decreasing dissociation rates, respectively. [b] Data were obtained at 100 mM of the test compound on rat striatal membranes at 25 °C. [c] Data were obtained at 1 mM of the test compound on rat striatal membranes at 4 °C. [d] Data were obtained at 10 μM of the test compound on membranes of CHO cells expressing

recombinant human A_{2A}AR at 0 °C. [e] Data were obtained at 2 mM of the test compound on membranes of HEK293 cells expressing recombinant human A_{2A}AR at 4 °C.

3.2. A_{2A}AR

Chemical structures of representative A_{2A}AR allosteric modulators are depicted in **Figure 8**. The allosteric activity of these compounds is summarized in **Table 6**.

3.2.1. Non-selective allosteric modulators.

The allosteric effect of sodium ions has been described for the A_{2A}AR as well.[181] The binding of sodium ions stabilizes an inactive conformation of the A_{2A}AR, suggested by both the high-resolution A_{2A}AR/ZM241385 crystal structure and several molecular dynamics simulations studies based thereon.[32,184,185] The dissociation rate of [^{3}H]-ZM241385 was significantly decreased from 0.24 min^{-1} to 0.15 min^{-1} in the presence of Na^+ at a physiological concentration.[181] In comparison, the binding of agonist and Na^+ appears to require mutually exclusive conformational states of the A_{2A}AR.[184]

Amiloride (**64**) and analogues have also been tested on the A_{2A}AR, where they increased the off-rate of [^{3}H]-ZM241385 from the A_{2A}AR. Among the derivatives HMA (**65**) proved to be the most potent allosteric inhibitor.[181] It produced a 12-fold enhancement of the off-rate of ZM241385 compared to the 1.2-fold enhancement by amiloride at the same concentration.

3.2.2. Emerging selective allosteric modulators

The first selective allosteric modulator of the A_{2A}AR was developed by Giorgi *et al.*[182] 1-[4-(3-Benzyl-5-phenyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7-ylamino)-phenyl]-3-(4-fluorophenyl)-urea (**66**) proved to be the most promising compound amongst the series of purine- and 8-azapurine-N^6-1,3-diphenylurea derivatives. It was observed that **66** at 10 μM increased the specific binding of both [^{3}H]-ZM241385 and [^{3}H]-CGS21680 in an equilibrium binding assay, and retarded the dissociation rate of [^{3}H]-ZM241385 from the A_{2A}AR (control, k_{off} = 0.082 min^{-1}; + **66**, k_{off} =

0.052 min^{-1}). Kinetics for the agonist radioligand were not reported, but further characterization of **66** in a functional study showed the compound was able to potentiate the A_{2A}AR-mediated action of vasorelaxing responses by CGS21680 in rat aortic rings precontracted by the adrenergic agonist noradrenaline. These results suggest **66** could be a lead for developing new allosteric modulators for the A_{2A}AR, although it is somewhat surprising that both agonist and antagonist seemed to be modulated in a similar way.[182]

Recently, Siegal and coworkers reported on a fragment screening campaign on the A_{2A}AR using biophysical methods to identify A_{2A}AR ligands with allosteric modulatory activity.[183] The authors used an approach called target immobilized NMR screening (TINS),[186] in which a target and reference protein are immobilized on a compatible solid support; binding of fragments to the immobilized protein can be detected by fluctuations in the 1D ^{1}H NMR spectrum of a ligand.[187,188] This approach was in combination with an antagonist-stabilized A_{2A}AR to overcome the instability and fragility of GPCRs during these time consuming experiments.[189,190] Since TINS selects for fragments that bind to the target regardless of binding sites, hits from the screen were then thoroughly characterized in follow-up binding and second messenger assays. Several hits were found with promising allosteric modulating profiles when tested in a kinetic radioligand dissociation assay using [^{3}H]-ZM241385 and [^{3}H]-NECA. As a result, three of 94 fragment hits were identified with significantly (at least 30%) increased k_{off} of the orthosteric ligand (negative allosteric modulators), and four with significantly decreased k_{off} (positive allosteric modulators). In addition, another four fragments allosterically enhanced either [^{3}H]-NECA or [^{3}H]-ZM241385 binding. One exemplary hit with detailed characterization is 3-(3,4-dihydroquinolin-1(2*H*)-yl)propanoic acid (**67**, ZB1854). It showed a concentration-dependent modulatory effect on the dissociation of [^{3}H]-ZM241385 from the A_{2A}AR (control, k_{off} = 0.0121 min^{-1}; + 0.5 mM ZB1854, k_{off} = 0.0102 min^{-1}; + 1 mM ZB1854, k_{off} = 0.0072 min^{-1}; + 2 mM ZB1854, k_{off} = 0.0058 min^{-1}).

In a cAMP assay ZB1854 potentiated the maximal effect (E_{max}) of CGS21680. This was consistent with the data from the kinetic radioligand binding experiment, suggesting ZB1854 acts via an allosteric mechanism.[164,191] It will be interesting to evolve the identified low potency fragment hits to lead-like compounds as novel A_{2A}AR allosteric modulators.

3.2.3. Binding sites for A_{2A}AR allosteric modulators

Recent advances in structural biological have greatly improved our knowledge of ligand-receptor interactions. The A_{2A}AR represents one of the most successful examples with several crystal structures available. In particular, the recently elucidated high-resolution structure of the A_{2A}AR in complex with the antagonist ZM241385 unveiled the binding site for sodium ions. It was observed that the positively charged ion has a direct interaction with the highly conserved aspartate residue Asp52$^{2.50}$, harbored in a cluster of structural water molecules.[32] Mutating Asp52$^{2.50}$ to either asparagine or alanine reduced or abrogated the allosteric effect of sodium ions on the A_{2A}AR and other GPCRs as well.[184] Furthermore, results from molecular dynamics simulations suggested Asp52A$^{2.50}$ directly affected the mobility of sodium ions, which readily migrated to another pocket formed by Glu13$^{1.39}$ and His278$^{7.43}$.[185] Amiloride and derivatives were shown to compete with sodium ions for the same binding site.[181] As evidence, the Asp52A$^{2.50}$ mutant also decreased the potency of amiloride with respect to ligand displacement but did not change orthosteric ligand affinity. Notably, the binding site for sodium ions and amiloride is deep in the 7TM cavity, different from the proposed or demonstrated site for other allosteric modulators on top of the binding cavity near the extracellular loops on other GPCRs such as the muscarinic receptors[111,192] or β_2 adrenergic receptor[110]. It is possible that several allosteric binding sites coexist on the A_{2A}AR, as reported in e.g., the gonadotropin-releasing hormone (GnRH) receptor.[173] One is conserved for non-selective allosteric modulators, such as sodium ions and

amiloride, to universally ‘lock’ the receptor in a ground, inactive state, while others are more diversely located, responsible for specific A_{2A}AR-mediated effects.

3.3. A_{2B}AR

The A_{2B}AR is the least characterized subtype in the AR family, partly due to the late availability of selective (radio-)ligands. Only one series of 1-benzyl-3-ketoindole derivatives as allosteric modulators for the A_{2B}AR has been reported, although originally designed as potential AR antagonists (**Figure 9**). Some of these compounds were shown to be putative allosteric modulators for the A_{2B}AR in a cAMP assay.[193] This was later confirmed in a follow-up study with detailed biological evaluations.[194] Interestingly, compounds from the same chemical class acted differently, showing either negative or positive allosteric modulating effects. In the presence of 2-(1-benzyl-1*H*-indol-3-yl)-2-oxo-*N*-phenylacetamide (**68**) the dissociation of [^{3}H]-NECA was slowed down from 0.0162 min^{-1} to 0.0086 min^{-1}, indicating a positive modulation of A_{2B}AR. In contrast, the presence of 1-benzyl-*N*-phenyl-1*H*-indole-3-carboxamide (**69**) increased the dissociation of [^{3}H]-NECA to 0.0481 min^{-1}, consistent with a typical profile of a negative allosteric modulator. Furthermore, in functional assays **68** increased agonist efficacy without changing its potency, whereas **69** reduced agonist potency and efficacy. Thus, these compounds may serve as leads for the discovery of other allosteric modulators for the A_{2B}AR.

3.4. A_3AR

Chemical structures of representative A_3AR allosteric modulators are represented in **Figure 10**. The allosteric activity of these compounds is summarized in **Table 7**.

3.4.1. Isoquinoline and quinoline derivatives

Table 7. Allosteric and antagonistic activities of A_3AR allosteric modulators.

