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

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Summary

Most of the small-molecular drugs are tailored to reversibly interact with their biological targets. There are concerns that the formation of a covalent bond with their target may yield off-target reactivity and this has led to these covalent drugs being disfavored as a drug class. Nevertheless, a common focus in modern drug discovery programs to maximize the strength of the desired drug-protein interactions has brought the concept of 'covalent interactions' back into the drug discovery track. These interactions may cause prolonged duration of action and improved potency and selectivity, providing advantages as both chemical probes and drug candidates. It has been recognized that the deficiencies of covalent modes of action may be tuned by designing compounds that carefully combine reactivity with specific complementarity to the target. 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.

Chapter 1 covers the main concepts studied throughout the thesis. The chapter starts with an introduction to G protein-coupled receptors (GPCRs), with a focus on the adenosine receptors. Subsequently, the concepts of covalent ligands for GPCRs and current state-of-the-art and challenges are described**. Chapter 2** continues with discussing the emerging molecular probes to profile adenosine receptors' biological effects and summarizes the utilization of the probes to characterize and interrogate adenosine receptor subtypes both *in vitro* and *in vivo*, and to study their behaviors in physiological and disease conditions. The following **Chapters 3-6** demonstrate the design of covalently binding ligands targeting adenosine receptors and highlight their utility to profile pharmacological effects.

In **Chapter 3**, LUF7445 was introduced and characterized extensively. This covalent antagonist was designed based on a high affinity non-xanthine antagonist, ZM241385. This ligand has been co-crystalized with $hA_{2A}R$, providing a clear blueprint of ligand-binding interactions. Analysis of the shape of the binding site shows that the phenylethylamine chain in ZM241385 is directed toward the more solvent-exposed extracellular region (EL2 and EL3), such that the ligand-receptor interactions offer us tremendous substituent flexibility to introduce the electrophilic fluorosulfonyl group responsible for the covalent interaction. Using time-dependent affinity shift and kinetic competition association assays, LUF7445 was likely identified as a covalent antagonist, which was further confirmed in wash-out assay. An *in*

silico A_{2A}-structure-based docking model combined with site-directed mutagenesis studies suggested the binding mode of LUF7445 with an anchor point at lysine amino acid $K153^{ECL2}$. Meanwhile, a functional assay combined with wash-out experiments was set up to investigate the efficacy of covalent binding of LUF7445. These findings advance the studies of covalent ligands to serve as a prototype for a therapeutic approach in which a covalent antagonist may be needed to counteract prolonged and persistent presence of the endogenous ligand adenosine.

Inspired by the successful design of LUF7445, **Chapter 4** starts with a structure-based design of covalent antagonists for human adenosine A_3 receptors, a case with few structural insights of the receptor binding pocket. Starting from the 1H,3H-pyrido[2,1-*f*]purine-2,4-dione antagonist scaffold, a series of ligands bearing a fluorosulfonyl warhead with a varying linker length was obtained. The follow-up streamlined workflow to characterize these ligands was adapted from **Chapter 3**, suggesting LUF7602 behaves as a covalent antagonist for the human adenosine A_3 receptor. Of note, in this research, a non-reactive methylsulfonyl derivative LUF7714 was developed as a reversible control compound. When combined with *in silico* hA_3R-homology modeling and site-directed mutagenesis, LUF7602 was used to characterize the spatial orientation and topography of the receptor ligand binding site, demonstrating that amino acid residue $Y265^{7.36}$ was the unique anchor point of the covalent interaction. In the end, using this structured approach a well-defined covalent ligand was quickly obtained and profiled.

Of note, this research workflow, including rational design of covalent ligands and non-reactive sulfonyl-bearing control compound, covalent interaction identification combined with *in silico* modeling and site-directed mutagenesis, may serve as a streamline to study the structural details of ligand recognition for other GPCR subtypes.

Subsequently, **Chapter 5** reports on the design, synthesis and application of a Capadenoson-based covalent partial agonist probe, LUF7746, bearing an electrophilic fluorosulfonyl moiety. Similarly, a nonreactive ligand bearing a methylsulfonyl moiety, LUF7747, was designed as a control ligand. In addition to the streamlined workflow to profile covalent ligands, a hA_1R-mediated G protein activation assay was used to identify LUF7746 as a partial agonist and it is resistant to blockade with an antagonist/inverse agonist. An *in-silico* structure-based docking study combined with site-directed mutagenesis of the hA_1R demonstrated that amino acid $Y271^{7.36}$ was the primary anchor point for the covalent

interaction, as it was for the adenosine A_3 receptor. In the end, a label-free whole-cell assay was set up to identify LUF7746's irreversible activation of an A_1 receptor-mediated cell morphological response.

After these demonstrations of covalent ligands to investigate adenosine receptor's pharmacological effects, **Chapter 6** reports on the development of A_{2A}R covalent ligands into an affinity-based probe. In detail, an irreversible ligand LUF7445 was equipped with a terminal alkyne to serve as an affinity-based probe, LUF7487. After binding to the purified hA_{2A}R, this probe was paired with a clickable fluorophore Cy3-azide, and irreversibly and concentration-dependently labelled purified hA_{2A}R, which was visualized by SDS-PAGE. This labelling of the purified hA_{2A}R by LUF7487 could be inhibited by both reversible and irreversible antagonists, provided that they target the same receptor binding site. In the end, a successful labeling of the receptor in cell membranes overexpressing hA_{2A}R was demonstrated, making LUF7487 a promising affinity-based probe that sets the stage for the further development of probes for GPCRs.

In summary, the approach to design and pharmacologically profile covalent ligands for adenosine receptors has been explored and detailed throughout this thesis. A universal strategy of covalent ligands' design has been investigated, and the findings highlight several elements that can profoundly influence the covalent ligand-receptor interaction. These factors manifest an optimal combination dependent on the affinity of the pharmacophore, the warhead's proximity to an appropriate nucleophilic amino acid residue and the warhead's reactivity. The overall conclusion from the results described in this thesis and emerging opportunities for drug discovery are discussed in detail in **Chapter 7**. In concert, the novel insights that have been obtained in this thesis may provide valuable information for drug discovery targeting the adenosine receptor as well as other GPCRs.