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Author: Doornbos, M.L.J.
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CHAPTER 7

Conclusions and future perspectives

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Over the last decades, the number of ligands for the metabotropic glutamate receptor 2 ($mGlu_2$) has vastly increased.[1] Initially the focus of discovery programs was on ligands targeting the orthosteric binding site, which later shifted to the allosteric site for which selectivity is more easily achieved.[2] Despite these tremendous efforts, no drug targeting the $mGlu_2$ receptor has reached the market so far. Therefore this thesis aimed to increase the understanding of the mechanism of action of the $mGlu_2$ receptor. To achieve this, a variety of pharmacological concepts are covered in this thesis, including allosteric modulation, binding kinetics, constitutive activity, inverse agonism and insurmountability. To study these concepts novel assays and tool compounds were developed. Ultimately, a better understanding of the *in vitro* parameters involved in *in vivo* efficacy contributes to the development of safe and efficacious drug therapies. This chapter combines the conclusions from this thesis, followed by the future perspectives that arise from these conclusions.

CONCLUSIONS

The concept of allosteric modulation has been widely evaluated in the field of G protein-coupled receptors (GPCRs).[2] Especially for class C GPCRs the allosteric binding site in the seven-transmembrane (7TM) domain is being explored thoroughly, as it provides better opportunities for selectivity compared to the orthosteric binding site located in the extracellular Venus Flytrap (VFT) domain.[1] Understanding of the architecture of the mGlu receptor allosteric binding site is based on crystal structures of the negative allosteric modulator (NAM)-bound 7TM domain of the $mGlu_1$ and $mGlu_5$ receptors.[3–6] The allosteric binding site of the $mGlu_2$ receptor has been elucidated using mutagenesis studies, which revealed overlapping binding sites for positive allosteric modulators (PAMs) and NAMs.[7–11] Moreover, these studies revealed a transmission switch (i.e. amino acid residues that relocate upon the conformational change from the inactive to active state of the 7TM domain or vice versa) that is located in the same receptor region as the agonist binding site in class A GPCRs.[12]

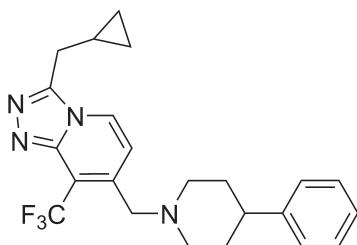

Figure 1. Structure of JNJ-46281222.

In **Chapter 2**, JNJ-46281222 (Fig. 1) was characterized as an $mGlu_2$-selective, highly potent PAM with nanomolar affinity. This ligand and its radiolabeled version were therefore used as tool compounds throughout this thesis. JNJ-46281222 increased the potency and affinity of agonists, which is typical for a PAM. Furthermore, it behaved as PAM agonist with a submaximal intrinsic activity, i.e. activity on its own. The maximum binding capacity of JNJ-46281222 was increased by the presence of the agonist glutamate without changing its affinity, which indicated a two-way mechanism of allosteric modulation (Fig. 2). On the other hand, JNJ-46281222's maximum binding and affinity were decreased by the presence of GTP (which initiates dissociation of the G protein), indicating its preference for a G protein bound state of the receptor. Using computational docking and molecular dynamics studies, the PAM binding mode was visualized. Subsequent binding experiments using mutant receptors confirmed this PAM's binding mode.[11,13]

