It's about time: novel drug discovery concepts for the molecular pharmacological characterization fo the cannabinoid CB2 receptor

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Chapter 7

Discussion and future perspectives

7.1 Conclusions

At least one third of all marketed pharmaceutical drugs interacts with G protein-coupled receptors[1,2]. Nevertheless, a major challenge in the extensive drug discovery and development process is a high attrition rate of drug candidates in clinical trials[3]. Almost half of the failures are due to lack of clinical efficacy, but also toxicity is a major cause for attrition[4]. To this end, novel concepts and approaches in preclinical development are gaining recognition to provide a more successful translational perspective[5]. In this thesis, we focused on the investigation of drug-target binding kinetics, allosteric modulation and biased signaling on the cannabinoid CB_2 receptor (CB_2R), an interesting GPCR for the treatment of inflammatory conditions. Here, the findings from the different chapters are combined and future perspectives and opportunities for drug discovery on CB_2R and other GPCRs are discussed.

7.1.1 Assay development: continuous improvements to provide novel insights on receptor pharmacology

To improve the preclinical to clinical translational perspective, it is important to develop and properly use biologically, physiologically, and pharmacologically relevant *in vitro* assays. Additionally, continuous adaptation of these assays and data analyses may provide novel insights beyond the initial application[6].

In **Chapter 2**, we provided a comprehensive protocol for the recruitment of ß-arrestin-2 to activated cannabinoid receptors (CBRs). In this chapter, we used the PathHunter® technology, which relies on the complementation of two enzyme fragments for the generation of an active ß-galactosidase that emits a luminescent signal relative to the amount of complementation. Agonist-mediated activation of CB_1R or CB_2R induced the recruitment of ß-arrestin-2 to the receptors, which resulted in complementation of the active ß-galactosidase and a luminescent signal (**Figure 2.1**). Furthermore, this assay could be used for the investigation of antagonists and inverse agonists by co-incubation with an agonist or prolongation of the incubation time, respectively. Altogether, the PathHunter® technology provided an easy-to-use and high-throughput assay for a quick screening of ligand-induced ß-arrestin-2 recruitment to CBRs. As such, we successfully used this assay in **Chapter 5** for a set of ligands to investigate orthosteric and allosteric activation of CB_2R.

In **Chapter 3**, we continued the development of a ß-arrestin-2 recruitment assay for CB_2R by the use of the NanoLuc Binary Technology (NanoBiT®). This technology also relies on the complementation of two enzyme fragments, but in this case an active NanoLuc luciferase (NLuc) is generated. The advantage of this system is that the complementation is reversible, in contrast to the irreversible complementation of the ß-galactosidase, and as such kinetic, real-time analysis of protein-protein interactions is possible. In our assay, CB_2R was C-terminally fused to a small complimentary peptide (SmBiT) and ß-arrestin-2 was N-terminally fused to the large peptide (LgBiT) (**Figure 7.1**). After agonist-mediated ß-arrestin-2 recruitment to CB_2R the two subunits interact and form the active NLuc. We combined this technology with the GloSensor™ technology for the detection of real-time

inhibition of cAMP production after CB_2R activation (**Figure 7.1**). This biosensor was developed by circularly permuting a firefly luciferase (Fluc) and inserting a cAMP binding domain[7]. Binding of cAMP to the sensor will cause a conformational shift to the active Fluc. In the presence of the two different substrates both luciferases generate a luminescent signal with different emission wavelengths. The combination of these two technologies presented, for the first time, a multiplex assay for the simultaneous and kinetic detection of cAMP production and ß-arrestin-2 recruitment in one well. In this assay, the influence of system or observation bias was reduced, i.e., all results were obtained at the same time and under the same conditions. The applicability of the multiplex assay was shown by screening a diverse panel of benchmark and clinically tested CB_2R agonists. The results were

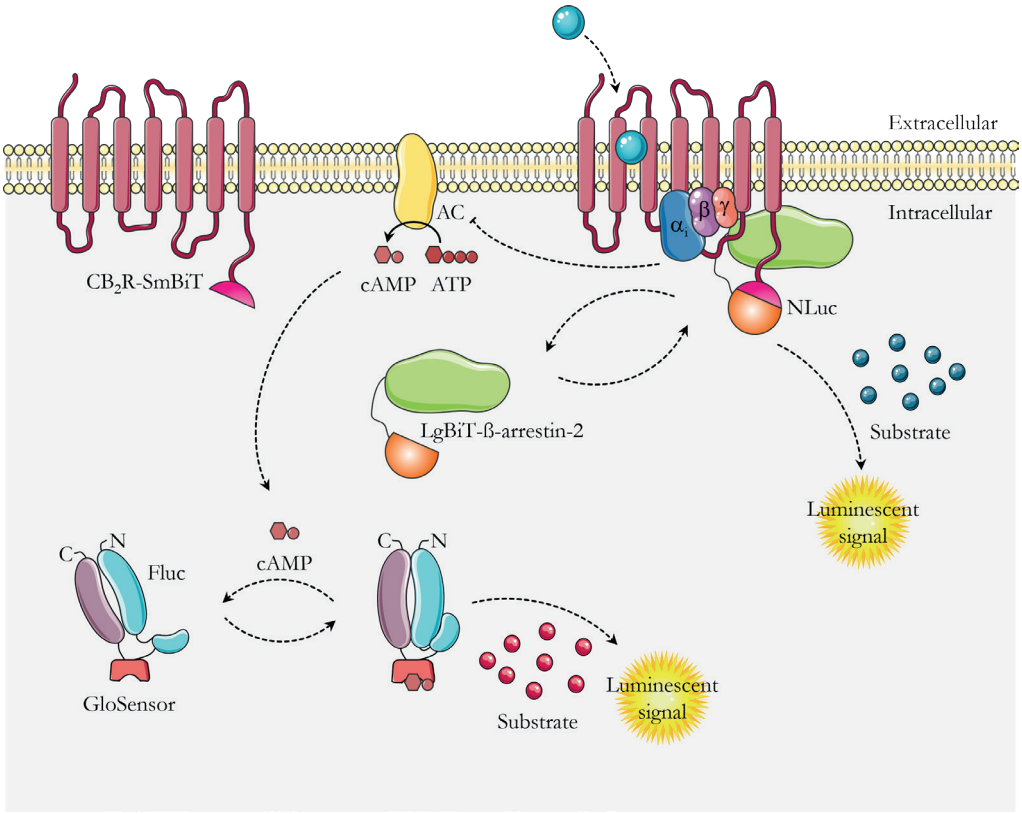

Figure 7.1 Schematic representation of the novel multiplex assay for ß-arrestin-2 recruitment and cAMP production.

