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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Chapter 8

Conclusion and future perspectives.

Conclusions

Yeast system is suitable for GPCR studies

Engineered yeast systems *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia pastoris* (*P. pastoris*) have been used during the past three decades as a synthetic "null" background for human GPCRs studies. They serve various purposes, including receptor purification, characterization of novel ligands and GPCR mutations, as a biosensor, and for receptor deorphanization[1]. These yeast systems are cheap, stable and versatile for GPCR expression and characterization. As reviewed in **Chapter 2**, we summarized the strategies of linking human GPCRs' expression and functionality to these yeast systems and highlighted the studies on adenosine receptors heterologously expressed in yeast. The *P. pastoris* system with high similarity to advanced eukaryotic expression systems is commonly used for GPCR expression with the purpose of receptor purification[2], while the *S. cerevisiae* system is often used for GPCR signaling research due to the similarity between the yeast mating pathway and human GPCR signaling[3]. Moreover, multiple modifications have been generated on the yeast pheromone signaling pathway in order to enhance human GPCR expression, to couple to the yeast signaling pathway, and obtain quantifiable read-outs[1].

The yeast strain used in this thesis (**Chapter 4, 5** and **6**), namely MMY24, contains one chimeric G protein subtype and the HIS3 reporter gene (Figure 1). In this yeast strain, the last five C-terminal amino acids of the yeast G_{α} protein were transplanted by the corresponding sequence of the mammalian $G_{\alpha i}$ protein. With the HIS3 reporter gene present, yeast cell growth on histidine-deficient medium can be used as a measurement of human receptor activation. We concluded that this yeast system was suitable for functional characterization of cancer-related mutations on the A_{2B}AR (**Chapter 4**) and for initial functional screening of cancer-related mutations on the A_1AR (**Chapter 5** and **6**).

Cancer-related mutations alter receptor pharmacology

GPCRs are the largest membrane protein family, and regulate divergent physiological and pathological activities throughout the human body[4]. They are targeted by around 30% of current therapeutic drugs for the treatment of various types of diseases. However, only a few members of this superfamily are currently being explored as oncological drug targets[5]. In **Chapter 3**, we discussed the role of GPCRs, their signaling pathways and their mutations in cancer, with a focus on adenosine receptors. In that chapter we summarized current existing evidence for the involvement of GPCRs in tumor biology, as well as the effect of mutations in receptor pharmacology, including receptor expression, receptor-ligand interaction and GPCR-G protein coupling. Moreover, we discussed the potential impact of GPCR mutations occurring in all stages of cancer development and progression. The accumulation of adenosine has been reported in the hypoxic tumor micro-

environment and regulates cancer hallmarks via its corresponding GPCRs, the adenosine receptors[6]. Therefore, adenosine receptors have attracted much attention as therapeutic targets for cancer treatment, although their exact roles in cancer progression still remain unclear[7]. Cancer-associated mutations in adenosine receptors have been identified from cancer patient isolates, their data stored in the Cancer Genome Atlas[8] and used by us.

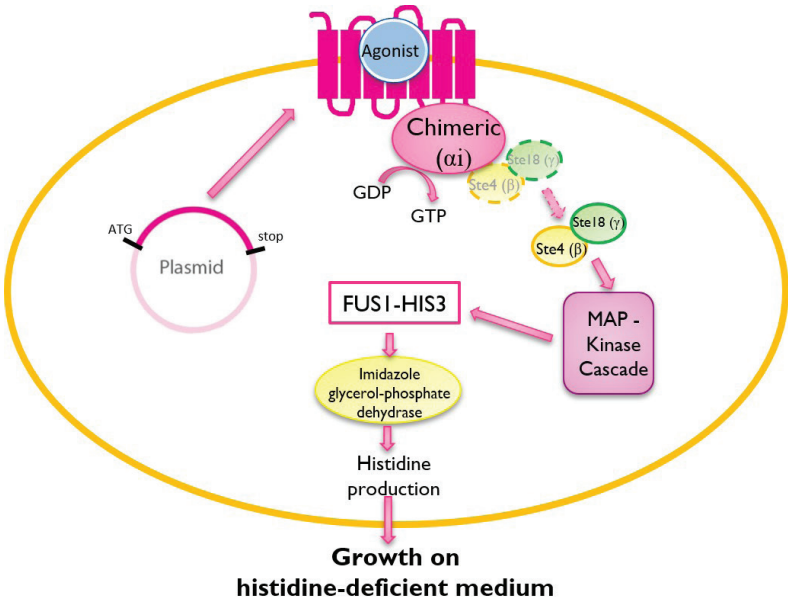

Figure 1. Schematic picture of human GPCR expression and activation in genetically modified yeast strain MMY24.

