# To bind or not to bind, that is an important question! : Development of covalent probes for adenosine receptors

Yang, X.

**Citation**

Yang, X. (2019, December 4). *To bind or not to bind, that is an important question! : Development of covalent probes for adenosine receptors*. Retrieved from https://hdl.handle.net/1887/81190

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**Author**: Yang, X.
**Title**: To bind or not to bind, that is an important question! : Development of covalent probes for adenosine receptors
**Issue Date**: 2019-12-04

# Chapter 1

## General Introduction

## About this thesis

Most small-molecule drugs are designed to interact with their biological targets under equilibrium binding conditions, whereby the desired drug-protein interaction is a rapid and reversible (non-covalent) process. However, for a drug to exert its effect, it needs to be bound to the target, and hence a common focus of modern drug discovery programs is to maximize the strength of these noncovalent molecular interactions. As an extension to this reasoning, a less conventional strategy termed 'covalent interactions' has recently gained reputation in the field of drug discovery [1, 2]. A covalent bond between ligand and receptor can be sufficiently long-lived that it is irreversible within the half-life of the target protein, resulting in a drug-protein complex that is not subject to classical equilibrium kinetics. Accordingly, such drugs were initially less favored owing to concerns over potential nonspecific side effects or off-target toxicity [1, 2]. However, one of the oldest drugs in current clinical practice, aspirin (acetylsalicylic acid), acts as a covalently binding inhibitor [3]. Hence, in this thesis a covalent strategy is applied and shown to be compatible with a target-directed, structure-guided discovery paradigm, with a focus on G protein-coupled receptors as drug targets.

Receptor proteins, located on both the cell surface and within the cell, are the targets through which many drugs produce their beneficial effects in various disease states. The receptor theory was once proposed as the underlying mechanism for drug action by the pioneering scientists Paul Ehrlich and John Newport Langley at the beginning of the 20$^{th}$ century. Whereas Langley referred to specific sites affected by nicotine as 'receptive substances' [4], Ehrlich simultaneously envisioned chemical 'side-chains' on the cell membrane to which toxins could bind [5]. Among the receptors, so-called G protein-coupled receptors (GPCRs) are one of the largest and most important drug target families [6]. This chapter provides a background for the covalent research strategy for GPCRs presented in this thesis, followed by the aim and outline of this thesis.

# 1. G Protein-coupled Receptors (GPCRs)

G protein-coupled receptors (GPCRs) represent a protein superfamily responsible for vision, olfaction, taste and signal transduction by hormones and neurotransmitters [7]. More than 30% of the current clinically used small-molecular drugs target GPCRs [8]. According to Fredriksson and colleagues, this large family in the human genome encompasses five subtypes, including *Rhodopsin* (class A), *Secretin* (class B), *Adhesion* (class B), *Glutamate* (class C), *and Frizzled/Taste2* (class F), shortened to GRAFS [9]. The main structural features of GPCR-family members are the seven hydrophobic, transmembrane, α-helices, termed as TM1-TM7 (Figure 1). The N terminus, considered a glycosylation site for most GPCRs, together with three extracellular loops (ECL1–ECL3) compose the extracellular section. The intracellular part of the receptor contains a C terminus, three corresponding intracellular loops (ICL1-ICL3) and an amphipathic helix (H8). Due to their similar role in receptor function, such as activation, the seven hydrophobic helices, located in the lipid bilayer, are highly conserved, especially in class A GPCRs.