Upon activation of SmBiT-tagged CB_2R by a ligand the LgBiT-ß-arrestin-2 is recruited to the receptor. This induces complementation to the active NanoLuc luciferase (NLuc), which results in a luminescent signal upon substrate addition. This process is reversible, and the LgBiT-ß-arrestin-2 can uncouple from the receptor, which will reduce the luminescent signal. Activation of CB_2R can also activate the $G\alpha_i$ pathway and subsequently inhibit the adenylyl cyclase, which will reduce the cAMP levels in the cytosol. These levels can be monitored by the GloSensor technology. Binding of cAMP to the sensor will cause a conformational shift to the active firefly luciferase (Fluc), which results in a luminescent signal upon substrate addition. The resulting two luciferases require a different substrate and as such emit light at different wavelengths, which allows combining them in the multiplex assay for the simultaneous and kinetic measurement of inhibition of cAMP production and ß-arrestin-2 recruitment after CB_2R activation. This figure incorporates drawings from Servier Medical Art (smart.servier.com).

interpreted via the use of an endpoint, semi-kinetic and kinetic analysis to investigate time-dependency of agonist-mediated activation as well as the determination of kinetic signaling parameters. Interestingly, the activation by certain agonists was time sensitive and the potency increased over time, whereas activation by other agonists was not affected over time. Time-dependency of activation was further highlighted in the bias determination, which indicated that agonists such as 2-AG may be (slightly) biased towards cAMP production at early time points but switch to ß-arrestin-2 recruitment bias after a longer incubation time. On the other hand, clinically relevant agonists Olorinab, S-777469 and ART-27.13 did not display changes in their bias profiles at different time points. Furthermore, novel mathematical models were applied to analyze the full time course and calculate kinetic parameters. Agonists Olorinab, PRS-211375, ART-27.13 and Tedalinab displayed higher efficacy in ß-arrestin-2 recruitment than commonly used full agonist CP55,940, classifying them as superagonists. These superagonists were characterized by faster signaling rate constants (k_1) than CP55,940, but not all agonists with faster k_1 values demonstrated superagonism. Nevertheless, independently of the analysis, none of the benchmark or clinically relevant agonists induced significant signaling bias in cAMP production or ß-arrestin-2 recruitment in our cellular system. This may suggest that the lack of detectable signaling bias could be the reason for the high attrition rate of CB_2R selective agonists in clinical trials. However, the mechanism of therapeutic effects at CB_2R and the potential importance of biased signaling is still largely unknown. Incorporation of the novel kinetic multiplex assay in early drug discovery programs may aid in a better and more extensive profiling of agonists prior to selection for (pre)-clinical models. Altogether, we hypothesize that combining the kinetic signaling parameters with target binding kinetics could provide a holistic overview of kinetic context for agonist-mediated receptor activation, which may be a better prediction for *in vivo* efficacy as they capture the early signaling responses.

7.1.2 Association rate constant: more than just diffusion

The investigation of drug-target binding kinetics gained attention over two decades ago when Copeland and colleagues presented it as a better predictor of drug efficacy and safety *in vivo*[8]. The initial focus has been on the investigation and optimization of target residence time (RT), calculated as the reciprocal of the dissociation rate constant (k_{off})[9]. Specifically since the association rate constant (k_{on}) was initially thought to be diffusion controlled and as such would be unaffected by the ligand. Nevertheless, this assumption has been rejected and the role of the association rate constant has become increasingly more important[10].

In **Chapter 3** a large and diverse panel of CB_2R agonists was screened in radioligand competition association assays. This yielded k_{on} and k_{off} values, which were converted into target engagement time (ET) at 1 μM of agonist and RT, respectively. The agonists displayed diverse kinetic profiles in which ETs ranged by 260-fold. It appeared that a fast agonist association was the driving factor for high affinity on CB_2R. Subsequently, all agonists were screened in the newly developed multiplex assay and kinetic signaling parameters were determined to obtain a complete overview of agonist-mediated CB_2R activation in a kinetic context. A fast engagement, i.e., k_{on} value, was significantly correlated with high kinetic

potency. Altogether, this indicates that high affinity and kinetic potency for CB_2R is driven by fast agonist engagement with the receptor.

In **Chapter 4**, we utilized the k_{on} value to predict a novel ligand entry mechanism for lipophilic agonists. In this chapter, we combined *in silico*, *in vitro* and *in vivo* methods to characterize the potent and selective CB_2R agonist LEI-102. Four cryo-electron microscopy (cryo-EM) structures were elucidated with LEI-102, CB_2R-selective agonists APD371 (Olorinab) and HU308, and non-selective agonist CP55,940. Based on these structures, the influence of several amino acids in agonist activation was explored via site-directed mutagenesis in functional [^{35}S]GTPγS binding assays. Although the overall structures of the CB_2R-$G\alpha_i$ bound complexes with LEI-102, APD371, HU308 or CP55,940 were similar, the agonists interacted with different amino acids in the orthosteric binding pocket. Furthermore, two potential ligand entry pathways at CB_2R, i.e., either via the extracellular loop 2 (ECL) or via a membrane channel between transmembrane domains 1 and 7 (TM1 and TM7), were investigated. By combining results from site-directed mutagenesis studies and the association rate constants of the agonists, we suggest that highly lipophilic agonist HU308 and the endocannabinoids (eCBs) may reach the binding pocket via a membrane channel, whereas more polar ligands LEI-102, APD371 and CP55,940 use an alternative route. Ultimately, the promising *in vivo* efficacy of oral administration of LEI-102 was shown in a chemotherapy-induced nephropathy model without inducing central nervous system (CNS)-mediated side effects.

7.1.3 Dissociation rate constant: more than residence time and efficacy

Additionally in **Chapter 3** and **4**, the k_{off} values of all benchmark and clinically tested CB_2R agonists were determined. Residence times ranged from 2.1 min in our assays for Dronabinol (Δ^9-tetrahydrocannabinol, Δ^9-THC) to 93 min for TAK-937. Nevertheless, the RTs only differed 44-fold in our assays, opposed to the 260-fold difference in k_{on} values. We observed no statistically significant correlation between k_{off} values and affinity, potency or efficacy. However, we found that slowly dissociating agonists exhibited slow deactivation of ß-arrestin-2 recruitment, which may suggest that extended agonist binding results in a longer receptor interaction with ß-arrestin-2. This clearly indicates that optimization of the dissociation rate constants as well as optimization of the association rate constants is valuable for CB_2R agonists. Together, these results emphasize the importance of understanding drug-target binding kinetics of CB_2R agonists and quantification of these kinetic parameters could be a valuable addition to drug discovery efforts for CB_2R.