Cmpd	Allosteric effect	K_i (μM) or % displ. at 10 μM	Ref.
VUF5455	k_{off} of [^{125}I]-I-AB-MECA was decreased 43% [a]	1.7	127
LUF6096	249% enhancement [b]	17%	195
DU124183	k_{off} of [^{125}I]-I-AB-MECA was decreased 46%	0.82	196
LUF6000	173% enhancement [c]	45%	197
74	196% enhancement [d]	17%	198
2-AG	k_{off} of [^{125}I]-I-AB-MECA was decreased 58%	0.79	199
HMA [e]	k_{off} of [^{125}I]-I-AB-MECA was decreased 47%; k_{off} of [^{3}H]-PSB-11 was increased 1.6-fold	5.7 [f]	154

Data were obtained at 10 μM of test compounds on membranes of CHO cells expressing recombinant human A_3AR at 25 °C unless mentioned otherwise. [a] Data were obtained on membranes of HEK293 cells expressing human A_3AR at 37 °C. [b] Expressed as % of [^{125}I]-I-AB-MECA dissociation at 120 min, control = 100%. [c] Expressed as % of [^{125}I]-I-AB-MECA dissociation at 30 min, control = 100%. [d] Expressed as % of [^{125}I]-I-AB-MECA dissociation at 60 min, control = 100%. [e] Data were obtained at 100 μM of the test compound. [f] IC_{50} value (μM) displacing [^{3}H]-PSB-11 from the A_3AR.

In 2001, Gao *et al*. reported on a series of 3-(2-pyridinyl)isoquinoline derivatives as the first selective allosteric modulators for the A_3AR.[127] Interestingly, this series of ligands was originally identified as potential antagonists for the A_3AR.[200] By examining their effects on the dissociation rate of the agonist radioligand, [^{125}I]-I-AB-MECA, from the A_3AR, the authors found that 4-methoxy-*N*-[7-methyl-3-(2-pyridinyl)-1-isoquinolinyl]benzamide (**70**, VUF5455) significantly retarded the dissociation rate of the radioligand in a concentration-dependent manner, consistent with a typical profile of an allosteric enhancer. Its effect on the dissociation rate of the antagonist [^{3}H]-PSB-11, however, was insignificant, suggesting VUF5455 was a selective enhancer of

agonist binding. In competitive binding studies VUF5455 displayed modest displacement of the orthosteric ligand (K_i = 1680 nM). Its analogue without 7-methyl substitution (VUF8504), however, showed little effect on the allosteric activity. Instead, the A_3AR affinity was increased (K_i = 17.3 nM). Furthermore, chemical modifications of the 4'-methoxy group of VUF8504 lowered its competitive binding affinity without a major loss of the AE effect. A similar effect was observed when the carboxamido group was replaced for an imino group. Taken together, it is tempting to speculate that further chemical modifications at the aforementioned positions might lead to a further separation of the orthosteric and allosteric actions on the A_3AR.

A series of 2,4-disubstituted quinolines was synthesized and evaluated as a new class of allosteric enhancers of the A_3AR.[195] Among these compounds, *N*-{2-[(3,4-dichlorophenyl)amino]quinolin-4-yl}cyclohexanecarboxamide (**71**, LUF6096) was the most potent enhancer. It was able to change the biphasic dissociation of [^{125}I]-I-AB-MECA from the receptor into a monophasic process, from the result of slowing down the agonist's kinetics in the fast dissociating phase (k_{off_fast} = 0.089 min^{-1} to 0.035 min^{-1}). This phenomenon suggests that LUF6096 can specifically stabilize the active conformation of the receptor. This is also supported by data from a functional assay, in which LUF6096 significantly enhanced the intrinsic activity of Cl-IB-MECA to 286% (control = 100%). In the absence of the orthosteric ligand, no inhibition of forskolin-induced cAMP production was observed, suggesting a "clean" profile of allosteric modulation. LUF6096 also displayed high target selectivity. As evidence, no enhancement was observed on the A_1AR. Additionally, negligible orthosteric competition on the four AR subtypes was shown for LUF6096.

3.4.2. Imidazoquinoline derivatives

Similar to the discovery of the isoquinoline derivatives, imidazoquinolines were originally defined as non-xanthine AR antagonists as well.[201] Later pharmacological characterization of

these compounds revealed their allosteric enhancer profile for the A_3AR.[196] 2-Cyclopentyl-*N*-phenyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine (**72**, DU124183) was the most potent compound from this series, and selectively enhanced agonist binding and function at the A_3AR. As evidence, DU124183 decreased the dissociation rate of the agonist [^{125}I]-I-AB-MECA from the A_3AR (control: k_{off} = 0.056 min^{-1}; + DU124183, k_{off} = 0.030 min^{-1}) but not for the antagonist [^{3}H]-PSB-11, or for [^{3}H]-R-PIA on the A_1AR and [^{3}H]-CGS21680 on the A_{2A}AR.

In a follow-up study, structural modifications were performed by Göblyös *et al*. at the 4-amino and 2-position of DU124183.[197] This effort led to the discovery of 2-cyclohexyl-*N*-(3,4-dichlorophenyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine (**73**, LUF6000), which enhanced agonist efficacy in a functional assay and decreased the agonist dissociation rate without influencing agonist potency. Surprisingly, LUF6000 was even able to convert an antagonist into an agonist on the A_3AR, representing a novel mechanism of GPCR activation. [202]

Further synthetic attempts on the imidazoquinoline derivatives were reported by Kim *et al.*.[198] The authors introduced divergent structural changes at the 2- and 4-positions of the scaffold. Only few substituents at these positions were tolerated to preserve the AE activity on the A_3AR. *N*-(3,4-dichlorophenyl)-2-(1-adamantanyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine (**74**) with a bulky adamantanyl group at the 2-position displayed an comparable AE activity to that of LUF6000 and yet minimal inhibition of orthosteric binding on the A_3AR. This study showed that allosteric and orthosteric inhibitory effects in the imidazoquinolines series are structurally separable.

3.4.3. Non-selective allosteric modulators

Compounds from different sources have also been reported with allosteric actions on the A_3AR. For instance, Lane *et al.* found that the endogenous cannabinoid agonist 2-arachidonylglycerol (**75**, 2-AG) was able to accelerate the dissociation of [^{125}I]-I-AB-MECA from the A_3AR,

consistent with an action as a negative allosteric modulator.[199] The same holds for amiloride analogues, a finding supported by data from both binding and functional assays.[154] HMA was the most potent compound, which significantly increased the dissociation rate of [^{3}H]-PSB-11 (control: k_{off} = 0.007 min^{-1}; + 100 μM HMA: k_{off} = 0.016 min^{-1}). In contrast, HMA displayed a positive modulatory effect on the dissociation rate of an agonist radioligand, [^{125}I]-I-AB-MECA, retarding its dissociation rate from 0.059 min^{-1} to 0.031 min^{-1}.

3.4.4. Binding site for A_3AR allosteric modulators

Several residues responsible for the allosteric modulation of the A_3AR have been identified by Gao *et al.*.[203] These residues include Phe182$^{5.43}$ and Asn274$^{7.45}$. Upon mutation of both residues to alanine DU124183 and VUF5455 lost their allosteric effects. However, the binding of the orthosteric agonist was not affected. The Asp107$^{3.49}$Asn mutant abolished the effects of DU124183, but not of VUF5455. In another study, Deganutti *et al*. used Supervised Molecular Dynamics (SuMD)[204], a computational method for the acquisition of the ligand-receptor recognition trajectory, to delineate the molecular mechanism of LUF6000's activity.[178] The authors suggested two possible mechanisms by which LUF6000 might exert its positive allosteric modulator effect. One is involving conformational changes in ECL2 triggered by LUF6000 that enables adenosine to form more energetically favorable interactions with the residues located deeper in the orthosteric binding site. Another possibility is that LUF6000 forms a ternary complex with the agonist and the receptor and thus acts as a cap for the orthosteric binding pocket. It was also noticeable that during the simulations LUF6000 was able to establish favorable interactions with conserved residues in the orthosteric binding site of the A_3AR, consistent with the experimentally observed modest orthosteric competition profile of the imidazoquinoline derivatives.[196,197]

3.5. Summary

Extensive structure activity relationship studies of allosteric modulators have been performed on the A_1AR and A_3AR, yielding novel chemical entities with convincing allosteric effects. On the A_1AR, the 2-amino-3-substituted thiophene scaffold is the most studied, whereas on the A_3AR isoquinoline and quinoline derivatives as well as imidazoquinoline derivatives have promising allosteric actions. In comparison, fewer studies and less convincing results have been reported on the A_{2A}AR and A_{2B}AR.

