Mutation-driven modulation of GPCR pharmacology in cancer: insights from adenosine and serotonin receptors

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Chapter 7

Conclusions and future perspectives

1. Conclusions

Cancer-associated mutations of GPCRs alter receptor pharmacology

Our studies have shown that cancer-associated mutations in GPCRs can alter receptor pharmacology either by disrupting ligand binding or by independently altering receptor conformation. This was demonstrated in case studies of the adenosine A_{2A}AR (**Chapter3**) and the serotonin 5-HT_{2C}R (**Chapter 6**).

Firstly, mutations adjacent to ligand entry path or the orthosteric binding pocket of a ligand can affect the affinity and consequently the potency of the ligand. For example, mutations F70L and H278N of A_{2A}AR the decreased affinity and potency of NECA, and mutations L209H and F328S of 5-HT_{2C}R decreased affinity and potency of 5-HT.

Secondly, mutations structurally distant from the binding pocket may affect conformational change during receptor activation, and in turn, alter ligand binding affinity. For example, I92M and P285L mutations in the conserved of motifs of A_{2A}AR were found to decrease the potency of NECA, of which the affinity was also decreased by I92M (not determined for P285L due to low receptor expression). Furthermore, $S132L^{4.53}$ and H278N may shift A_{2A}AR towards the inactive state, contributing to increased efficacy but decreased potency of agonist NECA, while they increased the inhibitory potency of ZM241385, despite the affinity of ZM241385 was decreased by H278N (not determined for S132L due to low receptor expression). Similarly, D151Y and P365H mutations in the conserved of motifs of 5-HT_{2C}R decreased the affinity of 5-HT, while E306K and E306A increased the potency and affinity of 5-HT by disrupting the conserved ionic lock and shifting the receptor towards an active state.

Thirdly, some other factors may decouple the direct correlation between ligand binding and receptor functioning. For instance, mutation V275A of A_{2A}AR was found to increase the affinity of NECA but decrease its potency, indicating a loss in the coupling efficiency of the intracellular signaling pathways. Last but not least, the inhibitory potency of an antagonist was found to be influenced by the presence and competitive binding of the agonist. For example, the inhibitory potency of ZM241385 at A_{2A}AR in the presence of NECA was not significantly affected by mutation A265V, when affinity of NECA and ZM241385 were both decreased, but was decreased by V275A, which increased the affinity of NECA but not ZM241385. Similarly, when the affinity of 5-HT and mesulergine at 5-HT_{2C}R were both decreased by mutation L209H and F325S, the inhibitory potency of mesulergine was not significantly affected.

Taken together, cancer-associated GPCR mutations alter receptor pharmacology in a context-dependent manner, with changes in ligand binding, receptor conformation, downstream coupling and receptor occupancy all contributing to their effects (**Figure 7.1**). The fact that a single mutation can exert multiple and sometimes opposing effects complicates the prediction of its overall impact, highlighting the need for case-by-case characterization.