In **Chapter 5** we described an alternative use of the dissociation rate constant to reveal allosteric interactions with CB_2R. All commercially available proclaimed allosteric modulators of the endocannabinoid system (ECS) were screened in a single point radioligand dissociation assay to reveal allosteric interactions. This suggested allosteric properties of cannabidiol-dimethylheptyl (CBD-DMH), but not for structural analog cannabidiol (CBD) or other compounds. CBD-DMH was further investigated in dissociation assays and was found to significantly reduce the k_{off} value of radioligand [^{3}H]RO6957022 in a dose-dependent manner. To this end, CBD-DMH was characterized as a positive allosteric modulator (PAM)

for an inverse agonist. In functional assays, it behaved as a negative allosteric modulator (NAM) for synthetic and endogenous agonists in G protein activation assays, but not in ß-arrestin-2 recruitment. Moreover, in these assays CBD-DMH itself behaved as orthosteric agonist and partially activated both the G protein and ß-arrestin-2 recruitment. Together, this suggests dual allosteric and orthosteric molecular pharmacology of CBD-DMH at CB_2R, which may provide a new class of molecules targeting CB_2R.

7.1.4 Precision medicine: keeping the patient in mind

Chapters 2, **3**, **4**, and **5** focused on assay development and targeting of wild type (WT) CB_2R. Yet, in several diseases, including cancer, GPCRs may contain somatic point mutations[11–13]. Despite this, the effect of GPCR mutations on cancer progression or druggability is largely unknown[14]. While targeting of WT CB_2R may provide a great therapeutic potential in cancer, mutations have been observed in cancer patient samples. Therefore, in **Chapter 6**, we aimed to investigate the impact of CB_2R cancer-associated mutations on the functionality of the receptor as well as the implications for drug targeting. We selected ten single point mutations in CB_2R from the Genomic Data Commons based on occurrence and proximity to the orthosteric binding pocket. Receptor expression and G protein activation by endogenous, synthetic and clinically tested CB_2R agonists was investigated for all ten mutant receptors. Binding affinity of a subselection of these agonists was further tested in radioligand displacement assays. We found that mutations in the binding pocket or structurally close to a conserved motif markedly affected receptor activation. Although the activation and binding were differentially affected dependent on the combination of CB_2R mutant and agonist. This effect was less pronounced on mutations located in the N- or C-termini. Altogether, this emphasized the importance of precision medicine, i.e., investigating patient CB_2R genotype, prior to administration of cannabinoid-based therapies.

In conclusion, by the development and application of a variety of assays we have increased the molecular pharmacological understanding of targeting CB_2R. The work presented in this thesis highlights the potential and importance of studying kinetic binding and signaling parameters for the elucidation of novel ligand entry pathways, allosteric interactions and the overall agonist-mediated CB_2R activation. Moreover, by combining and developing different biochemical and cellular assays along with the implementation of new methods of analysis, this thesis presents comprehensive procedures to improve agonist profiling during the initial phases of drug discovery. These findings could proof valuable for future drug discovery endeavors on CB_2R as well as other GPCRs.

7.2 Future perspectives

7.2.1 Kinetic traces as indication for mechanism of signaling regulation

The novel multiplex assay that we designed and validated in **Chapter 3** provided an elegant system for the simultaneous assessment of two signaling events after agonist-mediated CB_2R activation. Furthermore, the kinetic nature of this assay offered the opportunity to trace and analyze the full dynamics of agonist-mediated receptor activation. Equations to fit the time-trace data have been developed that enable the determination of kinetic parameters, which could display kinetic differences between agonists[15]. It has been hypothesized that the shape of the trace represents the complexity of signaling and regulatory mechanisms. Therefore, kinetic parameters could potentially shed light on the different regulation events. However, a good understanding of the regulation of signaling is crucial, which may vary depending on the target, agonist or cellular background. Utilizing specific inhibitors of certain processes or complementary assays may offer more insights into these signaling mechanisms, as further outlined below.

The best-known regulators of cAMP signaling are phosphodiesterases (PDEs), which belong to a superfamily consisting of eight different families[16]. PDEs play a role in the rapid degradation of cAMP to AMP[16]. PDE inhibitors prevent the degradation of cAMP, resulting in accumulation of cAMP. Often, competitive non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) or selective PDE inhibitors rolipram and cilostamide are used in (endpoint) cAMP assays since an accumulation of the cAMP signal is required for quantification of the effect[17,18]. Our kinetic assay does not require this accumulation and we therefore deliberately omitted PDE inhibitors from our setup to limit artificial modification of the system. Nonetheless, addition of IBMX or subtype-selective PDE inhibitors could be beneficial for studying agonist-mediated effects of G protein activation and subsequent adenylate cyclase activity independently of cAMP metabolism[7,19].

Alternatively, ß-arrestin recruitment to the receptor is only the first step in initiation of potential signaling or regulation mechanisms. As described in **Chapter 1**, this could terminate G protein signaling or cause internalization and trafficking of the receptor to endosomes prior to different receptor fates such as recycling or degradation. Complementary assays could shed light on the specific receptor fate after agonist-mediated ß-arrestin recruitment to CB_2R, and the difference between ß-arrestin isoforms. Investigation of trafficking to endosomes can be done by use of endosomal markers from the Rab-GTPase (Rab) family. Specially, Rab5 is a marker for early endosomes, Rab4 or Rab11 for the recycling endosome and Rab7 is used as marker for the late endosome[20]. Bioluminescence resonance energy transfer (BRET) assays have been developed to measure relative distances between a luciferase-tagged GPCR and green or yellow fluorescent protein (GFP or YFP)-tagged Rab5, Rab4 or Rab7[20,21]. These assays have already been described for CB_2R and could be used to investigate whether agonists, like the data set in **Chapter 3**, promote the same receptor fate or if there could be bias in internalization. Nevertheless, caution should be taken to check whether this process is actually ß-arrestin-dependent, and not G protein-dependent, since it was recently demonstrated that GPCRs may differentially rely on ß-arrestins or G proteins for internalization[22].

Additionally, ß-arrestin recruitment could trigger signaling cascades via mitogen-activated protein kinase (MAPK) and extracellular signal-related kinase 1 and 2 (ERK1/2)[23,24]. Activation of the ERK1/2 signaling cascade can be measured in a variety of endpoint assays, or a kinetic assay by the use of a BRET-based sensor[25–27]. However, whether this activation is ß-arrestin-dependent and G protein-independent, or if recruitment of ß-arrestin is nonessential requires a more thorough examination. To this end, CRISPR/Cas9 genome-edited cell lines with genetic ablation of ß-arrestins or G proteins, or in combination with pharmacological inhibition of G proteins by, for example, Pertussis Toxin (PTX) could help to discriminate whether there is a dependence on a specific pathway[25].