4. BINDING KINETICS OF BITOPIC LIGANDS

Recent studies have begun to explore the possibilities of linking orthosteric and allosteric pharmacophores to yield so-called "bitopic" ligands, or dualsteric/bivalent ligands in other terms.[20,205] Through concomitant engagement with both orthosteric and allosteric sites on a single target, the bitopic ligand may offer several advantages over the classical 'monovalent' ligand.[205] Firstly, it can show improved subtype selectivity, as exemplified by several bitopic ligands on the muscarinic receptors, when compared to non-selective orthosteric agonists for the different receptor subtypes.[206-209] Secondly, a bitopic ligand can promote biased signaling and hence cause the separation of on-target efficacy from adverse effects. An example is the A_1AR bitopic ligand VCP746 (**Figure 11**, **76**), which displayed biased agonism relative to prototypical A_1AR ligands in native rodent cells and isolated rat atria.[210] Additionally, bitopic ligands are good tool compounds for understanding the spatial geometry within the ligand-GPCR complex. For instance, Narlawar *et al.* designed a series of A_1AR bitopic ligands containing linkers with varying lengths to probe the distance between the orthosteric and allosteric binding sites.[179]

The special pharmacological profile of a bitopic ligand, as mentioned above, may be reflected in its unique binding kinetics. Notably, upon binding, each pharmacophore has its own

"micro-" binding kinetics that can induce synergistic effects (i.e. allosteric cooperativity) on the overall apparent kinetics of the compound—greater than simply combining two monovalent ligands.[211-213] More specifically, a dissociating allosteric or orthosteric pharmacophore of a bitopic ligand is obliged to remain in "forced proximity" to its cognate binding site—instead of dissociating further away—as long as the tethered, companion pharmacophore is still bound.[212] This favors its rebinding to that site, thereby significantly retarding the net dissociation process of the bitopic ligand.[214] As evidence, two A_1AR bitopic ligands (**Figure 11**), LUF6234 (**77**) and LUF6258 (**78**), exerted significantly increased receptor residence times compared to the monovalent parent orthosteric ligand LUF5519 either in the absence or presence of the allosteric modulator PD81,723.[57]

5. FUTURE PERSPECTIVES AND CHALLENGES FOR KINETIC CHARACTERIZATIONS

5.1. Structure-kinetics relationship (SKR) and structure-activity relationship (SAR) studies

SKR and SAR studies of orthosteric AR ligands are emerging, particularly for the A_1AR and A_{2A}AR.[72,73,106] Apparently, ligands in the same chemical series with similar affinities can display divergent binding kinetics. Such detailed SKR studies offer added value to the traditional metric of affinity. This is exemplified by LUF6632 (**32**), a high-affinity A_{2A}R ligand previously reported by Vu *et al.* (compound 11 in the original paper).[118] Further subjecting this compound to a kinetic characterization revealed its exceptional long RT (323 min). This type of information can support the selection of LUF6632 from its analogues with similar affinity for further testing. Similarly on the A_1AR, capadenoson (**13**) showed a RT of 28 min while its analogue LUF6941 (**14**) displayed a RT of 132 min. Both compounds have high affinity on the A_1AR (capadenoson, 1.4 nM; LUF6941, 5.0 nM). If affinity were the only factor taken into consideration, LUF6941 would not be selected among capadenoson and other analogues with better affinity. Now with its kinetic

results determined, LUF6941 may be an interesting candidate for further *in vivo* testing as well. Thus, the strategy combining both SAR and SKR provides a better understanding of a compound's molecular mechanism of action. It includes detailed characterization of not only the bound states under equilibrium (SAR) but also the entire ligand-receptor interaction that comprises metastable intermediate states and transition states (SKR).[215] Such knowledge may offer new rationales for efficient drug design and yield improved drug candiates.[45]

5.2. Signaling bias and ligand-receptor binding kinetics

GPCRs are pleiotropic with respect to the signaling protein to which they couple in a cell. It is now widely accepted that agonists of GPCRs do not uniformly activate all cellular signaling pathways linked to a given receptor—a phenomenon termed signaling bias.[216] "Biased" ligands – ligands that selectively activate one pathway over another – are therapeutically tempting, since they may deliver more precise therapeutic benefits with fewer side effects than current drugs.[217] This is exemplified by the biased opioid receptor agonists which might provide pain relief without the adverse side effects normally associated with the opiate narcotics, presumably due to the activation of the μ-opioid receptor rather than recruiting of the arrestins.[218] Studies probing biased signaling on the ARs are emerging as well.[219] The differential effects in various signaling pathways can be induced by an orthosteric ligand,[220] an allosteric modulator,[221] or more recently a bitopic ligand.[210] It has been proposed that these ligands exert their effects through stabilization of unique active receptor states to create a signal that is "biased" towards specific cellular pathways.[216,222] Such stabilization of different receptor states is likely caused by differences in strength of interaction between the receptor and a given ligand, hence linking with different binding kinetics. In this sense, slow binding kinetics might be diagnostic of conformational changes, as exemplified by maraviroc's long residence time at the CCR5, where reorganizations

of the initial maraviroc-receptor complex were involved.[223] Alternatively, different ligand-receptor binding kinetics might drive the stabilization of specific receptor states acting on varied downstream signaling pathways.

5.3. Equilibrium selectivity and kinetic selectivity profiling

An exploration of ligand binding kinetics at different targets will also help in selectivity profiling. This is complementary to the classical equilibrium selectivity profiling, i.e., determining the affinity of lead compounds against a number of potential targets under equilibrium conditions. Combining both aspects of selectivity profiling may ‘‘rescue” many potent yet nonselective compounds, increasing the number of potential candidate drugs moving to clinical trials and finally to the market. This is particularly useful in cases in which its ‘equilibrium selectivity’ is less than ideal. A study was recently reported in the context of adenosine receptors. The equilibrium K_i values and binding kinetics of six AR antagonists were examined on three adenosine receptors, namely A_1AR, A_{2A}AR and A_3AR.[224] It was found that XAC and 2,6-diphenyl-8-propyl-9*H*-purine (**79**, LUF5964, Figure 6) were kinetically more selective for the A_1AR and A_3AR, respectively, although they are non-selective in terms of their affinity. In comparison, 6-phenyl-2-(*p*-tolyl)-9*H*-purine (**80**, LUF5967, Figure 3) displayed a strong equilibrium-based selectivity for the A_1AR over the A_{2A}AR, yet its kinetic selectivity thereon was less pronounced. This study provides evidence that equilibrium and kinetic selectivity profiling can both be important in the early phases of the drug discovery process. The combined strategy could be considered for future medicinal chemistry efforts and aid the design and discovery of different or even better leads for clinical applications.[224]

5.4. Measuring the binding kinetics of allosteric modulators

Knowledge of the kinetics of an allosteric modulator interacting with its cognate binding site on the AR would allow us to compare an allosteric modulator's binding kinetics with its modulating effect. It is tempting to speculate that a long RT allosteric modulator might be able to trigger a more profound modulatory effect than a short RT ligand can do. However, due to practical issues such as their relatively low, micromolar potency, data for AR allosteric modulators are yet to be determined. The availability of one radiolabeled A_1AR allosteric modulator, [^{3}H]-T-62, suggests the use of it in kinetic radioligand binding assays. However, its low affinity (K_D = 4.6 μM) most likely precluded such experiments. High affinity allosteric modulators are therefore highly desired. An alternative approach may be SPR technology, as it worked in the kinetic characterization of low-affinity orthosteric A_{2A}AR ligands.[65] Another possibility may be to follow the BRET signal over time of a fluorescently labeled allosteric modulator in living cells in combination with a bioluminescent target, as demonstrated by Stoddart *et al*. to monitor ligand binding to a GPCR[225]. Needless to say that extensive assay optimizations are needed for both technologies to enable robust kinetic measurements of AR allosteric modulators.

5.5. Molecular mapping of the binding site for allosteric modulators

Molecular mapping of the allosteric binding site on the ARs is challenging. Although several mutagenesis studies have shed light on several functionally important residues in maintaining an AR allosteric modulating effect, there is still no direct evidence confirming these residues are interacting with the allosteric modulator. The bitopic ligands represent good tools to probe the allosteric binding site, however, due to the inherent flexibility of the linker and the lack of further structural information any conclusion where the allosteric pharmacophore binds remains speculative. Receptor crystallography in the presence of an allosteric modulator provides the

most direct structural evidence for the binding site. One example is the elucidation of the human M_2 muscarinic acetylcholine receptor structure in complex with both an orthosteric ligand, iperoxo, and an allosteric modulator, LY2119620.[177] As shown in Figure 12, the modulator is accommodated above the orthosteric ligand, engaging in extensive interactions with the extracellular vestibule including an aromatic stacking with Tyr177^{ECL2} and Trp422$^{7.35}$ as well as several polar interactions with Tyr80$^{2.61}$, Asn410$^{6.58}$, Asn419^{ECL3} and Glu172^{ECL2}. This allosteric binding site is directly superficial to the cognate orthosteric site, separated by a tyrosine lid, with Tyr426$^{7.29}$ interacting with both ligands. Reasoning from here, structure-based molecular dynamics simulations may provide a "computational microscope" to visualize the dynamic ligand-receptor interaction.[111,226] With recent advancements in structural biology, increased computational power and dedicated software, we envision that the location of allosteric sites on ARs can be unveiled in the near future.