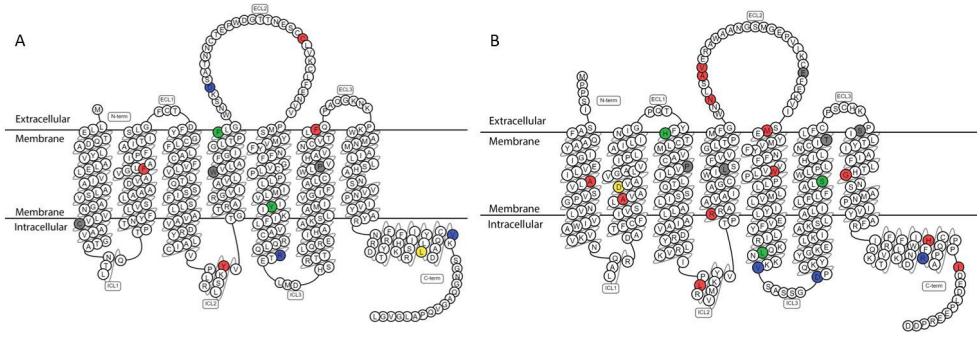

Figure 2. Snake plot of (A) A_{2B}AR and (B) A_1AR. Residues where cancer-related mutations were found are marked in colors. Yellow residues were found with more than 1 mutation. Residues identified with CAMs are colored in green, LAMs/CIMs in red, NEMs in blue and LFMs in grey. Most of the LFMs are located at the 7-TM domains. Half of mutant receptors with reduced agonist affinity or potency are at the extracellular region. Mutations positioned in the intracellular region lead to diverse effects in receptor activation.

In this thesis, we further investigated the effects of these mutations on receptor activation and ligand binding. The 15 cancer-related mutations on A_{2B}AR have been identified as cancer-specific, as they do not overlap with any point mutations from the natural variance (Figure 2A). The effects of these mutations on receptor activation have been reported in **Chapter 4**. We found that 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) by using the yeast system. 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.

In **Chapter 5 and 6**, we selected 27 point somatic mutations out of 48 total cancer-related mutations of A_1AR based on cancer types of interest (Figure 2B). In **Chapter 5**, we focused on the mutations located outside the 7-TM domains. By using the yeast system, we characterized 1 CAM, 7 constitutively inactive mutants (CIMs), 1 LFM and 3 NEMs. Mutant receptors found in ELs all 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 the mammalian system diverged from the yeast system, especially for mutations located at ILs namely L113F$^{34.51}$ and L211R$^{5.69}$. **Chapter 6** presents 13 cancer-related somatic mutations positioned within the 7-TM domains of A_1AR, 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. In summary, most of these cancer-related mutations in both A_{2B}AR and A_1AR influence receptor activation, and they might eventually alter cancer hallmarks where adenosine receptors play a key role.

Residue V6.51 is a selectivity hotspot in A_2 receptors

In **Chapter 7**, we investigated the stereospecific and selective recognition of a selective A_{2B}AR antagonist ISAM-140. Molecular modeling suggested that the structural determinants of this selectivity profile would be residue V250$^{6.51}$ on A_{2B}AR and (*S*)-ISAM-140 as the active stereoisomer. 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 radioligand binding assays. Higher affinity of (*S*)-ISAM-140 was obtained on A_{2B}AR, and 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. In summary, this study provides useful structural insights in the stereospecific binding mode of these novel A_{2B}AR antagonists, paving the way for future structure-based ligand design and optimization of selective antagonists as well as dual A_{2A}AR/ A_{2B}AR

ligands.