These strategies could be applied to the agonists studied in **Chapter 3** to investigate whether parts of the signaling time traces, and corresponding signaling rate constants, can be assigned to specific mechanisms. Furthermore, it would be highly interesting to investigate whether there is bias on another signaling level, which will be expanded upon in the following paragraphs. Eventually, a comprehensive understanding of signaling and regulatory mechanisms after agonist-mediated receptor activation is of the utmost importance to better exploit CB_2R, and other GPCRs, for therapeutic purposes. This becomes particularly valuable if a biased signaling approach has been confirmed as a therapeutic strategy. Alternatively, better profiling of agonists targeting novel receptors could contribute to a deeper understanding of the necessity for biased signaling. This may benefit from the inclusion of agonists with diverse bias profiles in *in vivo* studies to predict the most therapeutically relevant profile.

7.2.2 Alternative technologies to expand and further develop multiplex assays

In the multiplex assay from **Chapter 3**, we combined two luminescent technologies to measure cAMP production and ß-arrestin-2 recruitment after CB_2R activation. We employed the GloSensor™, a permuted firefly luciferase (Fluc) utilizing D-luciferin as substrate, and the NanoBiT®, which relies on complementation of two parts (BiTs) to form an active NanoLuc luciferase (NLuc) that requires furimazine as substrate (**Figure 7.1**). The luminescent signals could be distinguished due to the distinct emission spectra of the luciferases. We demonstrated, for the first time, that simultaneous and kinetic detection of two luminescent readouts was possible without the need for signal quenching or lysis of the cells. This encourages the exploration of adding more biosensors to expand the current multiplex assay or develop other new multiplex assays, which is explored in more detail in the following paragraphs. Ultimately, this could contribute to a more efficient and better screening of compounds and biased signaling for GPCRs.

7.2.2.1 Luciferase-based biosensors

Luciferase-based biosensors are widely employed in biochemical research due to their high signal-to-background ratio as they do not require excitation light energy like fluorescent assays[28]. Various luciferases have been used in biochemical assays, each requiring a specific substrate devoid of cross-reactions with other substrates (**Table 7.1**)[29]. Consequently,

Table 7.1 Examples of luciferases used in biochemical assays with associated substrate and emission wavelengths (λ_{em}), and the possibility to be used as split luciferase.

Luciferase	Substrate	Peak emission wavelength (λ_{em})	Split luciferase?	Ref.
Click beetle Green (CBGluc)	D-luciferin/ATP	540 nm	Yes	[28,34,35]
Click beetle Red (CBRluc)	D-luciferin/ATP	615 nm	Yes	[28,34,35]
Cypridina (Cluc)	Vargulin	465 nm	No	[29]
Firefly (Fluc)	D-luciferin/ATP	560 nm	Yes	[7,28,36]
Gaussia (Gluc)	Coelenterazine	460 nm	Yes	[28,37]
NanoLuc (NLuc)	Furimazine	453 nm	Yes	[30,32,38]
Renilla (Rluc)	Coelenterazine	480 nm	Yes	[28,39]

luciferases emit light at different wavelengths[28]. While the use of intact luciferase-based biosensors such as the GloSensor™ has been limited in GPCR research, split luciferase assays such as the NanoBiT® are gaining popularity. In **Chapter 3** we used the NanoBiT® for recruitment of ß-arrestin-2 to CB_2R. Moreover, this technology has been employed to investigate other signaling processes like G protein dissociation or GRK recruitment following agonist-mediated GPCR activation[30,31]. In the development of the split segments of NLuc, BiTs with different affinities for the LgBiT have been designed. For instance, the NanoBiT® LgBiT and SmBiT segments have a low intrinsic affinity (K_D 190 μM) for one another and consequently, complementation is driven by interaction of the tagged proteins. Conversely, the HiBiT segment exhibits a very high affinity for LgBiT (K_D 700 pM), and this complementation is used to monitor internalization of GPCRs (**Figure 7.2a**)[32,33]. In this case, a GPCR is N-terminally tagged with a HiBiT segment, which automatically complements with the extracellularly present LgBiT that is cell impermeable. Upon internalization of the receptor, a decreased NLuc signal is observed due to loss of GPCRs on the cell surface[31,33].

A similar approach was undertaken with click beetle luciferases (CBluc), which were split into C- and N-terminal segments[34]. Because of the overlap of the green and red CBluc (CBGluc and CBRluc) C-terminal segments and their distinct N-terminal segments, the CBGluc C-terminus could serve as a contact point for both CBGluc and CBRluc N-terminal segments. Consequently, mixing CBGluc and CBRluc fragments enabled simultaneous quantification of two pairs of interacting proteins or the interaction of two proteins with a shared protein[34]. The latter approach was recently successfully applied to monitor the simultaneous recruitment of ß-arrestin-1 and 2 to the δ-opioid receptor, which could be distinguished based on the different wavelengths (**Figure 7.2b**,**c**, **Table 7.1**)[35].

While split luciferases of Renilla luciferase (Rluc), Fluc and Gaussia luciferase (Gluc) have not yet been utilized in GPCR pharmacology, they have been designed and applied in research fields for other targets[37,39,40]. The complementation assays have been employed for the detection of a variety of protein-protein interactions proving their applicability across diverse systems. For instance, Rluc complementation assays have been conducted with Rluc segments tagged to heat shock protein 90 (Hsp90) and ATPase homologue 1 (Aha1), respectively, to monitor the disruption of these interactions[39]. On the other hand, a split firefly luciferase complementation assay has been explored for interactions between virus