5.6. Characterization of ligand-receptor binding kinetics in living cells

Characterization of ligand-receptor binding kinetics in living cells is of great interest and is essential to bridge the gap between the binding kinetics *in vitro* to the pharmacological response *in vivo*. However, the assay readout for the ARs is often complicated. For accurate data interpretation one should take into account several issues.

Firstly, it is now generally accepted that ARs can form homo- or heterodimers and thereby influence the kinetics of the ligand-receptor interaction. This is well illustrated by several studies on the A_3AR by Hill and colleagues. One example is the measurement of the dissociation kinetics of ABA-X-BY630 from the A_3AR.[64,227] In the absence of a competitive ligand its k_{off} value was 0.57 min^{-1}. This value was significantly increased by both orthosteric antagonist and agonist (antagonist, XAC: 15-fold; agonists, NECA: 9-fold and adenosine: 19-fold)—an observation not

compatible with that of a GPCR existing as monomer. The authors suggested that the kinetics of a ligand binding to one protomer can be modulated through negative cooperativity by targeting a binding site within the second, interacting protomer. Supporting this assumption, the effect was decreased when coexpressing a 'nonbinding' A_3AR mutant (Asn250$^{6.55}$Ala). Homodimerization of A_1ARs seems to occur as well at the surface of living cells, yet the extent of cooperative interactions was less pronounced than for the A_3AR. AR heterodimerization has also been observed and documented, e.g., between adenosine A_1AR and dopamine D_1R and between adenosine A_{2A}AR and dopamine D_2R in the central nervous system.[228] Franco and coworkers have shown that selective A_1AR agonists negatively affect the high affinity binding of D_1R. Likewise, activation of A_{2A}AR led to a decrease in receptor affinity for dopamine agonists acting on D_2R, specifically of the high-affinity state. These interactions have been reproduced in cell lines and found to be of functional significance.[229,230]

Another issue to take into consideration is target vulnerability, e.g., receptor desensitization, internalization, degradation and recycling.[88] The A_{2A}[231], A_{2B}[232] and A_3[233] ARs have all been observed with rapid desensitization upon agonist treatment, with A_3AR desensitization the faster process.[234] Posttranslational target modifications such as phosphorylation and ubiquitination also affect the target's half-life of A_{2A}[235], A_{2B}[236] and A_3[237] ARs leading to the recruitment of arrestins and subsequent sequestration from the plasma membrane. In contrast, A_1AR desensitization requires prolonged exposure to agonists and it is still controversial whether phosphorylation actually occurs.[234] As such, ligand-receptor binding kinetics will be significantly influenced with two possible yet opposite consequences: (1) internalization of a receptor via endocytic vesicles to the lysosome or vacuole for degradation leading to termination of the ligand-receptor residence time;[238] (2) internalized ligand-receptor complex "traps" the ligand within an intracellular compartment preventing it from diffusion and thereby inducing rebinding.[239] Both mechanisms

seem to be possible and have not yet been explored for the ARs. Thus, further experiments for different ligand-receptor pairs are needed. Additionally, it will be of great value to obtain information regarding the synthesis and degradation process of ARs, the rates of which may affect the impact of a ligand's binding kinetics.

6. CONCLUDING REMARKS

In this review we have shown that kinetics is an emerging parameter to probe ligand-receptor interactions which can provide additional information to direct medicinal chemistry efforts. It may support the triage and further advancement of compounds in the drug discovery pipeline that are otherwise chemically or biologically similar. This has been illustrated already in several SAR/SKR studies on the A_1 and A_{2A} ARs, in which equipotent compounds but with divergent kinetics were obtained.[72,73,106]

Investigations of binding kinetics also aid the identification and characterization of novel or known (AR) allosteric modulators. By comparing the kinetics of an orthosteric ligand in the absence or presence of an allosteric modulator, one can study the typical modulating effects such as "probe-dependence" or "concentration-dependency" of an allosteric modulator. It is also notable that an allosteric modulator can induce significant effects on not only the off-rate but also the on-rate of an orthosteric ligand, although the latter has been less investigated. Association rate constants may be important as well to delineate the molecular basis of GPCR allosterism.[57,74,240]

Taken together, examples presented in this review make a strong case for the value of measuring ligand-receptor binding kinetics in early phases of drug design and discovery. We anticipate that a fast accumulation of AR binding kinetics will take place in the near future, which hopefully will lead to the identification of novel and better-in-class candidate drugs, targeting both orthosteric and allosteric sites, for clinical applications.

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BIOGRAPHIES

Dong Guo, PhD obtained his PhD degree from Leiden University, The Netherlands, in 2014. Since then, he has worked as a postdoc fellow at the same university within the K4DD (Kinetics for Drug Discovery) consortium, financially supported by Europe's Innovative Medicines Initiative (IMI) program and major Pharma companies. His main research interest is the set-up of kinetic assays and investigation of ligand-receptor binding kinetics at the adenosine receptors, from both theoretical and experimental perspectives.

Laura H. Heitman, PhD studied Biopharmaceutical Sciences at the University of Leiden, The Netherlands. In October 2004, she started as a PhD student on the project "Allosteric modulation of 'reproductive' GPCRs" at the Division of Medicinal Chemistry of Leiden University in collaboration with Organon/MSD in Oss, The Netherlands. In January 2009 she was appointed 'tenure track' assistant professor molecular pharmacology, and became 'tenured' associate professor as of November 2014 at Leiden University in its Leiden Academic Centre for Drug Research. Her research interests are mainly focused on understanding and improving drug-receptor interactions, including the 'translational' concept receptor residence time.

Ad P. IJzerman, PhD obtained his pharmacy degree from Utrecht University, The Netherlands, in 1980. He then continued his career as a PhD student at the Vrije Universiteit of Amsterdam, The Netherlands. He obtained his PhD degree on the medicinal chemistry of the β-adrenergic receptor in 1985, after which he was appointed assistant professor of medicinal chemistry at Leiden University in its Leiden Academic Centre for Drug Research. Since 2000 he holds a full professorship of medicinal chemistry at the same university. His main research interest is in G protein-coupled receptors and in novel concepts to intervene with receptor function including allosteric modulation.

Figures

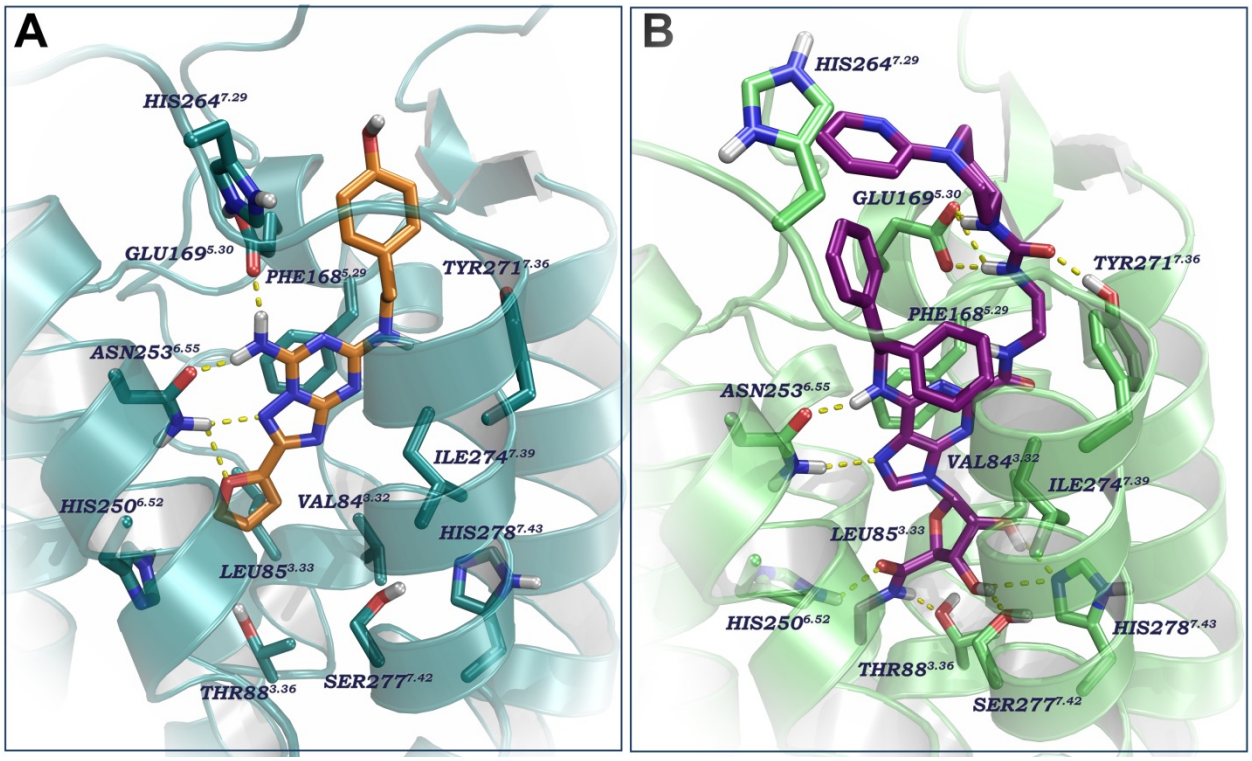


Figure 1. $A_{2A}AR$ crystal structures in complex with ZM241385 (A, PDB: 4EIY) and UK432,097 (B, PDB: 3QAK).