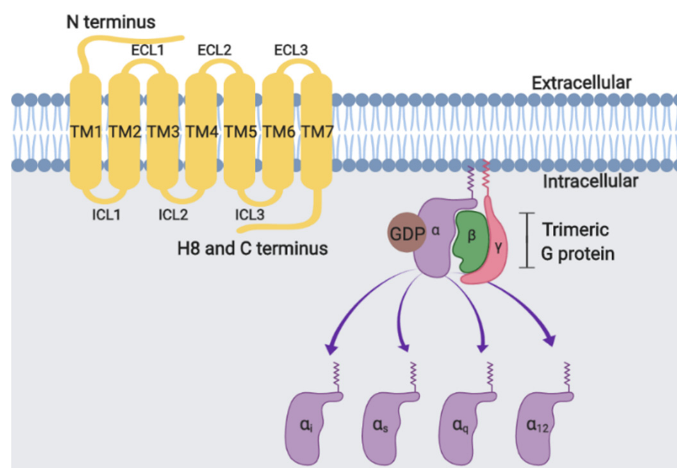


**Figure 1. A schematic representation of an inactive class A G protein-coupled receptor (GPCR).** Class A GPCRs consist of seven hydrophobic α-helices, termed as TM1-TM7, connected with extracellular loops (ECL1–ECL3) and three corresponding intracellular loops (ICL1-ICL3). The N terminus is directed to the extracellular side, while helix 8 (H8) and C-terminus are located at the intracellular side. Activated G proteins regulate diverse signaling cascades, depending on their subtype ($\alpha_s$, $\alpha_i$ , $\alpha_q$ , and $\alpha_{12}$ families).

The signaling from the extracellular to intracellular environment is mediated by the heterotrimeric G protein, having three subunits, α, β and γ (Figure 1). Activation of the receptor results in a conformational change, causing the G protein-bound guanosine diphosphate (GDP) to be exchanged for guanosine triphosphate (GTP), leading to dissociation of the βγ-dimer [10]. The α subunits of G proteins are divided into four subfamilies: $G\alpha_s$,

$G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$, and a single GPCR can couple to either one or more families of Gα proteins. Each G protein activates several downstream effectors (Figure 1).



### 1.1. Adenosine receptors

Adenosine receptors (ARs) belong to the rhodopsin, or class A subfamily of GPCRs. There are four recognized subtypes for ARs-named $A_1R$, $A_{2A}R$, $A_{2B}R$ and $A_3R$, each of which shows an individual pharmacological profile and tissue distribution [11, 12]. The classification of ARs is traditionally dependent on their differential coupling to adenylyl cyclase (AC), the enzyme responsible for increasing the intracellular concentration of the second messenger cyclic adenosine 5'-monophosphate (cAMP). Activation of the $A_1R$ and $A_3R$ will inhibit AC activity, caused predominantly by $G\alpha_i$ protein interaction, ultimately leading to a decrease of intracellular cAMP. The $A_{2A}R$ and $A_{2B}R$ are coupled to the $G\alpha_s$ protein, resulting in an increase of intracellular cAMP production. So far, only $A_1R$ and $A_{2A}R$ have been successfully subjected to structure elucidation, including both inactive [13, 14] and active states [15-17].

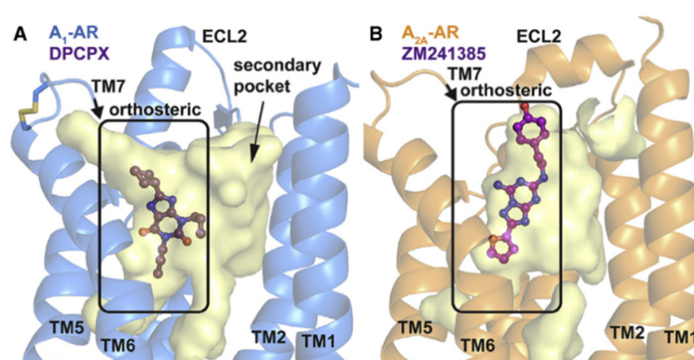


**Figure 2 Crystal structures of the indicated adenosine receptor.** (A) DPCPX, docked into the $A_1R$ structure, leaves a secondary binding site unoccupied. (B) The $A_{2A}R$ crystal structure with binding pocket occupied by ZM241385. Reproduced with permission [13].

Figure 2 includes the crystal structures of $A_1R$ (PDB: 5EUN) docked with antagonist DPCPX and $A_{2A}R$ co-crystalized with antagonist ZM241385 (PDB: 4EIY) [13, 14]. It was found that the amino acid residues in the orthosteric binding site are highly conserved for both receptor subtypes. However, the $A_{2A}R$ accommodates an elongated and narrow binding cavity, while in the $A_1R$, transmembrane helices TM1, TM2, TM3, TM7 and ECL3 brought a more open and wide binding cavity with a secondary binding pocket. These findings provide important structural insights into a highly valued GPCR target, supporting our research efforts in this thesis.

