Affinity-based profiling of the adenosine receptors

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Summary

The human body consists of a tremendous number of cells, classified into a wide variety of subtypes. Each individual cell is protected by a membrane bilayer that separates the intracellular compartments from the extracellular environment. Despite this 'barrier', cells must be able to respond towards external stimuli, such as small molecular drugs that float in the bloodstream. There are multiple mechanistic pathways that allow an intracellular response towards extracellular stimuli. One of the most important mechanistic pathways is the activation of G Protein-Coupled Receptors (GPCRs). GPCRs are receptor proteins that reside in the cellular membrane and consist of both an extracellular- and intracellular component. Upon binding towards an extracellular stimulus, e.g. a protein, peptide, small molecule or light, GPCRs will transduce a signal into the cell, 'telling' the cell how to respond to the specific stimulus. Malfunctioning GPCR signaling pathways can lead to severe states of disease. It is therefore no surprise that GPCRs have been of high interest in drug discovery programs of pharmaceutical companies, leading to some 25% of the currently marketed drugs.

A subfamily of GPCRs that has sparked the interest of drug discoverers is the set of Adenosine Receptors (ARs). This subfamily consists of four subtypes of adenosine receptors: the A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (A_1AR, A_{2A}AR, A_{2B}AR and A_3AR, respectively). Activation of the ARs through their natural ligand adenosine induces a wide variety of effects, strongly dependent on cell- and tissue type, but is often immunosuppressive. Caffeine is a famous drug that acts through the ARs, although via inhibition rather than activation. A more elaborated overview of the four ARs and their role in human physiology and pathology is presented in **Chapter 1**. In summary, the four ARs are involved in a plethora of cellular signaling pathways in both physiological and pathological conditions and therefore interesting targets from a drug discovery perspective.

Studying the ARs, however, bears many challenges. As mentioned in **Chapter 1**, there are multiple factors, inherent to GPCRs, that hinder biochemical detection of the ARs. These factors include poor solubility, low expression levels and complex cellular signaling pathways. To overcome, as well as to study the complexities mentioned above, we have put our attention to the development of chemical probes that bind irreversibly (covalently) to either one of the ARs. Covalent probes induce persistent binding to the receptor of interest, requiring the implementation of a reactive group ('warhead') for irreversible binding. Functionalization of covalent probes with reporter groups, e.g. a fluorophore, biotin or click moiety, allows detection of the ARs in a broad range of biochemical assays. Throughout the chapters of this thesis, we have transformed known AR ligands into functionalized and/or covalent probes, for their utilization in biochemical assays.

In **Chapter 2** an overview is given of all the recently reported functionalized covalent probes that target GPCRs. These include affinity-based probes (AfBPs), ligand-directed probes, glycan-targeting probes and metabolically incorporated probes. The warheads of these probes are compared, and their use in biochemical assays is evaluated. In this thesis, we have focused on the development of covalent probes, AfBPs and ligand-directed probes.

Chapter 3 shows the development of a covalent ligand for the A_{2B}AR. In this chapter, different types of warheads were chemically introduced onto a known scaffold for the A_{2B}AR, leading to a set of 12 potential covalent probes. Pharmacological investigation of time-dependent affinity and subtype selectivity showed strong differences between the various substitutions. One of the synthesized compounds, LUF7982, was found to be superior in terms of affinity and selectivity and was therefore further investigated for its possible binding mode towards the receptor.

While covalent ligands are interesting tools to study irreversible blockade of GPCRs, such probes do not allow direct detection of probe-bound receptors in biochemical assays. Therefore, in **Chapters 4** and **5**, we have put our efforts into the development of AfBPs for the A_1AR and A_3AR, respectively. In both chapters, a small set of clickable AfBPs was synthesized based on covalent ligands that had previously been developed in our lab. The potential AfBPs were pharmacologically evaluated on time-dependent affinity, AR selectivity and a covalent mode of binding. The best candidates were evaluated on their ability to fluorescently label the receptor in SDS-PAGE and confocal microscopy experiments. This yielded LUF7909 as AfBP for the A_1AR and LUF7960 as AfBP for the A_3AR.

The possibilities of using LUF7909 and LUF7960 as tools to detect the A_1AR and A_3AR were further explored at the end of the corresponding chapters. We managed to utilize LUF7909 as a tool to 'pull-down' the A_1AR from a complex mixture of proteins, as well as to detect possible protein off-targets using mass spectrometry. Unfortunately, we did not manage to detect the A_3AR in a similar attempt with LUF7960. Presumably the relatively low expression level and hydrophobicity of the A_3AR hinder detection in the current assay setup. Nevertheless, LUF7960 was shown to be a useful tool for the detection of the A_3AR in flow cytometry experiments. Altogether, this led to the detection of the A_1AR on rat adipocytes and the detection of the A_3AR on human granulocytes.

A drawback of AfBPs might be the irreversible occupancy of the receptor binding pocket. Therefore, in **Chapter 6**, we aimed to develop a ligand-direct probe for the A_{2B}AR. Such probes do not irreversibly block the receptor binding pocket and therefore can be used as tool to label to receptor prior to agonist-dependent activation. Two ligand-directed probes were synthesized based on covalent ligand LUF7982. These compounds showed to bind the A_{2B}AR with high affinity in pharmacological assays, but not in a time-dependent fashion, indicating a reversible mode of action. Preliminary SDS-PAGE experiments in A_{2B}AR-overexpressing cells showed labeling of the A_{2B}AR by one of these probes, LUF8019. However, other possible usages of LUF8019 in different biochemical assays and/or cell lines have yet to be explored.

In **Chapter 7**, a critical view is taken at the aspects that are of importance to covalent chemical probes: selectivity, reactivity and detectability. Also the usage of covalently functionalized probes in SDS-PAGE, fluorescent microscopy, flow cytometry and chemical proteomics is discussed, and conclusions are drawn based on the experiments reported in this thesis. Lastly, future prospects are given on possible usages of the herein synthesized probes in future biochemical assays.