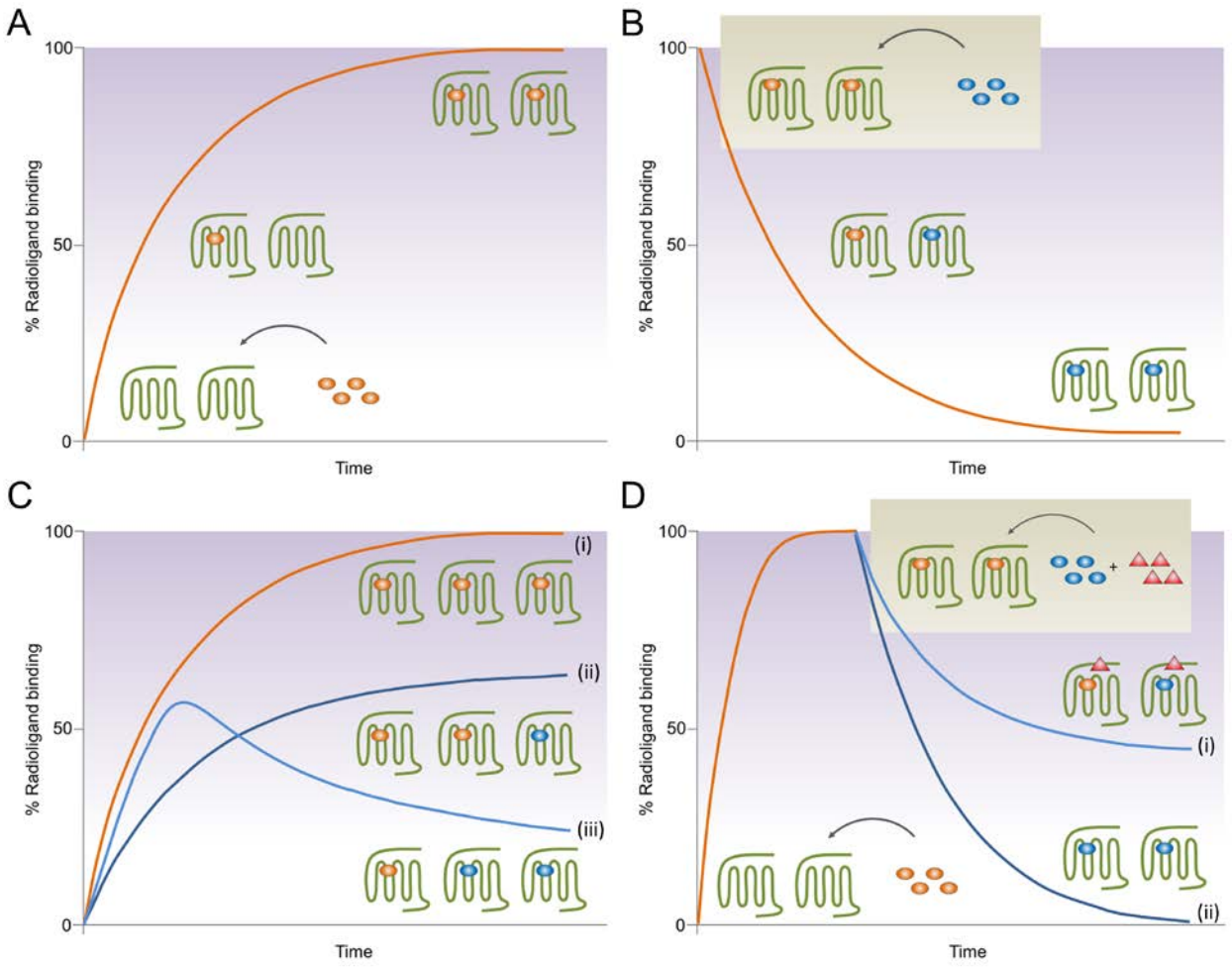


Figure 2. Current methods to measure binding kinetics on adenosine receptors. (A) Kinetic ligand association assay. The assay is initiated by incubating a labeled ligand (e.g., radioligand, orange spheres) with the receptor. After a period of incubation, the association approaches equilibrium caused by the formation of the ligand-receptor binary complex (B) Kinetic ligand dissociation assay. The receptor-bearing material is firstly equilibrated with the labeled ligand (e.g., radioligand, orange spheres) and then a saturating concentration of unlabeled competitor (blue spheres) is added to prevent the labeled ligand from reassociating to the receptor. After a period of incubation, the dissociation of the labeled ligand is complete with receptor binding sites fully occupied by the competitor. (C) Competition association assay. The association of a labeled ligand (e.g., radioligand, orange spheres) is initiated in the absence (i) or presence of an unlabeled

ligand (blue spheres, ii and iii). In the presence of a fast dissociating compound, the specific binding of the labeled ligand slowly and monotonically approaches equilibrium in time (ii). In the presence of a slowly dissociating compound, the specific binding of the labeled ligand consists of two phases starting with an ‘overshoot’ and then a decline until a new equilibrium is reached (iii). (D) Kinetic assay in the presence of an allosteric modulator. Formation of the ligand-receptor binary complex is initiated by preincubating the receptor-bearing material with the labeled ligand (e.g., radioligand, orange spheres). An allosteric modulator (red triangles) is then added, stabilizing the allosteric ternary complex, together with an excess amount of an unlabeled competitor (blue spheres) to start the dissociation of the labeled ligand. After a period of incubation, the allosteric activity is evaluated based on a comparison between the residually bound labeled ligand in the presence (i) or absence of the allosteric modulator (ii).