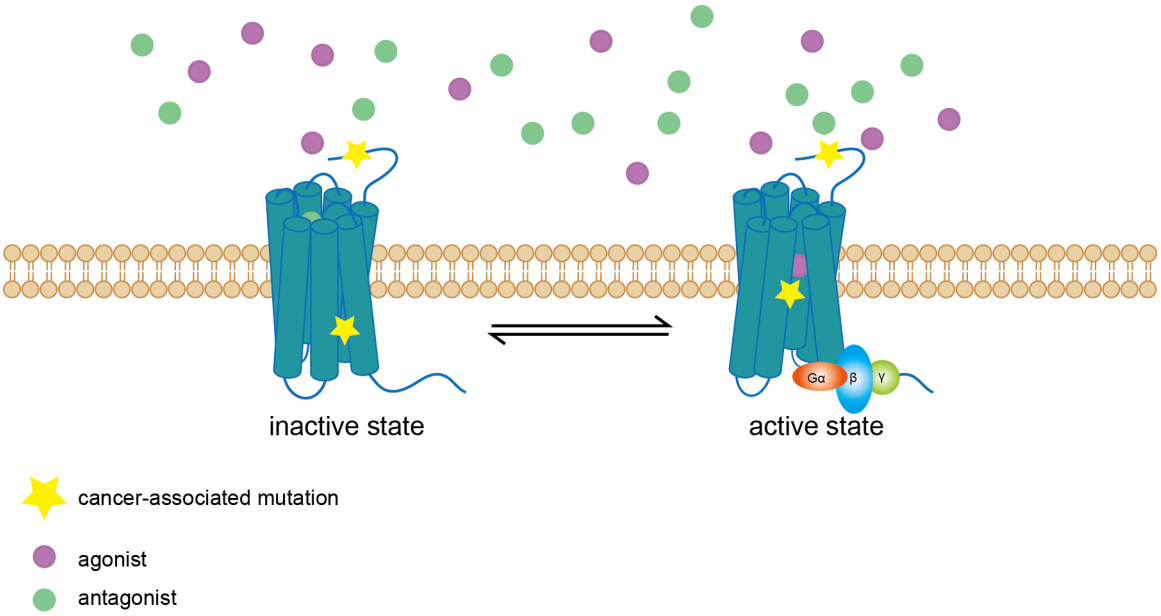

Figure 7.1. Schematic illustration of cancer-associated GPCR mutations that alter receptor pharmacology. Mutations in the extracellular loop of a GPCR are likely to directly affect ligand binding. Mutations in the transmembrane domain may induce conformational shifts toward the inactive or active state of the GPCR, thereby favoring the binding of antagonists or agonists, respectively.

Whether a GPCR mutation in cancer is a driver or passenger mutation is determined by receptor pharmacology and cancer biology

As discussed in **Chapter 2**, while numerous GPCRs have been implicated in cancer progression and immune regulation, and mutations have been shown to enhance or disrupt downstream signaling, a clear understanding of the functional roles of specific GPCR mutations in cancer remains largely lacking. Several challenges contribute to the difficulty in determining whether a given GPCR mutation acts as a driver or merely a passenger in tumor development, which are also evident in our investigations of A_{2A}AR and 5-HT_{2C}R.

Firstly, although GPCR mutations are commonly observed in cancer [1], mutations in individual GPCR members generally occur at low frequencies. In **Chapter 3**, we found in total 58 single-base missense mutations of the A_{2A}AR from 57 cancer cases out of ~13,000 cases in the Genomic Data Commons database (version 22.0; as collected by *Bongers et al.*) [2, 3]. Among the 57 patients, four harbored more than one mutation. Of the 58 mutations, only one mutation (S132L) was detected in four patients, and three other mutations appeared in two patients each, while the remaining 54 mutations were unique to individual cases. In addition, these A_{2A}AR mutations were distributed across a wide range of cancer types, showing no apparent tissue-specific clustering. Similarly in **Chapter 6**, we identified in total 128 single-base missense mutations of 5-HT_{2C} receptor from 153 cases of various cancer types, with each mutation occurring at a very low frequency. In contrast to highly prevalent kinase mutations, such as PI3KCA mutations in colorectal cancer [4], HER2 mutations in breast cancer [5], BRAF mutations in melanoma [6], EGFR mutations in lung cancer [7], and JAK2 mutation in

myeloproliferative disorder [8, 9], the largely dispersed GPCR mutations have shown limited potential as diagnostic biomarkers or therapeutic targets.

Secondly, the extent to which specific GPCR mutations influence cancer cell behavior through altered receptor pharmacology depends on both the metabolic conditions in the tumor microenvironment (TME) and the physiological role of the receptor in cellular function. For example, in **Chapter 3** we found that the potency of adenosine analogue NECA at A_{2A}AR (pEC_{50}=8.4 at wild-type) was decreased by 10-fold by mutations S132L (pEC_{50}=7.3) and H278N (pEC_{50}=7.3), and its efficacy (E_{max}%) was increased by more than 2-fold. Therefore, the state of (mutant) receptor activation in human body is determined by surrounding adenosine level. In the TME, extracellular adenosine accumulation is induced by release of ATP during cell death and hypoxia, and also the upregulated expression of CD39 and CD73 [10]. Although direct *in vivo* measurements of the adenosine concentration in human tumors are not yet available, *ex vivo* data has reported an overall at least twice higher level of adenosine in tumors than in healthy tissues [11, 12]. In addition, it was reported by *Blay et al.* using xenograft mice models of lung and colon cancers that the adenosine levels in the extracellular fluid of solid tumors ranged from 0.2 μM to 2.4 μM with a mean of 0.5 μM, which were 10 to 20 times higher than in adjacent normal tissues with a mean of 30 nM [13]. Based on these findings, we can infer that although the potency of adenosine at the A_{2A}AR-S132L and H278N mutants might be reduced, this effect is likely countered by the elevated adenosine concentrations in the TME, leading to a higher overall activation level of the mutant receptors than that of the wild-type. However, in **Chapter 5**, we observed that treatment with 1 μM or 10 μM NECA did not significantly promote the growth of the three breast cancer cell lines studied, suggesting that cells harboring A_{2A}AR-S132L or H278N mutations may not gain a growth advantage, and these two mutations act as passengers without directly driving oncogenic process. Importantly, the sensitivity of cell proliferation to cAMP accumulation may be influenced by several cell background conditions, such as the expression levels of cAMP effectors, cross-talk with other signaling pathways, cell cycle state and the compartmentalization of cAMP generation [14-16]. In summary, mutations that significantly alter receptor pharmacology may function as passenger mutations in specific cell types where the affected signaling pathway is not essential for proliferation, yet they may act as driver mutations under different biological conditions.