### 1.2. Adenosine receptor as drug target: focus on $A_1R$, $A_{2A}R$ and $A_3R$.

Adenosine receptors have distinct distributions through the human body and are important regulators of many different types of physiological and pathological processes. There is accumulating evidence that adenosine receptors are promising therapeutic targets for inflammatory, cardiovascular and neurodegenerative diseases, and cancer [18, 19]. In this thesis, we will only focus on the $A_1R$, $A_{2A}R$ and $A_3R$ subtypes as drug targets.

$A_1R$ is widely expressed throughout the body with particular high abundance at excitatory nerve endings [18]. The activation of receptors inhibits AC activity, activates potassium channels (including $K_{ATP}$ channels in neurons and the myocardium), blocks transient calcium channels and increases intracellular calcium ion concentrations and phospholipase C activity (PLC). Adenosine acting via $A_1R$ plays an important role in various pathological conditions, such as ischemia/hypoxia, epileptic seizures, excitotoxic neuronal injury and cardiac arrhythmias [20]. As a consequence, $A_1R$ agonists might be considered as therapeutic agents for the treatment of central nervous system (pain) and cardiovascular (arrhythmia) pathologies. Various $A_1R$ antagonists have been or are currently being explored for clinical applications targeting cognitive and renal dysfunction [19].

High expression levels of $A_{2A}Rs$ are found in the brain striatum, spleen, leukocytes and blood platelets [19, 21]. $A_{2A}Rs$ mediate vasodilation, modulate angiogenesis and protect tissues from collateral inflammatory damage. In the brain, the $A_{2A}R$ influences motor activity, psychiatric behaviors, the sleep-wake cycle and neuronal cell death. The $A_{2A}R$ has been validated as a therapeutic/diagnostic target by the clinical use of regadenoson, approved by FDA for myocardial perfusion imaging in patients with suspected coronary artery disease [22]. Furthermore, $A_{2A}R$ agonists are being investigated as agents to treat a number of conditions such as asthma, COPD and diabetic foot ulcers, while $A_{2A}R$ antagonists have emerged as an attractive approach for Parkinson disease [21] and, more recently, as adjuvants for checkpoint inhibitors in cancer [23] .

$A_3Rs$ have been recognized as a potential therapeutic target and biomarkers due to their overexpression in inflammatory and cancer cells compared to healthy cells [19, 24]. The $A_3R$ has been shown to couple to the $G_i$ and $G_q$ family and its anti-inflammatory activity correlates with the upregulation of nuclear factor-κB (NF-κB) signalling and the phosphoinositide 3-kinase (PI3K)–PKB–AKT signalling pathways. Earlier studies indicated an important role in the central nervous system, immune system, cancer, cardiovascular disease, and eye

disorder. Nevertheless, the medical relevance of the human $A_3R$ is enigmatic due to paradoxical effects in different therapeutic applications [25]. Currently, agonists are being considered as pharmacological agents for rheumatoid arthritis, psoriasis, glaucoma, and hepatocellular carcinoma.

### 1.3. Targeting adenosine receptors

From a drug discovery perspective, the wide distribution of adenosine receptors in mammalian cell types renders it mandatory to search for high-affinity and highly subtype-selective agonists and antagonists. The development of AR agonists started from structural modification of the endogenous ligand, adenosine, and hence structure-activity relationships for such ribose-containing compounds have been extensively investigated [12, 18, 26]. Besides these several non-ribose agonists have also been shown to reveal high receptor subtype selectivity, such as the $A_1R$ agonist capadenoson, a clinical drug candidate for atrial fibrillation [27]. Similarly, naturally occurring compounds caffeine and theophylline share a xanthine-based scaffold, contributing to the templates of antagonists for all AR subtypes, while non-xanthine structures have been explored for better water solubility and consequently improved bioavailability [12, 18, 26]. However, the major challenge in developing AR ligands for specific clinical applications is the ubiquitous receptor distribution and associated complex adenosine signaling, which exerts a broad spectrum of physiological and pathophysiological read-outs [19, 28]. The standard pharmacological assays of selectivity and efficacy are not sufficient to reveal a drug candidate's bio-distribution and pharmacokinetics, which may lead to potential side effects responsible for their failure in clinical trials. For example, the $A_1R$ antagonist rolofylline, a drug candidate for patients with acute heart failure with impaired renal function, failed in clinical trials due to $A_1R$-mediated ("on target") safety issues leading to an increased frequency of stroke and seizure activity [29]. Thus, it is desirable to decipher the distinct AR effects dependent on cellular and tissue specificity and disease phase. Moreover, repeated exposure to AR agonists may desensitize receptor activation or reduce receptor signaling over time, and consequently result in the development of ligand tolerance [19, 28]. Moreover, the daily consumption of AR antagonist caffeine, in coffee, tea, etc., undoubtedly complicates clinical trials studying AR-targeted agents [19, 28]. Therefore, despite its medical relevance in various diseases, only a few drugs targeting adenosine receptors have been approved for clinical indications [12, 22, 26].