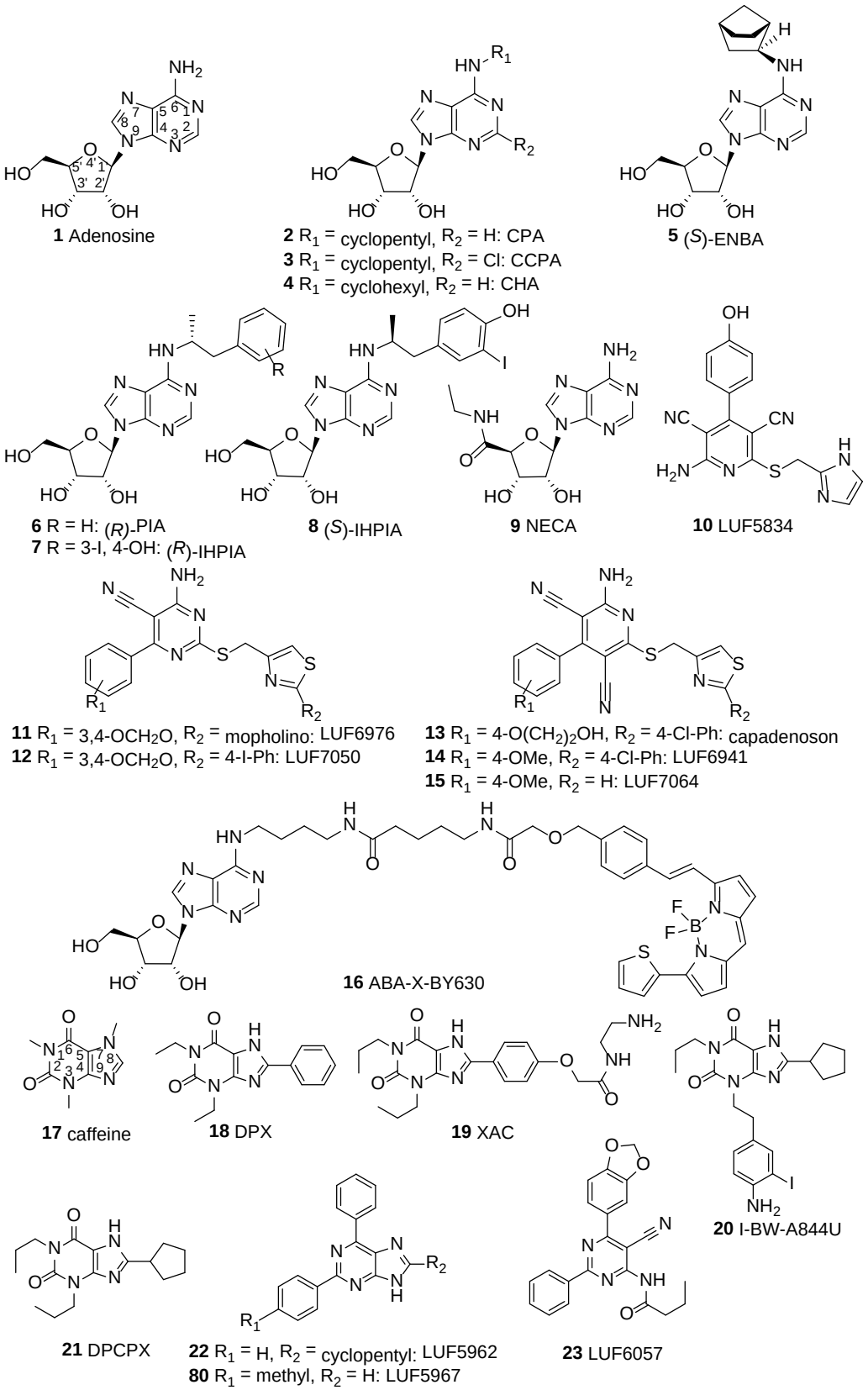


Figure 3. Representative A_1AR ligands with known binding kinetics

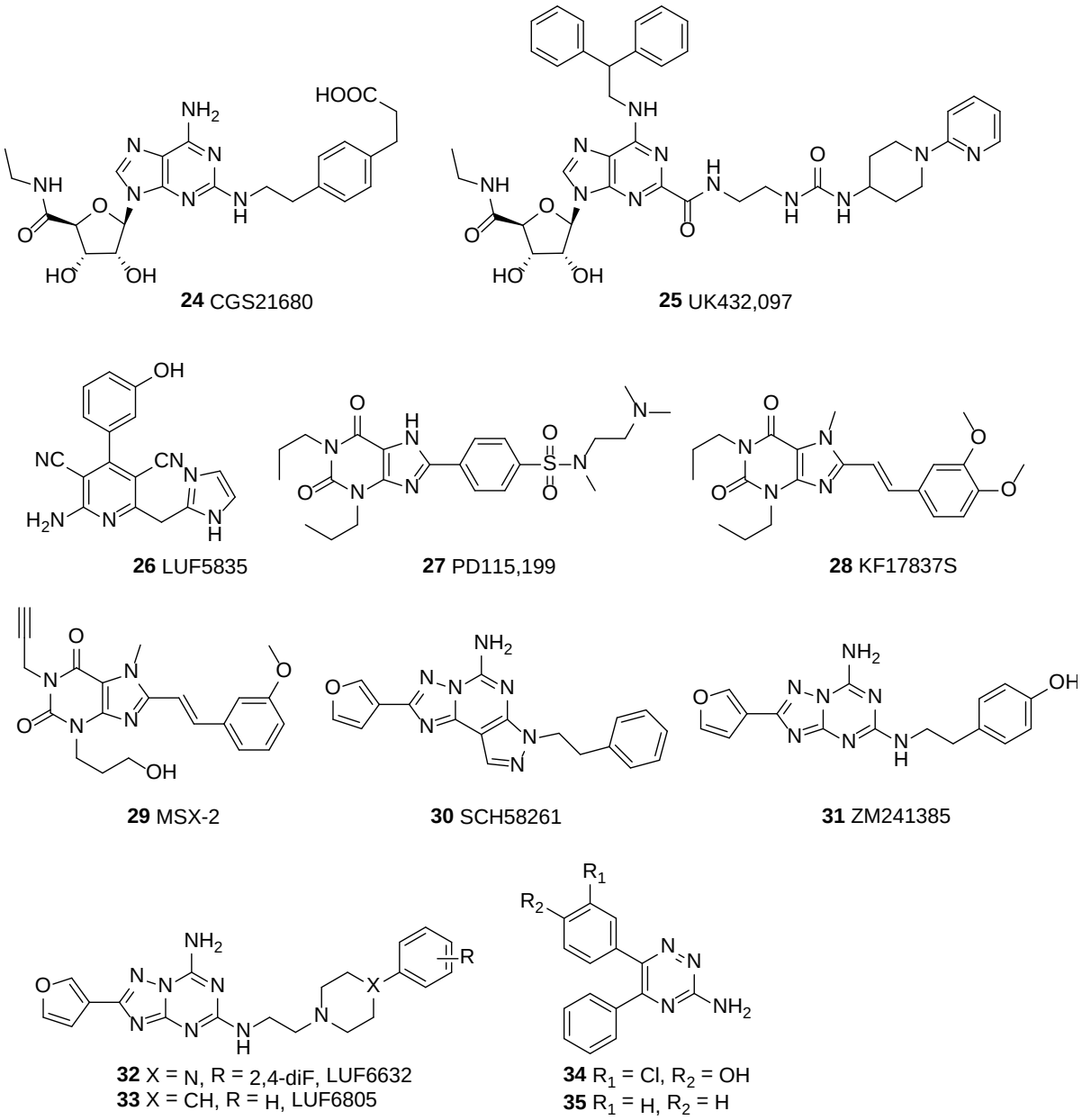


Figure 4. Representative $A_{2A}AR$ ligands with known binding kinetics

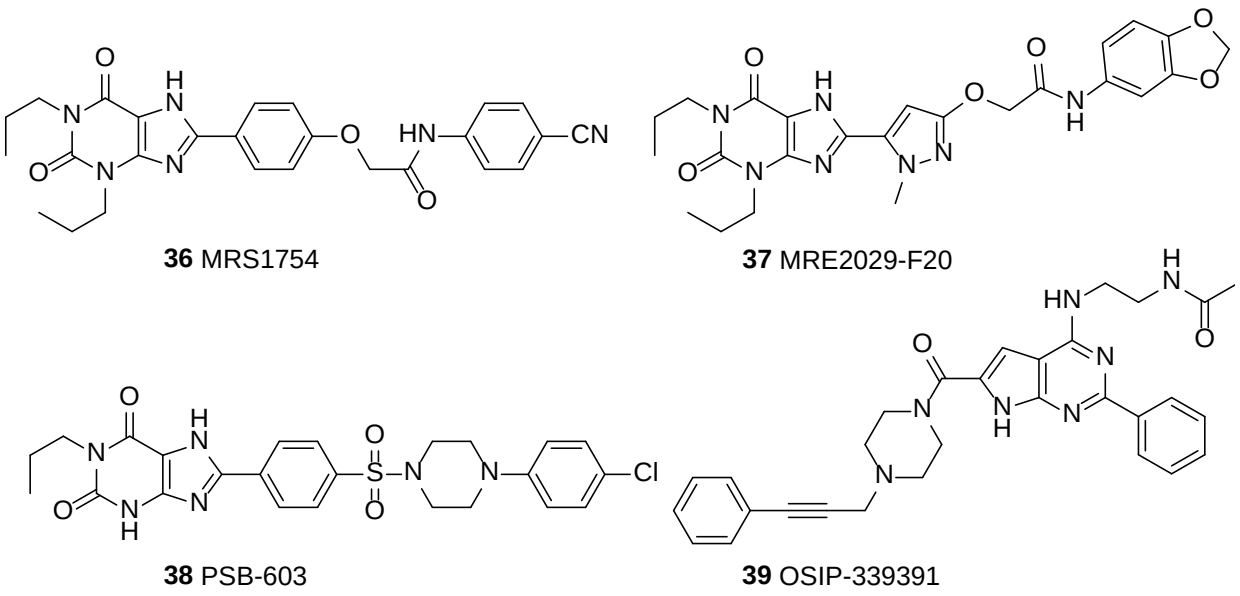


Figure 5. Representative $A_{2B}AR$ ligands with known binding kinetics