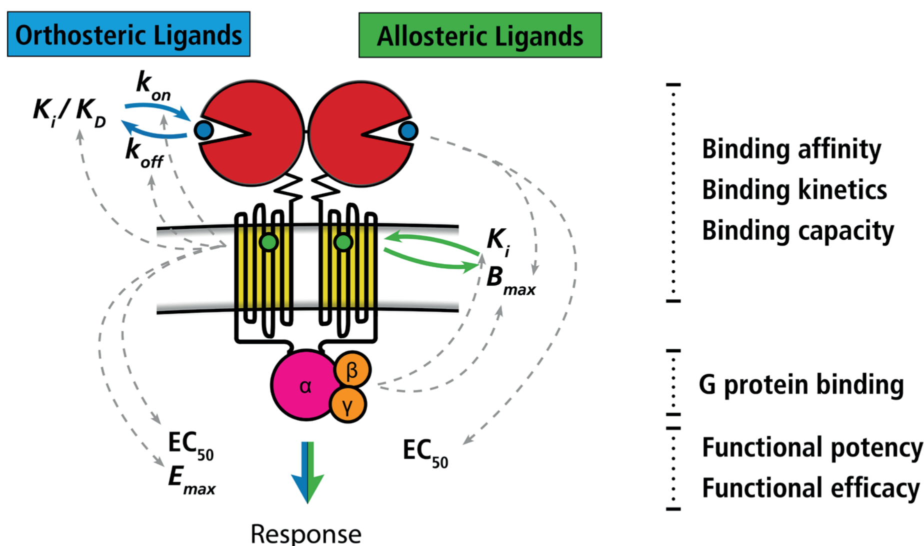

Figure 2. Summary of the findings obtained in **Chapters 2** and **3**. Allosteric modulators were shown to affect the potency, affinity and/or efficacy of orthosteric ligands (blue bar in figure): PAMs were shown to increase the affinity (K_i/K_D) and potency (EC_{50}) of orthosteric agonists, by reducing the dissociation rate constant k_{off}. Furthermore, they increase the efficacy (E_{max}) of orthosteric agonists. NAMs do not affect agonist affinity or potency, but decrease the efficacy drastically. Furthermore, NAMs may probe-dependently alter the kinetic parameters (k_{on}, k_{off}) of agonists leaving affinity and potency unchanged. Both PAMs and NAMs do not affect the affinity and potency of antagonists. Vice versa, orthosteric ligands were shown to affect the potency and maximum binding capacity (B_{max}) of allosteric ligands (green bar in figure). Agonists glutamate and LY354740 increase the maximum PAM binding capacity without changing the PAM affinity. Furthermore, the PAM potency is increased at increasing glutamate concentrations, whereas NAM potency is not affected by the presence of an agonist. GTP-initiated dissociation of the G protein (pink and orange) results in a decreased PAM binding affinity and binding capacity, indicating the importance of the G protein for PAM binding.

Chapter 3 describes the first kinetic study of orthosteric ligand binding at the $mGlu_2$ receptor from an allosteric modulation perspective. Kinetic radioligand binding assays were set up to determine the kinetic binding parameters for the endogenous $mGlu_2$ agonist glutamate for the first time. Its off-rate is fast, resulting in a residence time (RT) below 1 minute, whereas the on-rate is relatively high, resulting in an affinity ($K_D = k_{off} / k_{on}$) comparable to the K_i value obtained from equilibrium binding assays. Furthermore, the affinity and potency of the orthosteric ligands tested were strongly correlated to their association rate constants k_{on}, showing that on-rate is driving the affinity and thus target occupancy of orthosteric $mGlu_2$ ligands. The off-rates of the endogenous agonist glutamate and synthetic agonist LY354740 were decreased by the presence of a PAM, which resulted in increased affinities, whereas on-rates were unaffected. The NAM RO4491533 increased both on- and off-rates of glutamate without changing the affinity, but did not alter these kinetic parameters for agonist LY354740, indicating probe-dependency. Of note, the experiments had to be performed at 0°C to enable

recording of the association and dissociation of the radioligand used. Ideally, these should be done at 37°C, but this was practically unfeasible due to very fast binding events at that temperature.

The next step was to study the binding kinetics of a novel series of $mGlu_2$ PAMs. **Chapter 4** starts with classical determinations of *in vitro* affinity and potency of 41 novel 7–Aryl-1,2,4-triazolo[4,3–*a*]pyridines and 4 reference PAMs. All compounds behaved as functional and selective $mGlu_2$ PAMs with micromolar to subnanomolar affinity and potency. In addition to these classical parameters kinetic parameters k_{on} and k_{off} were determined and RTs were calculated subsequently. To do so, a kinetic radioligand binding assay using the scintillation proximity assay (SPA) was developed. In addition to typical structure-affinity/activity relationships (SAR), structure-kinetics relationships (SKR) of these novel allosteric modulators were obtained. The PAMs showed various kinetic profiles in which k_{on} values ranged over 3 orders of magnitude, whereas k_{off} and thus RT were within a small 10-fold range. As seen for orthosteric ligands in **chapter 3**, the affinity of all PAMs was k_{on}-driven, showing that this likely is a receptor-specific characteristic.