Furthermore, although passenger mutations typically do not confer growth advantages to cancer cells and are therefore merely regarded as contributors to broader genomic complexity, they still hold the potential to affect drug response in cancer treatment [17]. In **Chapter 5**, we found that A_{2A}AR antagonist ZM241385 significantly inhibited cancer cell growth, while **Chapter 3** demonstrated that A_{2A}AR mutation V275A and P285L markedly reduced the antagonist's ability to inhibit receptor activation. These findings suggest that cancer cells harboring either of these two mutations may exhibit increased resistance to ZM241385 treatment and potentially gain a proliferative advantage under such conditions, although this hypothesis requires further experimental validation.

2. Future perspectives

Understanding the role of GPCRs in cancer

We investigated the role of A_{2A}AR and A_{2B}AR in cancer cell growth regulation in **Chapter 5** and found that blockade and gene knock-down of these receptors led to varying degrees of growth inhibition. Notably, these effects only partially correlated with receptor mRNA expression levels, raising the possibility of off-target actions by the antagonists. To address this, receptor knock-out experiments would help to confirm target specificity, i.e., testing whether the antagonists retain their growth inhibitory effects in A_{2A}AR- or A_{2B}AR-deficient cell lines can distinguish on-target from off-target effects. Other approaches such as molecular docking, experimental validation of ligand-receptor interactions, and co-treatment with multiple agonists/antagonists in cell growth assays can also provide evidence toward identifying secondary targets. As discussed in **Chapter 2**, GPCRs are widely expressed across various tissues, and their functional redundancy increases the risk that a drug designed for one GPCR could act on others (including non-GPCRs) unintentionally. Although this phenomenon of poly-pharmacology may lead to opportunities for drug repurposing, current preclinical studies and clinical trials still consider the lack of selectivity a major challenge in development of GPCR targeted therapies [18, 19]. It is important to take this issue into account in future studies exploring the role of other GPCRs in cancer biology, for example the serotonin receptors addressed in **Chapter 6**. Moreover, cancer cell growth results from uncontrolled cell proliferation and resistance to cell death, and further clarification is needed on how GPCRs affect each of these processes. Lastly, the role of GPCRs in other critical aspects of cancer progression, such as angiogenesis, cell invasion and metastasis, dysregulated energy metabolism and tumor-promoting inflammation also warrant deeper investigation using relevant *in-vitro* and *in-vivo* models [20].

Underexplored opportunities for targeting GPCRs and their mutations in oncology

Despite growing genomic and pharmacological evidence suggesting their potential relevance in cancer progression and treatment, targeting GPCRs and their mutations in cancer remains a largely underexplored area. With each challenge faced in this emerging field, new opportunities arise.

First, the growing availability of sequencing datasets will facilitate the identification of hotspot GPCR mutations in cancer. Although GPCR mutations identified in cancer are in general weakly correlated with specific domains, *Bongers et al.* reported a higher mutational pressure in class-specific functionally conserved motifs in cancer patients than in healthy individuals [3]. In addition, The application of advanced computational approaches will also facilitate the GPCR targeted drug discovery in multiple stages [21]. For example, a machine learning model developed by *Matic et al.* was used to predict the consequences of 2140 missense mutations in 212 GPCRs on G protein coupling efficiency, and further efforts are being made to account for their effects on ligand interactions [22]. Such approaches can help identify mutations with greater functional relevance in cancer among large datasets, which can then be investigated *in vitro* and *in vivo* to understand their role in cancer progression and to guide the development of optimal therapeutic strategies. Furthermore, in-depth sequencing combined with mutation-guided patient stratification for clinical trials provide opportunities to uncover the potential of even rare mutations in personalized therapy [23].