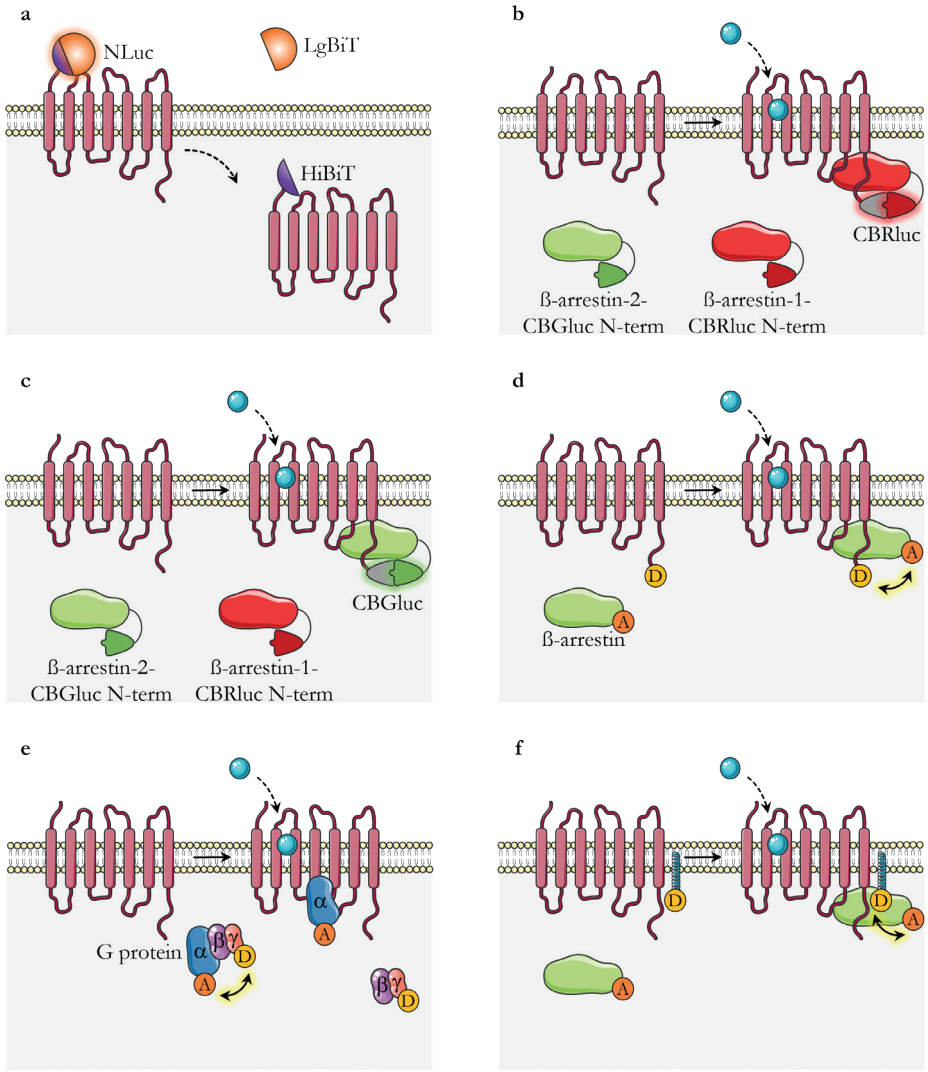

Figure 7.2 Schematic representation of currently available GPCR split luciferase-based and BRET-based biosensors.

(a) Split luciferase HiBiT to monitor the internalization of a GPCR. Extracellular LgBiT and a N-terminally HiBiT-tagged GPCR complement to an active NLuc. Upon internalization of the receptor, the NLuc signal will decrease. (b) Click beetle red luciferase (CBRluc) and (c) click beetle green luciferase (CBGluc) to simultaneously quantify the interaction of two proteins, in this case ß-arrestin-1 and 2, with a third protein, the GPCR. (d) BRET to measure the proximity between the donor (D)-tagged GPCR and acceptor (A)-tagged transducer, in this case ß-arrestin. BRET signal will increase upon recruitment of ß-arrestin to the GPCR. (e) BRET to measure the dissociation of the heterotrimeric G protein with donor-tagged Gα and acceptor-tagged Gγ, which will result in a decreased BRET signal. (f) Enhanced bystander BRET (ebBRET) to measure the proximity between the donor (D)-tagged membrane anchor and acceptor (A)-tagged transducer, in this case ß-arrestin. BRET signal will increase upon recruitment of ß-arrestin to the GPCR and thus membrane anchor. Luciferases (BRET donors) only emit light in the presence of substrate, but this is not shown for clarity reasons. This figure incorporates drawings from Servier Medical Art (smart.servier.com).

and host proteins in plant leaves[40].

Split luciferase assays may present an interesting strategy for multiplexing due to their high sensitivity for the quantification of protein-protein interactions. Complementation of NLuc and click beetle segments have already proven useful in GPCR pharmacology, while split luciferases of Rluc, Fluc and Gluc could open up new avenues for GPCR signaling. Moreover, novel substrates are developed to shift emission peaks and gain more distinct spectra. For instance, a new luciferin analogue, AkaLumine-HCl, was synthesized to shift the emission peak of Fluc to the near-infrared wavelengths (λ_{em} 677 nm)[41]. Nevertheless, multiplexing split luciferases may require some optimization to ensure no interference of the split luciferase segments with the GPCR of interest and the protein partner, such as G proteins or ß-arrestins.

7.2.2.2 BRET-based assays

Over the years, bioluminescence resonance energy transfer (BRET)-based assays have been widely used in GPCR research. BRET assays rely on the principle of energy transfer between a luminescent donor and a fluorophore acceptor, both fused to proteins or protein fragments of interest[42]. This energy transfer occurs when the donor and acceptor are brought into close proximity by ligand-binding, protein-protein interactions, or conformational changes. The resulting ratio between acceptor and donor emission is then used to quantify the effect[43].

A wide array of BRET donor and acceptor pairs have been documented in literature (**Table 7.2**). Initially, commonly used donors were Rluc variants (RlucII, Rluc8, Rluc8.6), which emit light between 400 and 535 nm in the presence of the required substrate[44,45]. These were typically combined with fluorescent proteins like enhanced yellow fluorescent protein (EYFP), green fluorescent protein (GFP) or its mutant variants (e.g., GFP2 or GFP10)

Table 7.2 Examples of BRET donor and acceptor pairs with associated substrate and excitation and emission wavelengths (λ_{ex} and λ_{em}) found in literature.

Luciferase (donor)	Substrate	λ_{em}	Fluorophore (acceptor)	λ_{ex}	λ_{em}	Ref.
RlucII	Coelenterazine h	480 nm	EYFP	511 nm	530 nm	[42]
RlucII	Coelenterazine 400a	400 nm	GFP2 or GFP 10	400 nm	510 nm	[42]
RlucII	Prolume Purple	405 nm	GFP2 or GFP 10	400 nm	510 nm	[42]
RlucII	Coelenterazine 400a	400 nm	rGFP	480 nm	508 nm	[44,45]
RlucII	Prolume Purple	405 nm	rGFP	480 nm	508 nm	[44,45]
Rluc8	Coelenterazine 400a	400 nm	GFP2	400 nm	510 nm	[46]
Rluc8	Coelenterazine	480 nm	mOrange	548 nm	562 nm	[47]
Rluc8.6	Coelenterazine	535 nm	TurboFP635	588 nm	635 nm	[48]
NLuc	Furimazine	453 nm	Venus	515 nm	528 nm	[50,52]
NLuc	Furimazine	453 nm	mVenus	515 nm	527 nm	[31,51]
NLuc	Vivazine	453 nm	mKATE2	588 nm	633 nm	[49]
NLuc	Vivazine	453 nm	EGFP	488 nm	507 nm	[49]

serving as acceptors with emission peaks around 510-530 nm[42,46]. However, enhanced energy transfer efficiency has been achieved by combining Rluc with other fluorophores such as rGFP, mOrange and TurboFP635[42,44,45,47,48]. Similarly, improvements on the luciferase donor have been made by introducing the brighter intact NLuc for NanoBRET assays[38]. This allowed pairing with red-shifted fluorophores, thereby enhancing the signal-to-noise ratio compared to early BRET pairs due to better spectral separation between donor and acceptor emission[31,49–52].