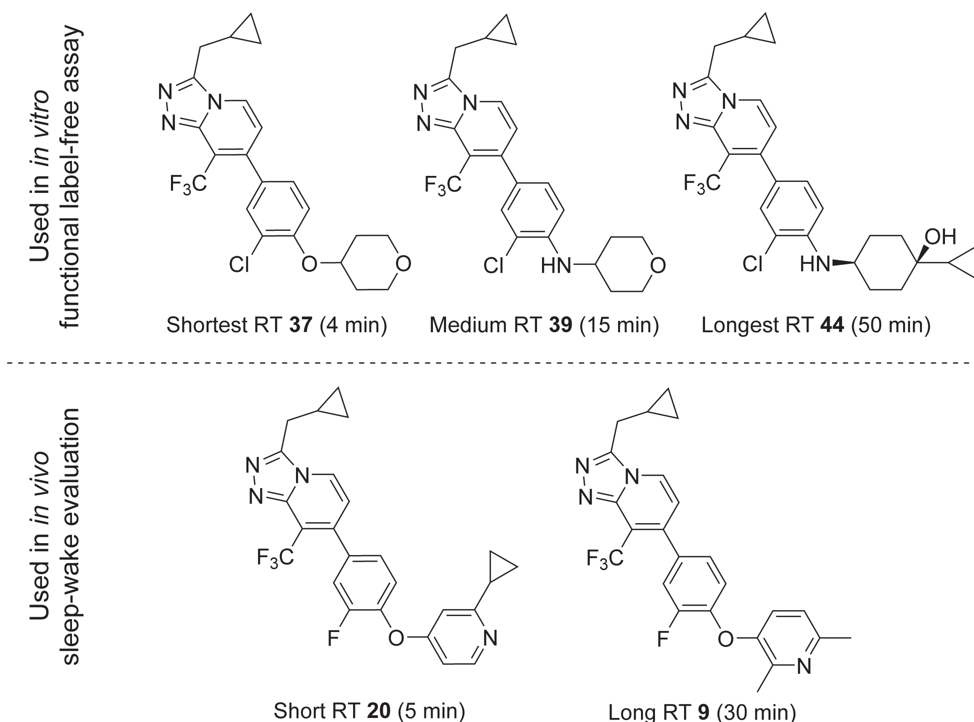

Figure 3. Structure of the shortest RT PAM (**37**), a medium RT PAM (**39**) and the longest RT PAM (**44**) used for evaluation in an *in vitro* functional label-free assay and the short RT PAM (**20**) and long RT PAM (**9**) used in *in vivo* sleep-wake evaluation, as described in **Chapter 4**.

Subsequently, the shortest RT PAM **37**, a medium RT PAM **39** and long RT PAM **41** (Fig. 3) were evaluated for their functional efficacy in the label-free xCELLigence assay detecting changes in cell morphology. The results obtained showed a correlation between RT and the duration of action of the PAM in this label-free assay. Ultimately, the translation from *in vitro* binding kinetics to *in vivo* efficacy was evaluated. Two PAMs with similar affinity, potency and pharmacokinetics (PK), but with divergent RTs were selected. Compared to short RT PAM **20**, the effects of the long RT PAM **9** on sleep-wake states were translated significantly better into sustained inhibition of rapid eye movement sleep, which is a well-established *in vivo* measure for $mGlu_2$ efficacy. This provides a first hint that *in vivo* $mGlu_2$ PAM efficacy benefits from a longer *in vitro* RT.

The association rate constant k_{on} is often believed to equal the diffusion rate limit. However, this thesis shows that for both orthosteric and allosteric ligands of the $mGlu_2$ receptor the k_{on} values are spread over multiple orders of magnitude whereas k_{off} values are within a small range. The diffusion rate limit is around 10^8 - 10^9 $M^{-1}s^{-1}$,[14] the k_{on} values obtained in this thesis range from $10^3 – 10^7$ $M^{-1}s^{-1}$, which clearly shows that the k_{on} value is a ligand-specific characteristic that is not equal between ligands and is not equal to the diffusion rate limit either.