Second, in **Chapter 5**, we focused on the potential role of GPCR in cell growth regulation, taking A_{2A}AR as a case study. Beyond this, GPCRs have also been implicated in anti-cancer immune

response [24]. For example, growing evidence has demonstrated A_{2A}AR as an emerging immune checkpoint in the TME, which mediates immunosuppression and also induces the expression of other immune checkpoints [25]. Deletion of A_{2A}AR using CRISPR/Cas9 in CAR T cell treatment has been found to enhance the *in vivo* efficacy in a mouse model of ovarian cancer [26], and in clinical trials, combination of A_{2A}AR with other immune checkpoint inhibitors has also displayed enhanced anti-cancer effects in multiple cancer types compared with monotherapy [27]. Currently, GPCR mutations in immune cells remain relatively underexplored. In a clinical trial for Waldenström macroglobulinemia, CXCR4 antagonist uloculumab was used in combination with Bruton's tyrosine kinase (BTK) inhibitor ibrutinib for patients harboring CXCR4 mutations, but this study was halted early and did not progress to phase II [28]. However, with the advances in GPCR pharmacology, single-cell genomics, and AI-driven functional prediction, this field still holds great promise in cancer immunotherapy.

Last but not the least, biased signaling and allosteric modulation are featured in GPCR pharmacology and warrant further investigation in the context of oncology. In addition to G protein mediated signaling, studies have revealed the engagement of β-arrestins mediated GPCR signaling network in tumorigenesis. On one hand, β-arrestins contribute to cancer invasion and metastasis by activating key oncogenic pathways such as MAPK, Wnt/β-catenin, NF-κB, and PI3K/Akt, thereby regulating cytoskeletal dynamics and enabling cancer cells to adapt and remodel the TME [29, 30]. On the other hand, β-arrestins serve as critical regulators of GPCR desensitization and internalization, preventing sustained receptor activation. This dual role underlies the functional consequences of β-arrestin–biased signaling in cancer. Biased signaling of GPCR is mostly related to specific ligands, but can also be induced by mutation. In a subset of uveal melanoma cases, mutation L129Q in cysteinyl leukotriene receptor 2 (CysLTR2) resulted in constitutive activation of $G_{q/11}$ signaling pathways, while β-arrestin was poorly recruited, contributing to malignant transformation [31]. This is the first known example of a highly biased constitutively active mutant GPCR acting as oncogene, suggesting CysLTR2-L129Q as a potential therapeutic target in uveal melanoma. Furthermore, allosteric modulators offer innovative strategies for targeting GPCRs with enhanced selectivity (reduced off-target effects) and insurmountability, particularly in cases where patients do not respond to orthosteric ligands or carry mutations that confer (orthosteric) resistance. For example, Boujut *et al.* developed a novel negative allosteric modulator (NAM) of A_{2A}AR with retained potency even in high adenosine concentrations, which was also found to restore the activity of $CD4^+$ T cells suppressed by NECA, showcasing the potential for cancer immunotherapy [32].

Targeting GPCR mutations using novel drug candidates with optimized binding kinetic profiles

In **Chapter 3**, we found that A_{2A}AR mutation A265V and H278N significantly decreased the binding affinity of antagonist ZM241385, while in the impedance-based cell morphology assay (xCELLigence) using transiently-transfected HEK293T cells, ZM241385 effectively inhibited the NECA-induced receptor activation of A_{2A}AR-A265V and A_{2A}AR-H278N. However, in **Chapter 4,** we found that mutation A265V and H278N significantly increased the dissociation rate of ZM241385 from A_{2A}AR by more than 5-fold and correspondingly shortened its residence time (RT). It has been reported in several studies that drug RT is positively correlated with the duration and efficacy of its pharmacological effects [33, 34]. As described in **Chapter 5,** ZM241385 significantly inhibited the growth of breast cancer cell lines endogenously expressing wild-type A_{2A}AR. Thereby the follow-up research question is, in cancer cells harboring A_{2A}AR mutations that shorten antagonist RT, whether ZM241385 can still inhibit

receptor activation in short-term and lead to long-term inhibition of cell growth. In our lab, we have also found that long-RT A_{2A}AR antagonist LUF6632 exerted insurmountable A_{2A}AR antagonism in a cAMP assay even in the presence of high agonist concentrations [35]. It will be of interest to further investigate if LUF6632 also acts as long-RT antagonist at mutant A_{2A}ARs and exert growth inhibitory effects on mutant harboring cancer cells in a more effective or prolonged way compared with ZM241385.