Taken together, this thesis contributes to a better understanding of cancer-related mutations in GPCR pharmacology and eventually will provide potential novel approaches of modulating their activities with medicinal products. Combinatorial strategies of computational and experimental techniques could provide further insight for structure-based ligand optimization.

Future perspectives

What's more with the yeast system?

Adenosine receptors are widely distributed throughout the human body and regulate various physiological and pathological processes including neurological, cardiovascular and inflammatory diseases, and cancer[9]. In this thesis, we successfully expressed human adenosine receptors in an engineered yeast system and performed functional characterization on the cancer-related mutations of these receptors (**Chapter 4-6**). Especially for A_{2B}AR, as mutant receptors cannot be expressed in mammalian cells using non-viral transfection methods as mentioned in **Chapter 7**, the yeast cells in this case are the alternative expressing system with a low cost of cultivation (**Chapter 4**). Apart from mutations identified from cancer patients, adenosine receptors are known to be mutated in neurological diseases[10–13]. These mutations have been reported to associate with disease development, some are even identified to be disease-causing[11]. In this case, the yeast system can also be used for rapid functional screening of mutant receptors, as well as high-throughput screening of novel ligands targeting these disease-causing mutations in adenosine receptors.

Up till 2020, nearly 100 of human GPCRs have been expressed in *P. pastoris*[14] and more than 50 have been functionally coupled to the pheromone pathway of *S. cerevisiae*[1]. Despite the many successes in human GPCR studies with the engineered yeast cells (**Chapter 2**), drawbacks of this system are still remaining. Firstly, compared to the membranes of mammalian cells, the yeast cell membrane contains less cholesterol and higher levels of ergosterol, which may dramatically change the conformation and thus functionality of human GPCRs with specific cholesterol binding sites[15]. As discussed in **Chapter 6**, some of the mutations located on the residues pointing towards the cell membrane showed diverged effects on receptor activation in between the yeast and mammalian expressing system. Humanized yeast strains with engineered cholesterol synthesis have already been applied to better express membrane proteins[16,17]. The same approach might also help in enhancing heterologous expression of functional human GPCRs in yeast. Secondly, in order to couple a human GPCR to the yeast signaling pathway, several different types of chimeric G_{α} protein have been investigated resulting in chimeric G_{α} proteins[18]. However, as discussed in **Chapter 5**, the yeast system used in this thesis

might 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. Key interactions between GPCRs and G_α proteins involve residues 12–20 of the G protein's α5-helix, although the strongest interactions are provided by the last 5 amino acids of the C-terminus[19]. Therefore, replacing only the last 5 amino acids from the yeast G_α protein might not be enough to precisely mimic human GPCR-G protein interactions. In this regard, heavily genome-modified yeast systems have been generated via the CRISPR/Cas9 technique with rational tuning of cell sensing, transcriptional regulations and various reporters[20–22]. The CRISPR technique may also be useful in generating a more humanized yeast expressing system for human GPCR studies.

How will the cancer-related mutations on GPCRs affect cancer hallmarks?

To obtain a better understanding of the complexity of cancer, "Hallmarks of Cancer" have been introduced as a useful conceptual framework to capture the complex biology of cancer in a few basic principles. The current framework consists of 10 hallmarks (Figure 3), including sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death, with the addition of two emerging hallmarks (i.e. deregulating cellular energetics and avoiding immune destruction) and two enabling characteristics (i.e. genome instability and mutation, and tumor-promoting inflammation)[23]. Kinases have been investigated as prominent therapeutic targets in preclinical oncology due to their critical involvement in protein phosphorylation[24], of which abnormal function has been linked to a driver or direct outcome of the disease[23,25]. Kinase signaling pathways have been proven to be the driver in many hallmarks of cancer indeed, such as cell proliferation, angiogenesis and evasion of antitumor immune response[23]. Receptor tyrosine kinases (RTKs) in particular have been intensively investigated as promising drug targets in different types of cancer during the last two decades[26]. Up till 2019, 43 inhibitors targeting RTKs have been approved by the FDA for cancer indications[27], however, drug resistance or adverse effects appear to limit the efficacy of these RTK inhibitors (RTKIs). The most common mechanism of drug resistance is the association of mutations occurring within RTKs, which diminish the binding of RTKIs[28]. Mutations of RTKs have been identified in around 46% of all cancers[29]. Moreover, notable cancer driver hotspots, such as mutants D1228H/N/V and M1250T of hepatocyte growth factor receptor kinases, have been identified in RTKs leading to abnormal cell proliferation and tumorigenesis, and possibly the rise of drug resistance upon treatment[30]. To overcome drug resistance caused by on-target mutations, various therapeutic strategies have been designed, including combinatorial treatments targeting single or parallel kinase pathways, other therapies addressing a hallmark phenotype[31], as well as third generation RTKIs (e.g. osimertinib) with higher selectivity towards mutant RTKs[32]. Similar approaches might also benefit drug design targeting GPCRs in cancer treatment, as the many findings in RTK aberrations seem to have