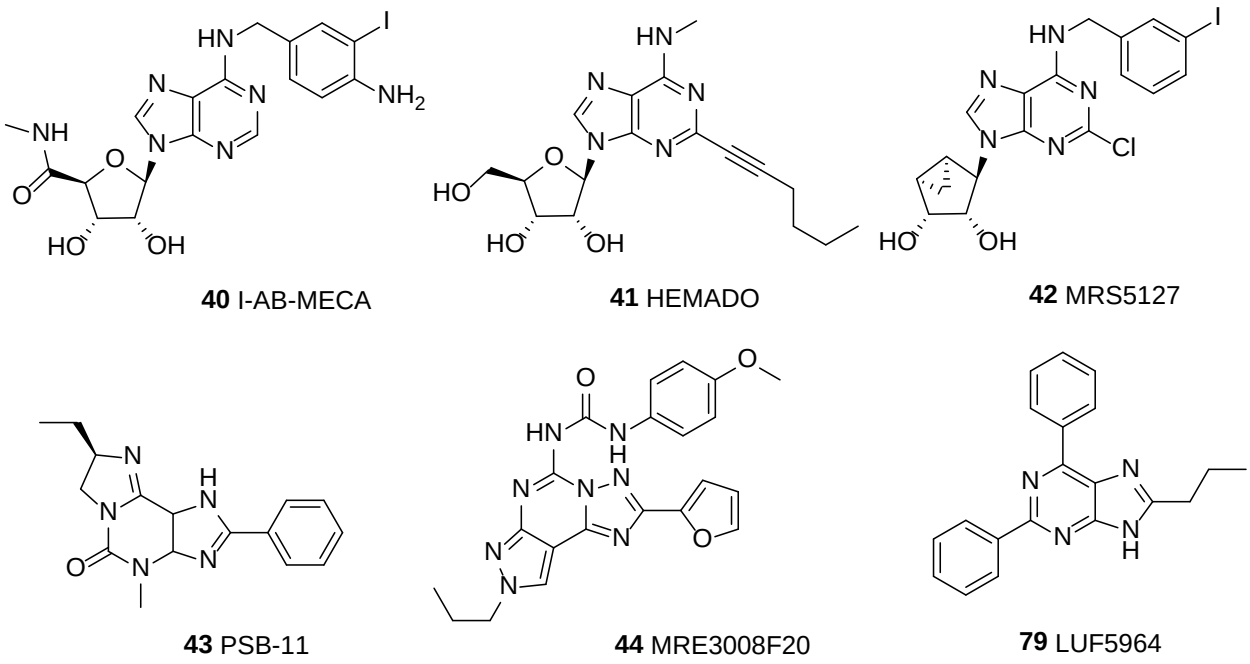


Figure 6. Representative A_3AR ligands with known binding kinetics

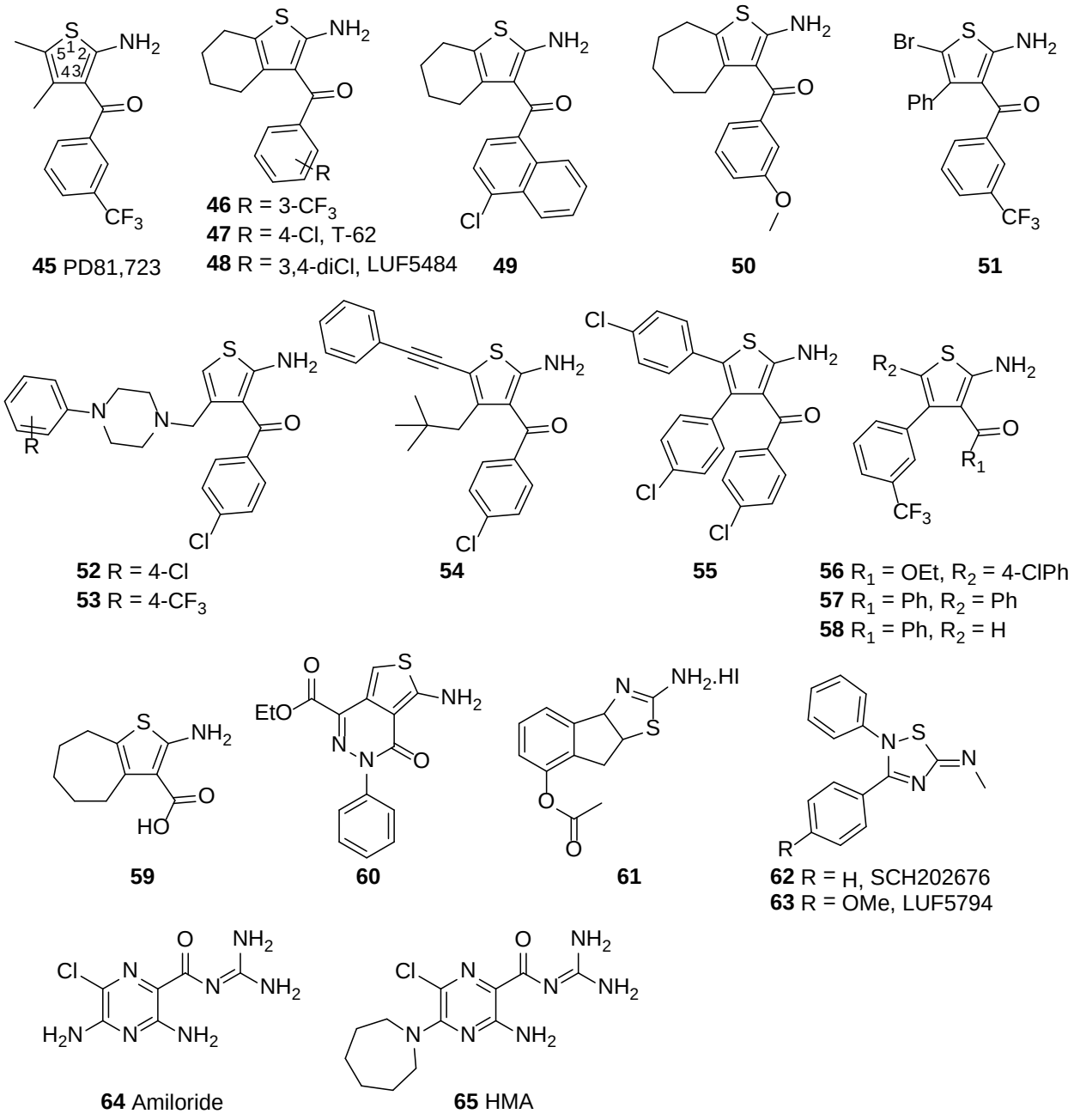
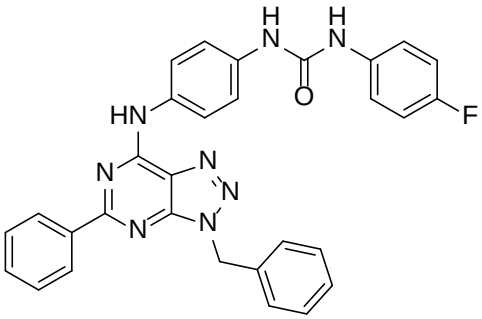


Figure 7. Allosteric modulators for the A_1AR



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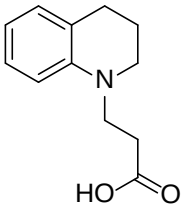
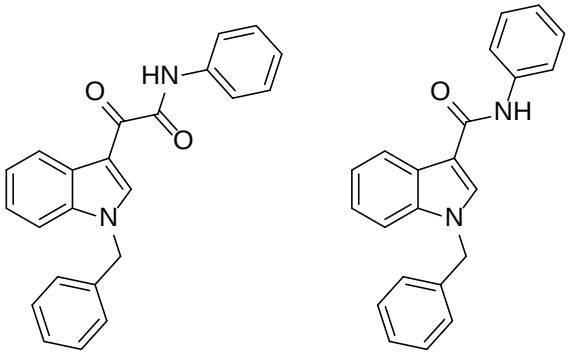