## 2. Covalent ligands for GPCRs

Covalent ligands, or affinity labels, are defined as compounds that target a specific receptor and feature a reactive moiety that forms a covalent interaction with amino acid residues at or near the binding pocket [30, 31]. Covalent tools should ideally be able to first bind to the receptor non-covalently with high affinity and then form an irreversible interaction with one or more nucleophilic amino acid residues. The archetypical example of a covalent ligand-GPCR complex is the visual pigment rhodopsin, having the 11-cis-retinal chromophore covalently bound via a protonated Schiff's base [32].

### 2.1. Applications of covalent ligands

Although being one of the largest families of membrane proteins, GPCRs are notoriously difficult to study due to their low expression levels, intrinsic fragility and flexibility, and often low affinity for their endogenous ligand [33]. Covalent ligands may have the potential to address some of these challenges, since they are able to irreversibly bind to GPCRs and contribute to the formation of a stable and conformationally homogeneous ligand-receptor complex [34]. In this way such ligands have emerged already as a promising strategy to facilitate the structure elucidation of GPCRs [31]. For instance, the structures of cannabinoid receptor $CB_1$ [35] and adenosine $A_1$ receptor [13] have been resolved in the presence of chemo-reactive ligands contributing to the formation of stable and functional ligand-receptor complexes. The fact that covalent probes are designed to interact irreversibly with residues near the binding site has widened their applications to investigate the topology of the GPCR-ligand binding pocket, receptor signaling and *in vivo* research [31]. For example, the covalent cannabinoid $CB_1$ receptor agonist, (-)-7-isothiocyanato-11-hydroxy-1,1-dimethylheptylhex ahydrocannabinol (AM841), was successfully applied to investigate the ligand binding site in the $CB_1$ receptor [36, 37], assess its potential use in physiological and pathophysiological *in vivo* studies [38] and demonstrate the differences in binding motifs between the two cannabinoid receptor subtypes, $CB_1$ and $CB_2$ [39]. Given the highly dynamic nature of GPCRs and the inherent flexibility of their ligand binding sites, the potential of "freezing" a conformational state of the receptor by a covalently linked agonist or antagonist is very attractive. Moreover, covalent attachment of the ligand/drug to the receptor will lead to an infinitely long residence time or receptor occupancy, and thus might contribute to a sustained on-target pharmacological efficacy and minimized off-target effect [1, 40]. From this perspective, covalent ligands will offer an advantage over conventional reversible ligands

with respect to increased biochemical potency and greater selectivity for the targeted receptors.

## 2.2. Current state and further challenges

More recently, covalent probes equipped with a ligation handle, termed affinity-based probes (AfBPs), have emerged as valuable tools for chemical biology or proteomics studies to gain further insight into receptor localization and target engagement [41-43]. This strategy was inspired by earlier activity-based protein profiling-click chemistry (ABPP-CC), which helped in visualizing and quantifying the activities of drug targets (mainly enzymes) in native biological systems [44, 45]. However, different from mechanism-based enzyme inhibitors, covalent ligands targeting GPCRs have no specific active center to target and are generally armed with a highly electrophilic moiety responsible for the covalent interaction. The abundance of nucleophilic groups in biological systems may cause insufficient target-specific cross-linking [46] and induce low coupling selectivity [47]. In addition, the low expression of GPCRs also hampers successful execution of this otherwise promising technique.