a correlate in GPCRs. Also, intervening with GPCR function may help to overcome resistance in RTK-based therapy.

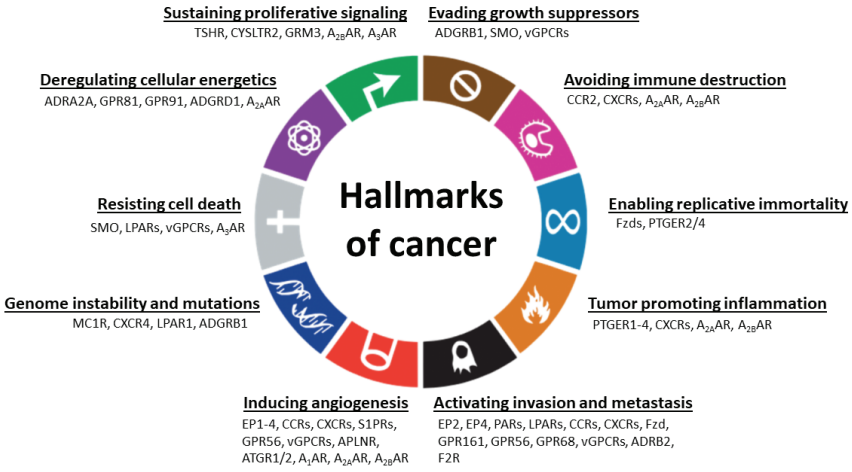

Figure 3. Examples of GPCRs in the hallmarks of cancer. Figure adapted from Arang and Gutkind[34] and Hanahan and Weinberg[23].

As summarized in **Chapter 3**, GPCRs, due to their remarkable centrality in various cellular and physiological processes, have also been identified as key participants in facilitating the hallmarks of cancer (Figure 3). Moreover, mutated GPCRs have been revealed in approximately 20% of all cancers covering various tumor types[33], which is comparable with the mutation frequency of RTKs[29]. However, for many of the GPCR mutations, their biological effects in cancer are largely unknown due to the complexity in the prediction of cancer-driving mutations[34]. Luckily, structure-function analysis of cancer-associated GPCR mutations has been developed to provide a better understanding of the functional effects of these mutations from a structural point of view[34]. An accumulation of cancer-related mutations has been observed in several highly conserved receptor sequence motifs (e.g. “DRY” and “NPxxY”) as well as the highly conserved positions of TM domains (3.50, 4.50 and 7.50 according to BW numbering[35]) in comparison to other residues[36]. These conserved motifs and residues are known as key regulators in ligand binding, G protein-coupling and/or receptor stability[37]. Mutations in these locations may lead to disabled receptor function, which has been shown in **Chapter 4** where mutant A_{2B}AR W130C$^{4.50}$ resulted in a complete loss of receptor activation.