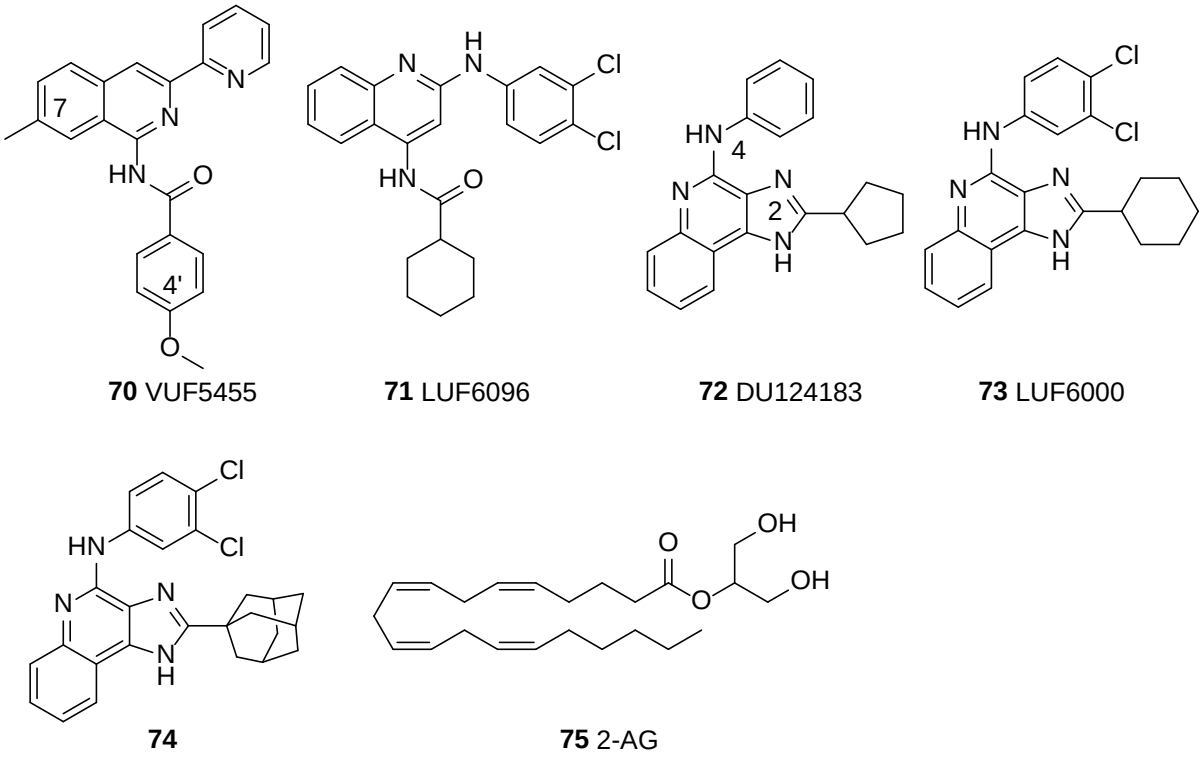
67 ZB1854

Figure 8. Allosteric modulators for the $A_{2A}AR$



68 **69**

Figure 9. Allosteric modulators for the $A_{2B}AR$



70 VUF5455 **71** LUF6096 **72** DU124183 **73** LUF6000

74 **75** 2-AG

Figure 10. Allosteric modulators for the A_3AR

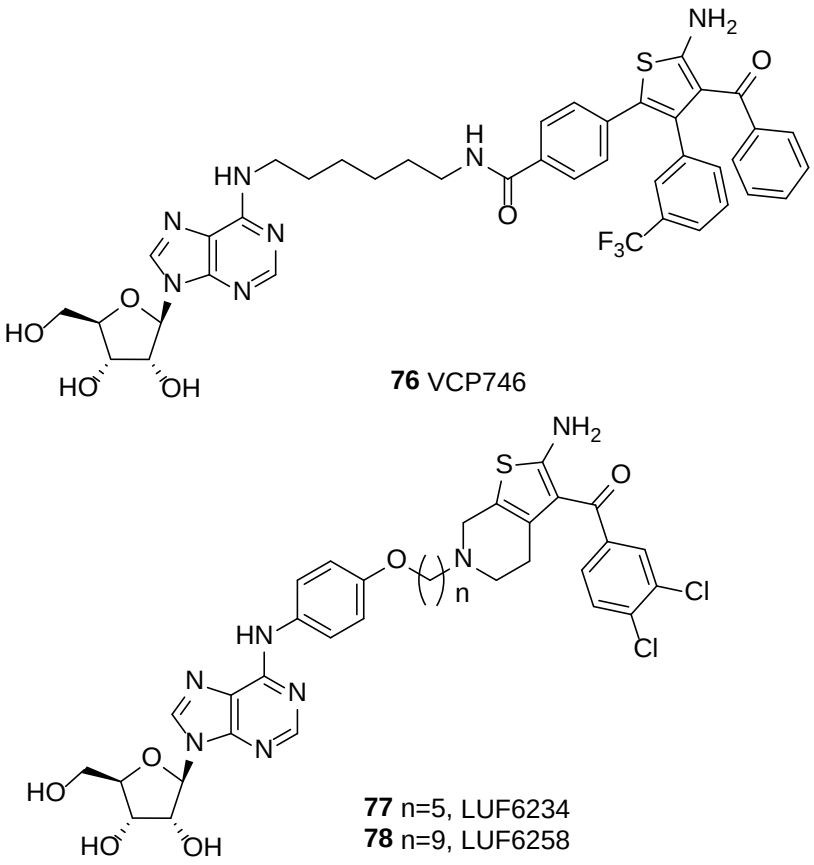


Figure 11. A_1AR bitopic ligands

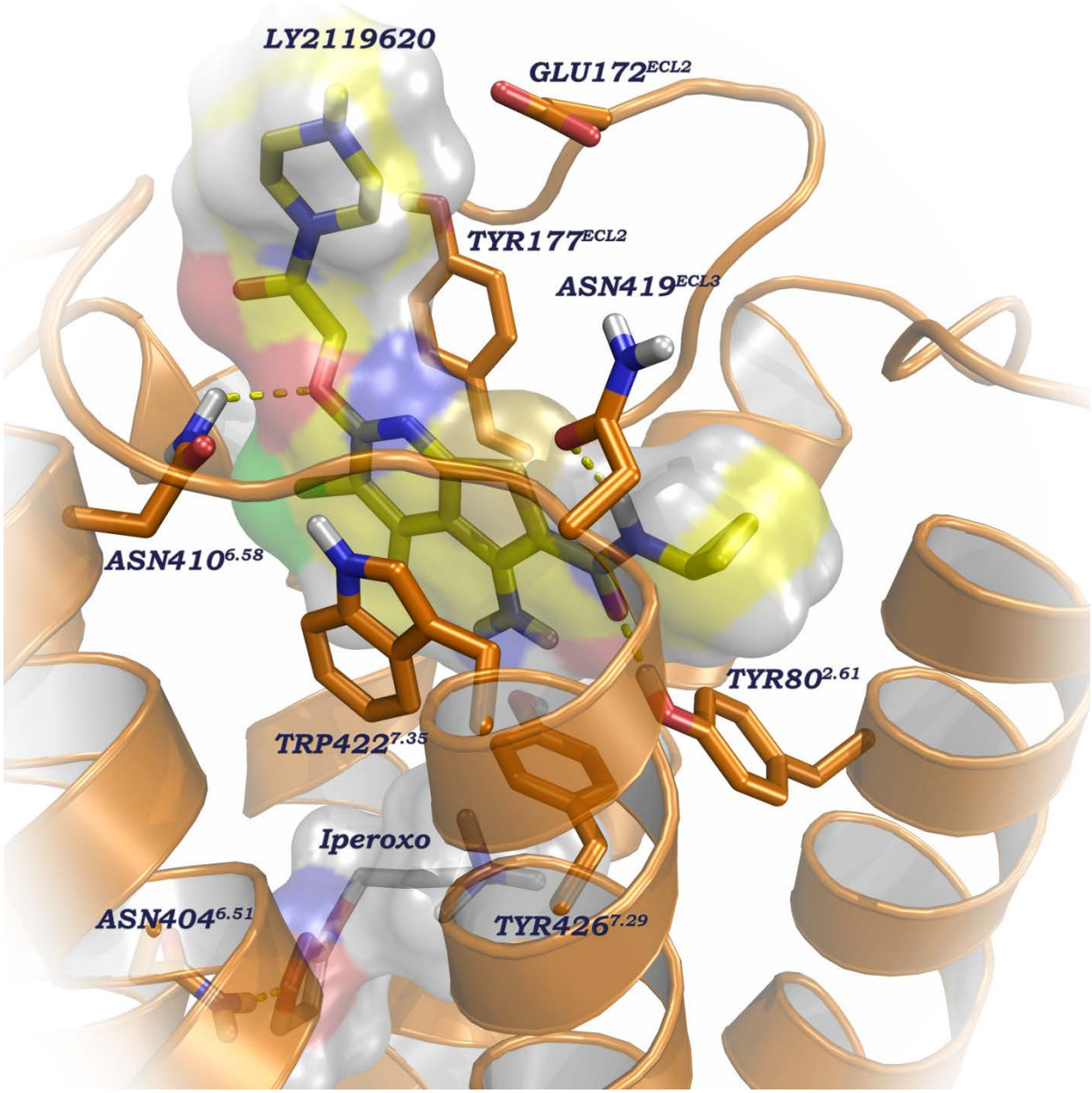
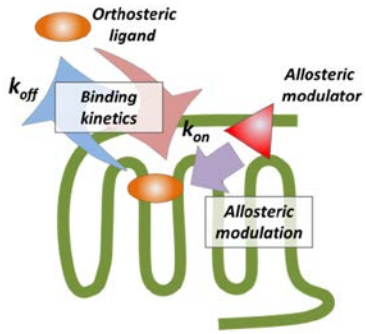


Figure 12. The structure of the human M_2 muscarinic acetylcholine receptor in complex with an orthosteric ligand, iperoxo, and an allosteric modulator, LY2119620 (PDB: 4MQT).

TOC graph


Orthosteric ligand
k_{off}
Binding kinetics
Allosteric modulator
k_{on}
Allosteric modulation