Together, these results showed the importance of understanding binding kinetics. Affinity-only driven selection will result in high affinity PAMs with a high k_{on} but not necessarily an optimized k_{off}, which is key for *in vivo* efficacy. Therefore, experiments that enable quantification of kinetic parameters k_{on}, k_{off} and RT will be a valuable addition to the experimental set-up used in drug discovery.

Covalent labeling of GPCRs by small molecules is a powerful approach to establish further understanding of receptor binding modes, mechanism of action, receptor expression patterns, receptor pharmacology and even to facilitate structure elucidation.[15,16] **Chapter 5** describes the first covalent PAM probe for a class C GPCR. Based on previous $mGlu_2$ medicinal chemistry efforts three novel putative covalent PAMs were designed, synthesized and pharmacologically characterized. All three compounds still behaved as selective $mGlu_2$ PAMs with good affinity and potency. Using an equilibrium-based affinity-shift assay and a kinetic competition association assay, compound **2** was identified as covalent PAM, which was confirmed in wash-out assays. The receptor binding mode of **2** was studied using computational modelling which also identified 5 nucleophilic amino acid residues potentially responsible for formation of the covalent bond with compound **2**. Receptor mutagenesis studies identified $T791^{7.29x30}$ as the likely position of covalent interaction.

Together, compound **2** was identified as a valuable tool compound to study the $mGlu_2$ PAM binding mode and binding kinetics. These findings advance the understanding of the $mGlu_2$ PAM interactions and suggest that compound **2** is useful for structure elucidation of the active state 7TM domain.

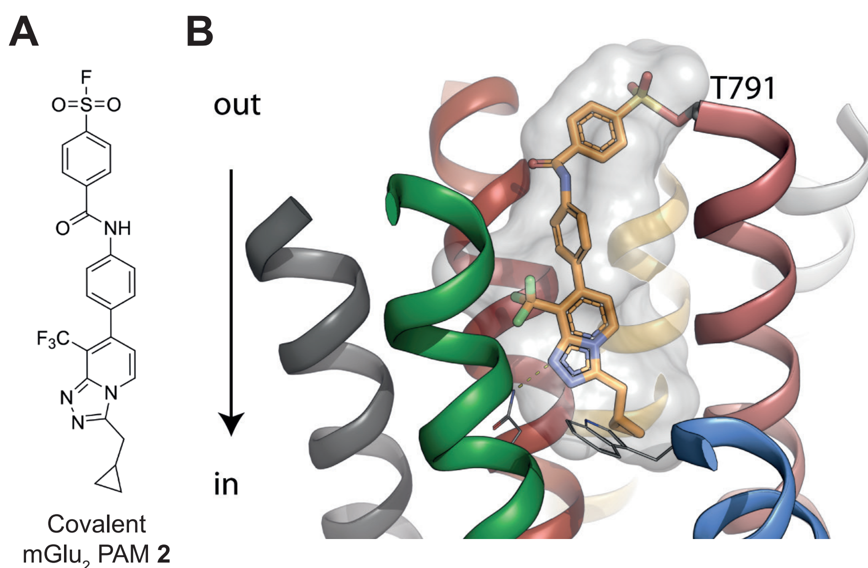

Figure 4. A) Structure of covalent mGlu$_2$ PAM **2**. B) After binding to the allosteric binding pocket, **2** forms a covalent interaction with residue T791$^{7.29x30}$, as described in **Chapter 5**.

Label-free assays using biosensors to study GPCRs have emerged over the last decade.[17,18] These assays provide new opportunities, as compared to conventional *in vitro* assays they determine integrated receptor-mediated responses on whole cells that are recorded in real time without the need for any labels. **Chapter 6** describes the set-up of such a label-free biosensor assay (i.e. xCELLigence) to study mGlu$_2$ receptor pharmacology and continues on the basic set-up that was established in **chapter 4**. The xCELLigence technology records cellular impedance that is generated by adhesion of cells to gold-coated electrodes.