The possibilities for BRET-based biosensors to study different components of GPCR pharmacology are endless and continuous development of BRET-based biosensors has led to the development of multiple generations, which have been reviewed previously[42,43,53–55]. For example, the earliest biosensors used donor-tagged GPCRs while transducers, such as ß-arrestins or G proteins, were tagged with an acceptor (BRET^1, BRET^2). Depending on the mechanism studied, BRET signals could either increase after agonist-mediated receptor activation, e.g., ß-arrestin recruitment, or decrease, e.g., dissociation of Gα and Gβγ subunits as a proxy for G protein activation (**Figure 7.2d**,**e**)[25]. The latter has been upscaled in the TRUPATH platform, which enables the detection of fourteen G protein pathways by tagging various Gα, Gβ and Gγ subunits with donor and acceptor pairs in separate assays with the same cellular background[46]. Enhanced bystander BRET (ebBRET) is the improved BRET-based biosensor technology, which does not require modification of the GPCR (**Figure 7.2f**). In this case, the BRET donor is tethered to a cellular compartment and the translocation of an accepter-fused protein to this compartment can be measured[42]. The ebBRET can be used for characterization of trafficking or localization of GPCRs and/or transducers, as the donor-anchors can be targeted to the plasma membrane but also endosomal or other membranes[44,45]. This offers the opportunity to explore agonist-mediated signaling across different cellular compartments, a phenomenon referred to as 'location bias' by activation of distinct signaling pathways in various subcellular locations[56,57]. Altogether, BRET-based biosensors have facilitated the study of numerous events following GPCR activation including G protein activation, GRK and ß-arrestin recruitment, desensitization, internalization, recycling and dimer formation[31,43].

The versatility of BRET-based biosensors renders them highly appealing tools for studying GPCR pharmacology. Nonetheless, multiplexing of BRET-based biosensors in cellular assays remains unexplored. This could prove very challenging given that two different emission spectra are measured in BRET-based assays. Incorporating a second biosensor would require meticulous optimization of donor and acceptor pairs to effectively distinguish between the different emission spectra.

7.2.2.3 Expanding the multiplex assay

Expanding the multiplex assay as described in **Chapter 3** by addition of a biosensor for dissociation of the heterotrimeric G protein, serving as proxy for G protein activation, may be a valuable strategy. However, incorporating more biosensors poses various significant challenges due to the emission spectra of the luciferases. First, no luciferase and substrate pairs with emission spectra >700 nm have been discovered, which would be required

since NLuc and Fluc signals range from approximately 380 to 580 nm and 500 to 700 nm, respectively. Secondly, switching the substrate for Fluc from D-luciferin to AkaLumine-HCl shifts the light emission to between 600 and 800 nm, and as such creates a small opportunity for integration of a third biosensor. In this case, integration of a CBGluc (λ_{max} 540 nm) biosensor may be the only possibility. However, CBGluc requires D-luciferin as substrate, which will move the Fluc emission spectrum back to 500 to 700 nm (**Table 7.1**). Thirdly, using complemented NLuc as donor for a BRET-based G protein dissociation biosensor renders challenges as this would require constant interaction of CB_2R with ß-arrestin for complementation to the active luciferase. Furthermore, this would require ß-arrestin recruitment to occur prior to G protein dissociation and remain at a constant level to prevent reduction of BRET signals due to reduced NLuc emission. Altogether, expanding the current multiplex presents various limitations. Consequently, exploring novel biosensor combinations may offer more opportunities for enhancing our understanding of GPCR pharmacology with particular regard to biased signaling. Moreover, this approach holds promise for elucidating multiprotein interactions or unraveling the sequence of signaling/trafficking events for which a few examples are outlined below.

To capture the effect of agonist-mediated GPCR activation on the ß-arrestin level and G protein level, opposed to the downstream cAMP in our multiplex, one potential strategy may involve multiplexing the CBluc complementation biosensors CBGluc (λ_{max} 540 nm) and CBRluc (λ_{max} 615 nm, **Figure 7.2b**,**c**) with NanoBiT® (λ_{max} 460 nm). In this case, ß-arrestin-1 and 2 could be tagged by CBGluc and CBRluc, respectively, which would show the preferred isoform recruitment after activation since either ß-arrestin-1 or ß-arrestin-2 can complement the CBGluc C-term segment. The LgBiT segment could be fused to the Gα subunit and the SmBiT segment to the Gβγ subunit consequently dissociation of the heterotrimeric G protein, and thus attenuated luminescence, can serve as proxy for G protein activation[35,58].

Alternatively, to better comprehend the effect of isoform-specific ß-arrestin recruitment and subsequent receptor internalization, the ß-arrestin CBluc complementation biosensors as described above could be combined with the HiBiT complementation assay by N-terminal fusion of the HiBiT to the GPCR (**Figure 7.2a**). This would capture the internalization of the GPCR and may be correlated to the recruitment of a specific ß-arrestin isoform.

In addition to multiplexing split luciferase assays, exploring the potential for multiplexing two BRET-based biosensors presents an intriguing avenue. While this approach may not be suitable for simultaneous detection of two transducer proteins, like the G protein and ß-arrestin, due to their likely proximity to one another and to the GPCR, it may hold promise for determining receptor localization after activation over time. A combination of NLuc with EGFP and mKATE2 may be a promising starting point due to the far-red shifted emission spectrum of mKATE2 (**Table 7.2**). However, careful consideration is required to prevent that the emission of EGFP causes excitation of mKATE2. In this case, the trafficking of a NLuc-tagged GPCR to the early endosome could be followed by increased BRET signals for EGFP if in close proximity to Rab5-EGFP. Subsequently, increased mKATE2 BRET signals would indicate proximity to mKATE2-tagged Rab7 and thus receptor trafficking to the late endosome (**Figure 7.3**).