In this thesis altogether, we have characterized 40 cancer-related mutations in A_{2B}AR and A_1AR (**chapter 4**, **5** and **6**) with the aim to contribute to a biological understanding from a molecular pharmacological point of view. These mutations are located all over the receptor structure (Figure 2), while interestingly enough their effects are in line with the role of receptor structure in receptor functionality. For

instance, the 7-TM domains including conserved motifs and residues are known to maintain receptor conformation upon activation[38]. Most of the LFMs in this thesis have been identified within this region; of note, all mutants located at the conserved residues are LFMs. Half of the mutant receptors with decreased agonist potency or affinity are positioned in the extracellular region, which regulates ligand binding[37,39]. Moreover, diverse mutational effects in receptor activation have been observed for mutations within the intracellular regions of which the conformational change influences G protein coupling[38]. Unfortunately, the effect of these cancer-related mutations on allosteric modulation still remains unclear, awaiting complete characterization of these mutations in receptor functionality. As a side note, among 40 mutations involved in this thesis, 7 of them are leucine mutations, which might be due to the higher amount of codons encoding leucine.

Although the roles of some GPCRs in cancer progression have been published, more research on their mutations and signaling pathways is warranted to fully understand their involvement in cancer hallmarks. Further studies in combination with structure-function analysis may benefit the identification of cancer driver hotspots within GPCRs. Of note, specific inhibitors targeting protein products of some passenger mutations could enhance the metabolic deleteriousness in cancer cells[40]. Taken together, addressing both driver and passenger mutations may provide strategies for the design of personalized therapeutics.

What can we improve in ligand optimization for drug discovery in oncology (and beyond)?

The first crystallographic structure of a GPCR was elucidated in 2000 for bovine rhodopsin[41]. In 2007, the first crystallographic structure of a human GPCR was published, i.e. the β_2-adrenergic receptor bound to an inverse agonist[42]. Thereafter, more high-resolution crystal structures of human GPCRs have been deciphered, paving the way for a more detailed structural insight of receptor-ligand interactions in GPCRs. Knowing the architecture of the ligand binding site is very helpful for structure-based drug design[43]. Unfortunately, huge numbers of GPCR structures still remain unresolved due to difficulties in pure protein isolation, crystal diffraction and many other technical problems. Homology/comparative modeling in this case could be used in structure-based studies when 3D structures are not available for the GPCR of interest[44].

A homology model predicts the 3D structure of an unknown protein based on known 3D structures of proteins with homologous sequence [45,46]. It has been shown that the usage of multiple templates provides better homology models when templates share low sequence identity with the target protein[47–49]. A homology model of A_{2B}AR, developed using crystal structures of hA_{2A}AR as templates, has been used in this thesis to provide a structural explanation of the impact the cancer-related mutations may have in receptor activation (**Chapter 4**). Docking is a computational method used to predict ligand-protein interactions and relative measures of affinity for series

of ligands binding to a protein of interest[50]. Having an A_{2B}AR homology model, we were able to predict the selectivity hotspot for stereoselective antagonist recognition in A_{2B}AR, which was confirmed by site-directed mutagenesis experiments (**Chapter 7**). Additionally, receptor ensemble docking studies have shown promising results supporting the application of a homology model in virtual screening for the discovery of novel GPCR ligands[51]. Moreover, key residues in A_3AR and A_{2A}AR for covalent interaction between ligand and receptor were predicted via assigning the docking mode towards a specific amino acid residue, and the hypothesis was further confirmed by mutagenesis study[52,53]. An increasing number of crystal structures of human GPCRs are expected to be resolved in the near future, and be used for *in silico* drug discovery studies and homology model development. More recently, a novel neural network AlphaFold has been published with atomic accuracy in predicting protein structures based on sequence combined with machine learning, providing a complementary approach to homology modeling[54]. With the help of various computer-aided techniques, more accurate homology modeling for other GPCRs will be developed in order to increase the chance of novel ligand identification, as well as ligand design and optimization for GPCRs in general.

Final notes

All in all, this thesis is focused on characterizing cancer-related somatic mutations in adenosine receptors with respect to receptor activation and ligand binding. We also confirmed that the yeast system is well suitable for the rapid and initial functional screening of these mutations on A_{2B}AR and A_1AR. The results obtained in the thesis contribute to a better understanding of receptor functionality at the structural level, as well as at the pathologically relevant level. Additionally, with the combination of computational and experimental approaches, we expanded the insight of structure-based selective ligand design and optimization. Finally, we hope that the findings from this thesis can provide potential strategies in cancer therapeutics and further drug development.

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