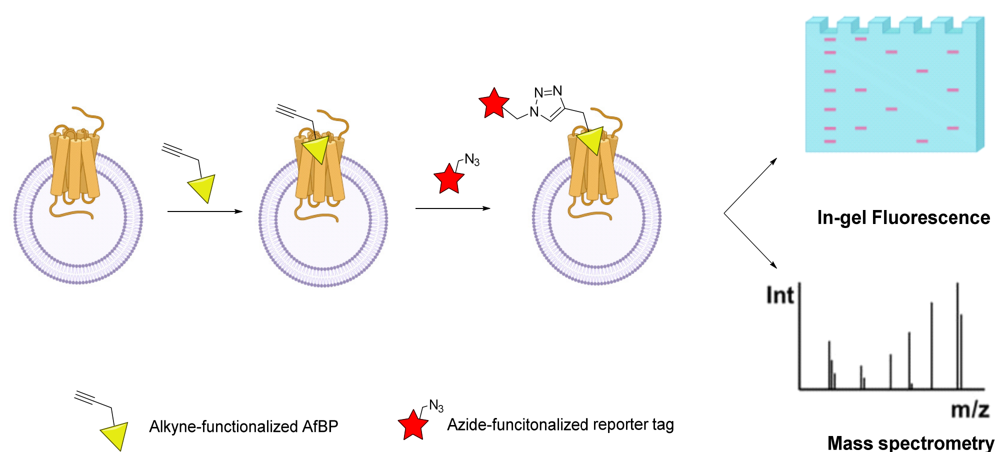


**Figure 3 Schematic representations of two-step affinity-based labeling strategy.** A small bio-orthogonal "ligation handle" is incorporated into a covalent ligand for a GPCR. After binding of the probe to the target receptor the tag is introduced by "click chemistry". The tagged probe-treated receptors are either visualized/quantified by in-gel fluorescence scanning or mass spectrometry analysis.

As illustrated in Figure 3, AfBPs provide the opportunity to label specific receptors, as they are functionalized probes paired with "clickable" reporter tags (e.g., biotin and/or a fluorophore), via a Huisgen 1,3-dipolar cycloaddition, to form a stable triazole-linked product. In this affinity-based protein profiling (AfBPP) approach the labeled protein targets are separated, visualized or affinity-purified, analyzed via the reporter tag using fluorescent SDS–PAGE, Western Blotting or enriched for multiple LC–MS platforms. It allows monitoring of

endogenous GPCR expression and engagement and may provide engineered biomarkers for translational drug discovery. Even though the entire GPCR family contains over 800 members, until recently only three subtypes including mGlu5 receptor, cannabinoid $CB_2$ receptor and adenosine $A_{2A}$ receptors have been subject of investigation [41-43], of which the $A_{2A}R$ will be the showcase in this thesis.

## 3. Aims and outline of this thesis

In this thesis the development and application of chemical tools and strategies are described to study three subtypes of ARs, $A_1R$, $A_{2A}R$ and $A_3R$. Combined, this research approach may ultimately aid in the discovery and development of novel adenosine receptor-based therapeutics that lack potential side effects as much as possible.

While **Chapter 1** serves as an introduction, **Chapter 2** provides a comprehensive overview of the molecular probes that have been reported to study adenosine receptor distribution, expression levels, occupancy, internalization and pharmacology in both *in vitro* and *in vivo* models. **Chapter 3** describes the development and pharmacological profiling of covalent ligands targeting $A_{2A}R$. The rational design of covalent ligands and a research flow chart to investigate irreversible ligand-receptor interactions have been applied to the $A_1R$ and $A_3R$ characterization in **Chapters 4** and **5**. **Chapter 4** describes a design and pharmacological profiling of the first non-ribose covalent partial $A_1R$ agonist. In **Chapter 5**, a structure-based approach was developed for a compound library based on the existing $A_3R$ antagonist 1H,3H-pyrido[2,1-*f*]purine-2,4-dione scaffold. The aim of this chapter is to identify a covalent ligand targeting the $A_3R$ and shed light on the details of its binding site. **Chapter 6** reports on the development of $A_{2A}R$ covalent ligands into an affinity-based probe. In the end, two-step affinity labeling with a ZM241385-based probe for $A_{2A}R$ target identification was established. **Chapter 7** summarizes the work described in this thesis and presents future prospects and challenges

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