Firstly, assay conditions had to be optimised as the culture medium contained a high level of endogenous glutamate which interferes with exogenous agonist signalling. Glutamate pyruvate transaminase (GPT) was used successfully to reduce these glutamate levels. Subsequently, detailed pharmacological assays were performed, yielding potencies for agonist LY354740, antagonist LY341495, PAM JNJ-46281222 and NAM RO4491533, which were comparable to literature values. Responses of the agonist and PAM showed a dose-dependent increase in impedance, whereas stimulation by the antagonist or NAM resulted in a dose-dependent decrease in impedance. This showed that opposite pharmacological responses also resulted in opposite responses on cell morphology and thus impedance measured. Interestingly, constitutive receptor activity was observed that could be inhibited by LY341495 which is commonly considered a classical antagonist for the mGlu$_2$ receptor but behaved as inverse agonist in this system. This was confirmed by concentration-dependent negative modulation of LY341495 potency by the PAM JNJ-46281222.

Thus, the label-free xCELLigence system was validated as a useful methodology to study $mGlu_2$ pharmacology. This is the first class C GPCR extensively characterized by a label-free biosensor assay, opening new avenues to study receptor pharmacology and novel concepts of receptor activation.

In conclusion, by a variety of techniques ranging from *in vitro* binding experiments to *in vivo* efficacy studies, we have shed light on the mechanism of action of the $mGlu_2$ receptor at a molecular level. The obtained insights are valuable for the design of ligands for the orthosteric binding site (**Chapter 3**) and particularly for the allosteric binding site (**Chapters 2, 4, 5**). The results were generated using novel tool compounds (**Chapters 2 and 5**) and novel assays to study binding kinetics and receptor pharmacology (**Chapters 4 and 6**). Together, these insights are valuable in future discovery projects for drugs targeting the $mGlu_2$ receptor as well as other GPCRs.

FUTURE PERSPECTIVES

Allosteric modulation and its potential for drug therapy

There is an urgent need for the development of new drug therapies for devastating neurological and neurodegenerative diseases such as schizophrenia and Alzheimer's disease, respectively. While the development of new medicines is effortful for all therapeutics areas, CNS drug discovery efforts have been particularly challenging resulting in high attrition rates during the various phases of the drug development process.[19] Many of the marketed CNS drugs exert their effect via a GPCR and currently multiple GPCRs are being evaluated as novel targets for treatment of CNS diseases with an unmet medical need, illustrating their vast and remaining potential for drug therapy.[20] Historically, drug discovery efforts have focused on the development of classical agonists and antagonists that target the orthosteric binding site of the receptor. However, targeting this orthosteric binding site of the mGlu receptor family presents challenges for subtype selectivity and drug-like properties that are essential for CNS drug candidates.[2] For these reasons most drug discovery efforts for mGlu receptors as well as other class C GPCRs have shifted towards the development of allosteric modulators. These are molecules that bind to a binding site that is topographically distinct and less conserved than the orthosteric glutamate binding site. Together this has resulted in promising subtype selective lead compounds that have been or are currently in clinical trials.[21]

Since allosteric modulators show little or no intrinsic activity by themselves and do not have to compete with the endogenous agonist, they provide an enhanced efficacy and safety

profile over typical orthosteric ligands. This has been widely acknowledged in the field resulting in vast discovery efforts and increasing numbers of allosteric modulators for GPCRs reaching clinical trials over the last decade.[20] Currently, two allosteric modulator drugs are on the market: the chemokine receptor CCR5 (CCR5; class A GPCR) NAM Maraviroc for treatment of HIV infection[22] and the calcium-sensing receptor (CaSR; class C GPCR) PAM Cinacalcet for treatment of chronic kidney disease involving hyperparathyroidism.[23] Current research efforts that increase the understanding of the mechanism of allosteric modulation will result in increasing numbers of candidate drugs which will ultimately yield an increased number of allosteric modulator drugs accessing the market in the next decade.

Allosteric modulation especially holds promise for class C GPCRs, as illustrated by the success of Cinacalcet. These receptors provide an allosteric binding site in their 7TM domain which is located in a similar location and uses a similar transmission switch mechanism compared to class A GPCR binding sites.[13] The allosteric binding sites are well explored, resulting in highly potent allosteric modulators for all major receptor families within this class – mGlu, $GABA_B$, sweet and umami taste and CaSR receptors. Multiple of these compounds have reached clinical development stages already.[19,28] Together, class C GPCRs provide promising drug targets for novel allosteric modulators and within the next decade novel allosteric modulator therapies may be provided in addition to Cinacalcet.