Nevertheless, multiplexing of biosensors may remain very challenging, and technical and biological considerations should be made. On the technical side, this would require careful optimization of protein constructs to prevent interference of (split) luciferase or fluorophore tags on the intrinsic protein function. Moreover, consideration of the appropriate substrate or combinations of substrates is essential and spectral overlap should be minimized via the proper use of suitable equipment to separate the detection of different excitation and emission wavelengths. Currently, most split luciferase and BRET-based assays are not applied to physiologically relevant systems as they require modification of proteins and the modified proteins need to be expressed in large excess to prevent interactions of native proteins. The competition between modified and native proteins may shield or reduce the luminescent or BRET signals[52]. Additionally, endogenous signaling may be altered by overexpression of these proteins and loss of biased signaling at GPCRs has been reported in overexpressed systems[59]. Solutions are presented in the form of endogenous protein modification by the CRISPR/Cas9 technology, which maintains the endogenous expression levels and stoichiometry[60]. For instance, NLuc fragments have been introduced on native proteins like ß-arrestin-2 in HEK293 cells or atypical chemokine receptor 3 in HeLa cells for

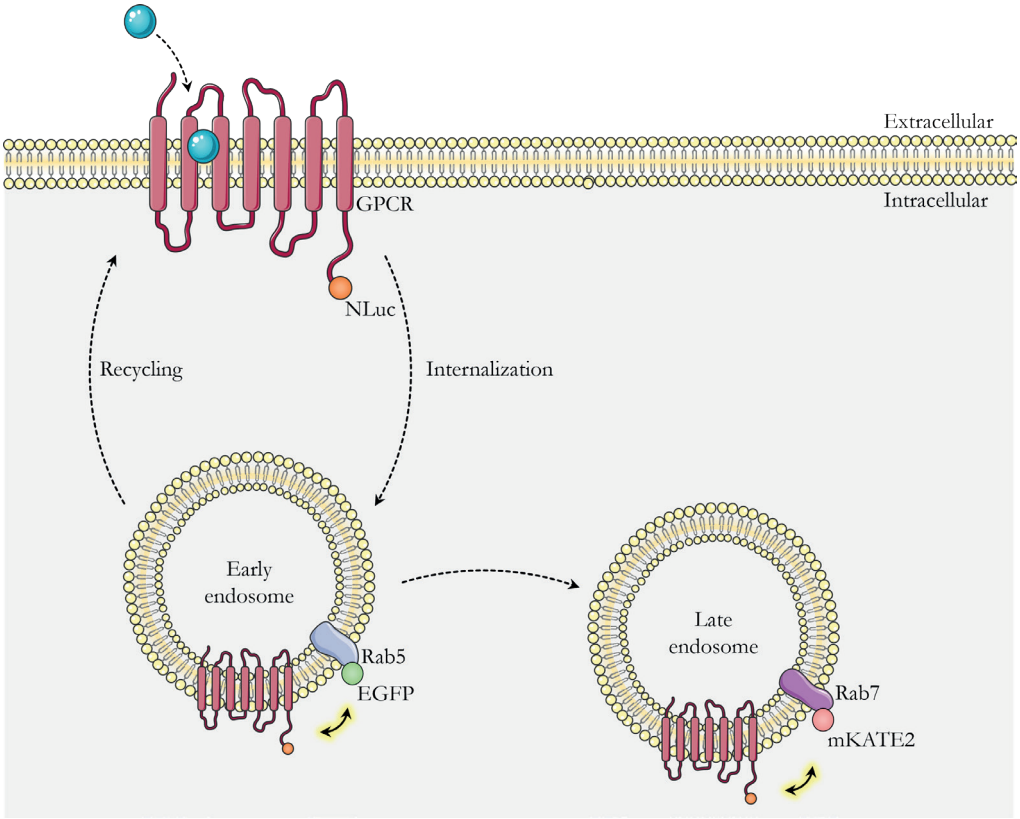

Figure 7.3 **Possibility for multiplexing two BRET-based biosensors.**
Possible multiplex assay setup with NLuc-tagged GPCR, which upon internalization in the early endosome may increase BRET signals with EGFP-tagged Rab5. Transition to the late endosome would be reflected by increased BRET signaling with mKATE2-tagged Rab7. This figure incorporates drawings from Servier Medical Art (smart.servier.com).

NanoBRET/NanoBiT purposes[61,62]. While endogenous expression levels and stoichiometry are maintained in these engineered cell lines, it does not always reflect the heterogeneity of the human population or the relevant disease tissue. The use of human induced pluripotent stem cells (hiPSCs) in GPCR pharmacology research is emerging to further increase the physiological relevance [60]. The use of biosensors in hiPSCs was first demonstrated by Avet and colleagues, where ebBRET was used to detect the translocation of (heterologous) $G\alpha_i$ proteins to the endogenous sphingosine 1 phosphate receptor-1 ($S1P_1$) in hiPSC-derived cardiomyocytes[63]. Nonetheless, in case of successful implementation of these technical and biological challenges, multiplex assays could contribute greatly to novel insights into agonist-induced GPCR pharmacology and concepts such as biased signaling.

7.2.3 Intertwining novel concepts to improve drug discovery

As described in **Chapter 1** there is great potential for integrating novel concepts in the early phases of drug discovery to enhance the translational perspective, and thus decrease clinical attrition rates. In **Chapter 3**, the drug-target binding kinetics of CB_2R agonists were investigated and related to their signaling profiles. However, allosteric modulation (**Chapter 5**) and the impact of single point mutations (**Chapter 6**) were approached as individual concepts. Recent findings on other GPCRs suggests that these concepts may be intertwined in diverse manners and offer new therapeutic possibilities, which will be expanded upon in the following paragraphs.

7.2.3.1 Biased allosteric modulation

Combining allosteric modulation and biased signaling is a newly emerging approach in drug discovery that provides spatial, temporal and signal pathway specificity[64]. Biased allosteric modulators (BAMs) exert their effect by selectively modulating one pathway activated by an orthosteric ligand over another pathway while binding to the allosteric binding site[65–69]. The feasibility of designing and employing BAMs has recently been demonstrated for several GPCRs in *in vitro* and *in vivo* studies, of which two are further presented below.

For example, a selective ß-arrestin-biased NAM was identified for the $ß_2$-adrenoceptor ($ß_2AR$) which in the presence of endogenous agonist antagonized the interaction with ß-arrestin-2 without affecting cAMP production via $G\alpha_s$ signaling[65]. Current clinical treatments for asthma or chronic obstructive pulmonary disease (COPD) rely on balanced clinical $ß_2AR$ agonists[70]. However, it has been described that the therapeutic effects are mediated via activation of the $G\alpha_s$ pathway, whereas ß-arrestins may contribute to the pro-inflammatory and pathogenic effects in asthma mouse models[65]. To this end, ß-arrestin-biased NAMs may provide a novel class of drugs that modify endogenous $ß_2AR$ activation with improved selectivity on receptor binding and signaling effects.

In the case of the neurotensin receptor 1 (NTSR1), a ß-arrestin biased PAM has shown promise for the treatment of drug addiction in *in vivo* studies[68]. Activation of this receptor offers therapeutic possibilities by restoring homeostatic dopamine signaling, but clinical

applications have been precluded by the occurrence of severe side effects due to NTSR1's involvement in regulation of fundamental physiological processes, such as regulation of body temperature, blood pressure and motor control[68]. Nevertheless, preclinical data showed that the regulation of addiction-associated behavior in rodents was primarily mediated via ß-arrestin-2. A screening effort led to the discovery of a ß-arrestin-2 biased NTSR1 activator, SBI-553. This compound selectively antagonized $G\alpha_q$ signaling in the presence of endogenous neurotensin (NTS), while ß-arrestin-2-mediated pERK generation was stimulated. These promising effects of SBI-553 were further exhibited by the attenuation of psychostimulant-associated behavior in mouse models of drug abuse, without introducing side effects seen with balanced agonists[68].

