The good? The bad? The mutant! Characterization of cancer-related somatic mutations and identification of a selectivity hotspot in adenosine receptor

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Summary

G protein-coupled receptors (GPCRs), one of the largest families of membrane proteins, are responsive to a diverse set of physiological endogenous ligands including hormones and neurotransmitters. Due to the various GPCR ligand binding domains present on GPCRs and their sensitivities to a diverse array of ligands, these proteins have shown to be very 'druggable' as they are the main target for an estimated 30% of approved drugs. A growing body of evidence shows a prominent role of GPCRs in all phases of cancer with a mutation frequency of approximately 20% in all cancers. Mutations occurring in GPCRs can severely alter their normal function and may ultimately convert their physiological and pathological roles. One particular class of rhodopsin-like GPCRs included in this thesis are the adenosine receptors (ARs). Due to the accumulation of adenosine in the tumor microenvironment, all four subtypes of ARs might be targets for the development of novel approaches for the treatment of cancer. For each of the four subtypes, a number of somatic mutations have been identified in patient isolates. In this thesis, we examined them on receptor activation and ligand binding using reference adenosine receptor ligands, and determined the impact mutations have on these pharmacological readouts.

Chapter 1 serves as an introduction covering the main concepts in this thesis. **Chapter 2** continues with the strategies of using yeast systems in human GPCR studies with a focus on adenosine receptors. The chapter starts with general features of budding yeast with multiple modifications in the yeast pheromone signaling pathway to be used for human GPCR studies. Subsequently, highlighted studies on ARs expression and functionality in yeast expressing systems are described. **Chapter 3** provides an overview of current existing evidence for the involvements of GPCRs and their signaling pathways in tumor biology, as well as the effect of mutations in receptor pharmacology and their potential impacts in cancer development and progression. Furthermore, evidence for ARs in cancer development is discussed in detail.

As mutations of ARs have been identified from cancer patient isolates, **Chapter 4**-**6** provide information on the impact of these mutations in receptor functionality. **Chapter 4** focuses on receptor expression and activation of cancer-related mutations on adenosine A_{2B} receptors (A_{2B}AR) using an engineered yeast system, MMY24. The 15 cancer-related mutations included in this chapter have been identified as cancer-specific. These mutations resulted in 3 constitutively active mutants (CAMs), 5 less active mutants (LAMs), 4 no effect mutants (NEMs) and 3 loss of function mutants (LFMs). Among the CAMs, mutant receptor Y202C$^{5.58}$, located on a GPCR activation switch, locked the receptor in an active conformation. All 3 LFMs are located on/near the most conserved residues of the transmembrane helices, indicating the important roles of these residues in receptor functionality of A_{2B}AR.

The effects of cancer-related mutations on adenosine A_1 receptors (A_1AR) on receptor activation and ligand binding are described in **Chapters 5** and **6**. **Chapter 5** describes twelve mutations located at the loop regions. By using the same yeast system, we characterized 1 CAM, 7 constitutively inactive mutants (CIMs), 1 LFM and 3 NEMs. All mutant receptors found in extracellular loops (ELs) showed decreased constitutive activity and/or potency of reference agonist CPA, as well as decreased affinity of DPCPX, a prototypic antagonist. However, the findings of mutational effects on receptor activation when we used a mammalian system diverged from the yeast system, especially for mutations located at intracellular loops (ILs), namely L113F$^{34.51}$ and L211R$^{5.69}$. The yeast system used in this thesis might therefore not be suitable for the investigation of mutations located in the receptor-G protein interaction interface, due to the lack of similarity to the human G_α protein. **Chapter 6** focuses on 13 mutations positioned in the 7-transmembrane (7-TM) domains, resulting in 2 CAMs, 5 CIMs and 6 LFMs. Similar to A_{2B}AR, mutations located on or near conserved residues in GPCRs showed abolished receptor activation. The CAM H78L$^{3.23}$ locked the receptor in an active conformation with an extremely high constitutive activity. Some of the mutations located on the residues pointing towards the cell membrane showed divergent effects on receptor activation between the yeast and mammalian expression system. Most of the investigated cancer-related mutations in both A_{2B}AR and A_1AR influence receptor activation, and they might eventually alter cancer hallmarks where adenosine and adenosine receptors play a key role.

Chapter 7 reports the approach for the identification of a stereoselectivity hotspot in A_{2B}AR antagonist recognition from both computational and experimental aspects. Having an A_{2B}AR homology model, we were able to predict the selectivity hotspot for stereoselective antagonist recognition. Molecular modeling suggested that the structural determinants of this selectivity profile would be residue V250$^{6.51}$ on A_{2B}AR and the (*S*)-stereoisomer of the ligand ISAM-140. The enantiomers of ISAM-140 were separated and their absolute configurations were unequivocally assigned via a combination of semipreparative chiral HPLC, circular dichroism spectroscopy and X-ray crystallography. The stereospecific binding mode was then confirmed by site-directed mutagenesis experiments and radioligand binding assays. Higher affinity of (*S*)-ISAM-140 was obtained on A_{2B}AR, and a partially recovered affinity for both stereoisomers was observed on the L249V$^{6.51}$ A_{2A}AR mutant (the A_{2B}AR-like mutation). This effect was explained on the basis of structure-energy modeling via rigorous free energy perturbation (FEP) calculations.

The overall conclusion from the results of the individual experimental chapters are discussed in detail in **Chapter 8**. This chapter also provides future prospects and challenges that emerge from the research presented in this thesis.