The potential of the $mGlu_2$ receptor for drug therapy

The $mGlu_2$ receptor is a potential drug target for various neurological disorders such as schizophrenia, anxiety, depression and cognition.[29–32] Therefore, many $mGlu_2$ PAMs and NAMs with *in vivo* efficacy have been developed, resulting in multiple clinical studies so far. In recent years $mGlu_2$ PAMs AZD8529 and JNJ-40411813 and the NAM Decoglurant have been in clinical studies, but unfortunately they were withdrawn due to a lack of efficacy. Despite the lack of clinical success so far, the use of allosteric modulators at the $mGlu_2$ receptor still provides promising opportunities for drug treatment. Together with current fundamental research efforts on the mechanism of action of the $mGlu_2$ receptor, the clinical results provide novel insights that will determine the next development steps towards an $mGlu_2$ allosteric modulator-based therapy. Such a next step may be the repurposing of the clinical compounds for specific patient populations or a different therapeutic indication, as suggested for JNJ-40411813, which demonstrated effect in a small population of schizophrenic patients with residual negative symptoms[33] and AZD8529, which was evaluated in a clinical trial for smoking cessation.[34] More importantly, novel $mGlu_2$ allosteric modulators will be designed and developed based on novel molecular strategies, including those that are discussed in this thesis. These future molecules will bear enhanced properties and therefore hold a strong promise for future allosteric drugs targeting the $mGlu_2$ receptor.

The promise of drug-target binding kinetics

The interest in drug-target binding kinetics has increased tremendously over the last decade.[35,36] The lack of technologies able to record binding kinetics has long been a limiting factor for the quantification of this parameter. Over the recent years many novel assays, such as a qualitative kinetic screening method using radioligand binding,[37] SPA assays,[38] FRET assays[39] and label-free binding assays[40] have been developed to determine kinetic binding and activation parameters.[41] Importantly, many of these assays are now ready to be implemented in larger scale drug discovery programs. The enhanced interest in binding kinetics of both academia and pharmaceutical industry is illustrated by the successful IMI project Kinetics for Drug Discovery (K4DD) that incorporated researchers from universities and different industrial partners.[41] Recently, the European Medicines Agency changed its requirements for first in human studies of new investigational products and now also demands information on the duration of *in vitro* action – and thus binding kinetics.[42] Given the increased number of techniques available to determine binding kinetics in high throughput and the awareness that is raised across the drug discovery community, the importance of assessing binding kinetics is anticipated to increase tremendously in the coming years. We will most likely enter an era in which medicinal chemists will design not only for affinity and potency, but also for kinetic parameters. This in itself will be a valuable and promising addition to the current discovery process. For the $mGlu_2$ receptor in particular we have shown that *in vitro* binding kinetics and specifically residence time is linked to *in vivo* efficacy of PAMs. This opens promising avenues for novel $mGlu_2$ PAMs whose chemical design is based on *in vitro* kinetic parameters in addition to classical affinity and potency parameters. Ultimately, this will increase the chance of desirable *in vivo* and in the end *in human* efficacy.

Covalent ligands as tool compounds for GPCRs

Covalent ligands for GPCRs have shown to be valuable tool compounds to study their mode of action.[16] The use of covalent ligands in structure elucidation of GPCRs has shown its potential and this will be used more and more in the near future. Together with other recent advances in the GPCR structure elucidation field - increased throughput of crystal structures using crystal soaking[43] and structure elucidation using cryo-electron microscopy[44,45] – the use of covalent ligands will contribute to increased understanding of GPCR structure and function. In the field of mGlu receptors an active state 7TM structure would be a valuable addition to the current NAM-bound inactive state structures of $mGlu_1$ and $mGlu_5$.[3–6] The covalent PAM **2** described in **chapter 5** could be a valuable tool to determine such active-state 7TM domain of the $mGlu_2$ receptor.

In addition, covalent ligands for GPCRs and other drug targets have been developed as promising useful probes for chemical biology applications, such as fluorescent labelling of receptors in native tissues.[46] Further research on the covalent $mGlu_2$ PAM **2** described in **chapter 5** could focus on the functionalization of the molecule for example by addition of a

'click-handle' that can be used to link the covalent molecule to a fluorescent dye in order to visualise the receptor in cells and tissues *in vivo* and ultimately even *in human*.