Altogether, the development of BAMs presents a promising strategy for the design of more selective drugs for GPCRs that target therapeutic relevant pathways while minimizing side effects via other pathways[64]. To date, the mechanism for the desired therapeutic effects at CB_2R and the potential importance of biased signaling is largely unknown. Here, BAMs could serve as tool compounds to provide more insight into the mechanism of receptor modulation.

7.2.3.2 Mutations introducing biased signaling

Biased signaling not only refers to the possibility of ligands inducing differential signaling, as studied in **Chapter 3**, but it also extends to biased receptors. Single point mutations, either natural variants in the population or associated with disease, can modify a receptor to adopt a specific conformation, thereby favoring stimulation of one signaling pathway over another[71]. Such mutations may play an important role in the disease progression by stimulation or inhibition of certain pathways. Consequently, genetic variation, and as a result variation in signaling, could contribute to variations in drug efficacy and toxicity[71]. To this end, it is imperative to investigate the impact of mutations on signaling.

Several natural variants in CB_2R have been described, of which a glutamine to arginine point mutation on position 63 (Q63R) is widely reported and has been suggested to affect several psychiatric disorders[21]. Additionally, a substitution of tyrosine for histidine was found in the C-terminal at position 316 (H316Y) and a high mutant allele frequency of leucine to isoleucine mutation (L133I) was found in bipolar disorder patients[21,72]. *In vitro* studies showed that the CB_2R variants Q63R and L133I had similar $G\alpha_i$ activation and consequently cAMP production as WT but showed distinct GRK and ß-arrestin-2 binding. Specifically, the Q63R mutant showed increased GRK2 and GRK3 binding compared to WT and consequently increased ß-arrestin-2 binding, whereas GRK2, GRK3 and ß-arrestin-2 binding was decreased for CB_2R-L133I[21]. Another study found compromised agonist-mediated inhibition of cAMP production on Q63R and H316Y receptors and the constitutive activity of H316Y, but not Q63R was increased compared to WT[72]. Of note, all experiments in this thesis were carried out on CB_2R with Q63, L133 and H316.

In the case of the cysteinyl leukotriene receptor 2 (CysLTR2), it was found that a leucine to glutamine mutation on position 129 ($L129^{3.43}Q$) was a recurrent hotspot in uveal melanoma

(UVM) patients[73]. Furthermore, the mutated receptor served as a driver oncogene in UVM and other melanocytic tumors[73]. Closer examination revealed that mutant receptor CysLTR2-L129$^{3.43}$Q was constitutively active with stronger $G\alpha_q$ coupling, while recruitment of ß-arrestins was attenuated compared to the WT receptor, and thus the receptor escaped down-regulation mechanisms associated with this pathway[73].

Studies on disease-associated mutations in extracellular loop 3 (ECL3) of the adhesion G protein-coupled receptor G1 (ADGRG1) revealed that mutated receptors ablated the serum response factor (SRF) response, while the signaling to nuclear factor of activated T cells (NFAT) pathways was unaffected[74]. Further elucidation of these signaling events uncovered mechanistic differences in these two pathways, which were initially brought to light by studying the disease-associated mutations.

While the precise implications of disease-related mutations in disease progression may not be fully understood, studying them offers an opportunity for obtaining a fundamentally better understanding of receptor signaling and their role in pathophysiology. Furthermore, this may lead to a potentially improved pharmacological strategy for conditions influenced by these mutations.

7.2.3.3 Mutations altering target-binding kinetics

While natural variants or disease-associated mutations in GPCRs can drastically influence downstream signaling, ligand binding may also be impacted which was described in **Chapter 6**. The impact of disease-associated mutations on receptor targeting by agonists and antagonists is generally investigated on the level of equilibrium binding affinity[75–80], while the effect on kinetic parameters association and dissociation rate constants (k_{on} and k_{off}) is less understood. Nevertheless, various studies have reported that single point mutations in GPCRs, introduced to better understand binding mechanisms, may affect one or both of these rate constants, and consequently the binding affinity.

A study with mutations introduced into the adenosine A_{2A} receptor ($A_{2A}R$) demonstrated the differential impact of single point mutations on antagonist dissociation, which was either decreased, increased or not affected[81]. Specifically, mutations in the binding pocket on amino acids typically involved in hydrogen bonding with the ligand prevented the formation of the hydrogen bonds and as such opened up the pocket and decreased the RT[81]. On the other hand, mutations on residues that are involved in the formation of a salt bridge with the ligands increased the dissociation rate constant of long RT ligands, while the effect on short RT ligands was less pronounced[82]. Similarly, in the muscarinic M_3 receptor, mutations on residues that were involved in locking the ligand into the receptor drastically decreased the RT[83].

While these studies only focused on the effect of mutations on ligand RT, a study by Swinney *et al.* also explored the effect of mutations in the human CC chemokine receptor 5 on the association rate constant[84]. They identified a kinetic fingerprint of residues that differentially affected k_{on} and/or k_{off} values of the ligand. Similarly, a study on mutations in $A_{2A}R$ demonstrated that changes in k_{on} values were observed, but that differences in binding

affinity were often derived from altered k_{off} values. Interestingly, while both k_{on} and k_{off} values of ligands for some mutated receptors were affected, the overall binding affinity of the ligands remained unchanged[85].

Altogether, these studies emphasized the influence single point mutations may have on kinetic binding parameters, which are overlooked when only reporting binding affinity. Accordingly, investigating target binding kinetics on mutated receptors, natural variants or disease-associated mutations, contributes to the overall understanding of receptor targeting and downstream signaling. Ultimately, this could contribute to more accurate selection of drugs in the application of precision medicine.

7.3 Final notes

In essence, this thesis explored the molecular pharmacological mechanisms of targeting CB_2R via investigation of novel drug discovery concepts such as target binding kinetics, allosteric modulation and biased signaling. Central to the investigation of CB_2R pharmacology was developing new assays and providing an overall kinetic view, aimed at bringing fresh insights that could be further integrated into the field of GPCR research. To this end, the development and application of state-of-the-art and novel cellular and biochemical assays contributed to a better understanding of agonist-mediated CB_2R activation and signaling, which can advance drug discovery efforts for treatments of diseases that involve CB_2R. Finally, *it's about time* that novel concepts for GPCRs are incorporated into early drug discovery programs, where a kinetic view is applied to provide a better translational perspective.

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