Moreover, since the A_{2A}AR mutations V275A and P285L markedly reduced the inhibitory potency of ZM241385 in xCELLigence assay [36], it is plausible that these mutations may also impair the compound's ability to suppress cancer cell growth, which warrants experimental confirmation. In such cases where mutant A_{2A}ARs confer cancer cells resistance to ZM241385, antagonists with optimized binding kinetic profiles, such as extended RT or covalent binding properties could offer a promising strategy to overcome this resistance and restore therapeutic efficacy. The irreversible binding of covalent inhibitors can lead to insurmountable receptor antagonism with enhanced selectivity and potency, especially when mutations create new nucleophilic sites [37]. In 2021, FDA approved the first KRAS-G12C inhibitor Sotorasib for treatment of non-small cell lung cancer (NSCLC), which works by covalently binding to the cysteine mutation site [38]. Later on, Sotorasib was approved for treatment of KRAS G12C-mutated metastatic colorectal cancer in 2025. Moreover, the third-generation EGFR inhibitor Osimertinib has been developed to bind covalently at C797 of EGFR-T790M mutant with selectivity over wild-type EGFR, and to counter the T790M-dependent resistance to earlier EGFR kinase inhibitors, while C797S mutation in patients can abolish the covalent binding and cause resistance to Osimertinib [39]. Although there are no currently approved covalent GPCR inhibitors for cancer, this area remains a promising field of research. Successful attempts have been made to develop covalent ligands of CC chemokine receptor 2 (CCR2) [40], β_2-adrenergic receptor (β_2AR), dopamine D_2 receptor (D_2R), 5-hydroxytryptamine 2A receptor (5-HT_{2A}R) [41], and A_{2A}AR [42, 43]. For all cancer-associated A_{2A}AR mutants examined in **Chapter 4**, we observed that the covalent antagonist LUF7445 displayed varying apparent affinities. Further investigation is required to determine whether LUF7445 still forms covalent bonds with these mutants, particularly the more resistant variants V275A and P285L, and to assess whether its use yields sustained growth inhibition in cancer cells.

Paying attention to G protein mutations in cancer

As discussed in **Chapter 2**, the challenges in investigating or targeting GPCRs in cancer partly come from the intricate signaling networks where multiple receptors can activate similar downstream pathways, and thus the loss or alteration of a particular GPCR can be compensated by alternative receptors and ligands. In this case, a specific GPCR and its mutations may not exert a unique or critical influence on cellular function [44]. However, G proteins are the primary mediators of most GPCR signaling pathways, playing a pivotal role in the intricate signaling network, and several studies have demonstrated a causal link between G protein mutations and human diseases, including cancer [45]. For example, in human pituitary tumors, mutations of the G protein subunit $G_{\alpha s}$ can lead to loss of its GTPase activity, and thereby constitutive activation of adenylyl cyclase, resulting in increased cAMP production and uncontrolled cell proliferation independent of growth hormone–releasing hormone (GHRH) [46, 47]. In addition, *Wilson et al.* reported that $G_{\alpha s}$ activating mutation R201C alone was not sufficient to induce tumorigenesis, but cooperated with inactivation of APC to promote colorectal cancer in mice model through activation of Wnt and ERK/MAPK pathways [48]. Furthermore, compared with the widespread but individually rare mutational landscape of

GPCR in cancer, G protein mutations, especially in the Gα subunits (GNAS, GNAQ, GNA11, GNA13) are less numerous but more recurrent and clustering in specific cancer types [49]. Taken together, G protein mutations are more tissue-specific and potentially more pathogenic, which calls for further attention for their prognostic and therapeutic value in cancer.

3. Final note

This thesis presents a systematic exploration of GPCRs and their cancer-associated mutations, combining molecular pharmacology, ligand binding kinetics, and functional cell-based assays. Beginning with a general overview and a comprehensive review of the mutational landscape of GPCRs in cancer, subsequent chapters focus on two representative receptors, A_{2A}AR and 5-HT_{2C}R, to investigate how specific mutations affect receptor function, ligand binding dynamics, and potential resistance to antagonists. By characterizing the cellular responses to agonists and antagonists, this work also reveals the potential role of adenosine receptors in cancer cell proliferation. Moreover, the thesis underscores the therapeutic potential of binding kinetics–driven drug optimization in overcoming mutation-induced resistance. Collectively, the findings advocate for a more nuanced approach to targeting GPCRs in oncology, considering not only receptor affinity but also residence time, signaling bias, and mutational context to advance precision medicine strategies.

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