Covalent ligands as GPCR therapeutics

Application of a covalent mode of action is commonly avoided in the pharmaceutical industry to prevent off-target toxicity.[47] Specifically, reactive electrophilic moieties are avoided in drug design, as exemplified by the restraint use of moieties that are included in the so-called "pan-assay interference compounds" (PAINS).[48] On the other hand, drugs that bind their biological target covalently have a long history as illustrated by the 39 approved covalent drugs[49] including well-known and commonly used medicines such as aspirin (COX1,2-inhibitor),[50] omeprazole (H^+/K^+ ATPase-inhibitor)[51] and clopidogrel ($P2Y_{12}$ receptor antagonist).[52]

Current discovery efforts on covalent ligands focus on so-called "targeted covalent inhibitors".[15] Such molecules possess a high affinity for a specific target and avoid off-target toxicity by using a weakly reactive electrophilic warhead. This approach has been studied extensively in the field of kinase inhibitors for cancer therapy,[53] and is nowadays increasingly evaluated for drug targeting of GPCRs.[16] These molecules are receptor inhibitors, though when it comes to molecules that should activate GPCRs, PAMs provide a clear benefit over orthosteric agonists as they only activate the receptor whilst an agonist is bound and thus do not activate the target receptor permanently.[54] In a therapeutic context, covalent PAMs may result in less frequent drug dosing without a loss of efficacy. For these reasons, covalent PAMs targeted at mGlu receptors provide a promising therapeutic strategy. Successful development of covalent kinase drugs as safe and efficacious cancer therapies will support the efforts towards other targets such as GPCRs. Overall, if the selectivity and thus the safety of covalent molecules can be guaranteed these molecules provide valuable opportunities for future drug therapy where they will be added to the already marketed covalent drugs.

The application of label-free technologies for GPCR drug discovery

The number of biosensor-based label-free technologies in the drug discovery field has increased vastly over the last decade and is still increasing.[18,55] Since these assays measure integrated cellular responses of whole cells in real-time they can be used to study activation kinetics in addition to classical receptor pharmacology parameters. Further automation of label-free assays will enable implementation of these techniques in drug discovery programs. Another important advantage of biosensor-based label-free assays over most classical assays is that cells with low receptor expression can be used such as cell lines endogenously expressing the receptor of interest or even patient-derived cells like lymphoblastoid cell lines (LCLs).[56] By using these cells, the effects of interindividual genetic differences in receptor sequence on the drug efficacy can be studied, which is an important step towards precision medicine.[57] Currently, huge progress is being made in the use of stem cells to study responses of individual patients. These human induced pluripotent stem cells (iPSCs) are generated

from adult cells which are subsequently reprogrammed to the cell type of interest using the right cellular matrix.[58] This provides valuable progress in the use of patient-derived material, since in this way specific cell types, or even organ-like structures – so-called 'organoids' - can be used.[58] The number of different cell types that can be obtained from iPSCs is increasing tremendously at this moment. Of great importance for the field of $mGlu_2$ receptor research is the development of neuronal cultures based on iPSCs which can be used to study disease mechanisms of for example schizophrenia.[59] These cells provide a radical step forward for *in vitro* experimentation as only a very limited number of neuronal cell lines was available so far. Given the tremendous progress that is being made in the development of iPSC-derived cell types, powerful developments may be expected in the coming years when combining these cells with the advantages of label-free assays. As an example, such experimental set-up was applied to cardiomyocytes derived from iPSCs that were studied using the label-free xCELLigence assay.[60] Altogether, development of label-free assays based on cell types from patient-derived iPSCs will result in more focused insights in disease mechanisms and as such this experimental set-up holds great promise for the study of the $mGlu_2$ receptor as well as other GPCRs.

Final notes

Throughout this thesis the molecular mechanism of action of the $mGlu_2$ receptor has been studied to better understand the key *in vitro* parameters that drive *in vivo* efficacy. To obtain these insights, various pharmacological concepts were studied, including allosteric modulation and binding kinetics and to be able to do so multiple tool compounds and assays were developed. Together, the results obtained in this thesis contribute to the improved understanding of the molecular processes involved in GPCR drug action. This improved understanding will ultimately contribute to increasing the number and the quality of drug candidates that will hopefully become novel, safe and efficacious drug therapies for the $mGlu_2$ receptor as well as for other GPCRs in the near future.

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