# Chemical tools to study the cannabinoid receptor type 2

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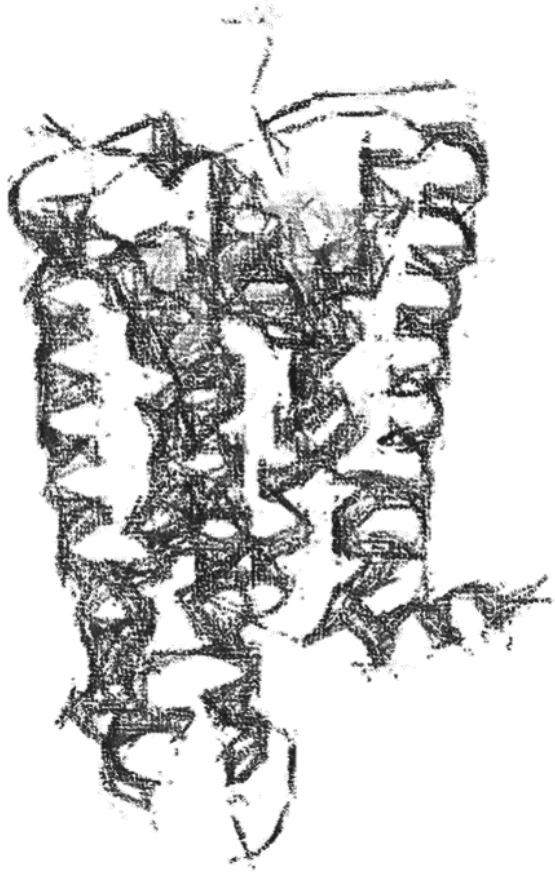
# Chapter 8

**Summary and Future Prospects**



The cannabinoid receptor type 2 ($CB_2R$) is, together with the cannabinoid receptor type 1 ($CB_1R$), an established target of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), the psychoactive substituent of the plant *Cannabis sativa*.[1] Preparations of the cannabis plant have been used throughout history as a medicine for a wide variety of conditions.[2] The $CB_2R$ is predominantly found in immune cells and has been proposed as a therapeutic target for several neuroinflammatory diseases.[3-9] The $CB_1R$ is predominantly located in the central nervous system,[10] and is responsible for the psychoactive side effects associated with cannabis use. Drug discovery efforts have focused on the development of $CB_2R$-selective ligands,[11-13] because it is anticipated that these drugs will lack the $CB_1R$-mediated side effects.[10, 14-16] Since not all THC-induced biological effects are abolished in $CB_1R$ KO mice, research efforts have also focused on the identification of other (non-CBR) protein targets of THC.[17] Ultimately, these developments could lead to the discovery of novel $CB_2R$ therapeutics with fewer side effects.[18] The work described in this thesis has focused on the discovery, synthesis and application of chemical tools and strategies to study the $CB_2R$ (**Chapters 3-6**) as well as on the identification of unknown protein targets of THC (**Chapter 7**). This chapter summarizes the work described in this thesis and provides possible future directions for the research towards $CB_2R$-based therapeutics.

## 8.1 Development of chemical tools to study the $CB_2$ receptor

**Chapter 1** provides a general introduction to the drug discovery concepts applied in this thesis (i.e. target identification, target validation, hit-to-lead optimization, target engagement), followed by a general description of G protein-coupled receptors (GPCRs) and their signaling pathways. The cannabinoid receptors type 1 and 2 ($CB_1R$ and $CB_2R$) are introduced, which are both members of an endogenous signaling system, i.e. the endocannabinoid system (ECS). Both cannabinoid receptors (CBRs) are targeted by endogenous ligands, such as anandamide (AEA) and 2-arachinoylglycerol (2-AG). Despite two decades of drug discovery efforts on the development of $CB_2R$-selective ligands, no $CB_2R$-selective drugs have yet reached the market.[11-13] More information on $CB_2R$ distribution, expression level, occupancy, internalization and pharmacology, both *in vitro* and *in vivo* models, is essential to guide the development of novel $CB_2R$-based therapeutics. Therefore, new chemical tools ("probes"), such as radioligands, PET tracers, fluorescent or biotinylated small molecules as well as covalent probes, like electrophilic or photo-activatable ligands, are required to aid drug discovery and development efforts.[19, 20]

**Chapter 2** provides an extensive summary of various types of $CB_2R$ probes reported in the literature and discusses their advantages and limitations. In general, $CB_2R$ probes suffer from low metabolic stability,[21-26] low $CB_2R$ selectivity,[27, 28] significant losses in receptor affinity, or high non-specific binding.[29-33] Covalent probes for $CB_2R$ have been successfully applied in $CB_2R$ binding site mapping when used in conjunction with site-directed mutagenesis.

The covalent probes do not possess a detection element, which makes it difficult to use them to study $CB_2R$ expression and ligand engagement. Previously, drug-target engagement in living systems has been successfully studied using two-step photoaffinity-based protein profiling ($pA_fBPP$).[34] This technique combines the covalent addition to the receptor's active site with a detection element, but avoids the problems associated with large reporter groups (see **Figure 11** in **Chapter 2**). It has previously been predominantly applied to soluble proteins, but in this thesis it was used to study $CB_2R$ expression (i.e. a membrane-bound protein) and ligand engagement (**Chapter 6**) as well as for target identification studies (**Chapter 7**).

In **Chapter 3**, the development of the PathHunter® β-arrestin recruitment assay is described.[35, 36] In this assay, β-arrestin activity is measured in live CBR-overexpressing cells using enzyme complementation and a chemoluminescent read-out. These CBRs are tagged at their C-terminus with a small fragment of β-galactosidase. This cell line stably co-expresses β-arrestin that is fused to a catalytically inactive N-terminal deletion mutant of β-galactosidase.[36] Recruitment of β-arrestin to the activated CBR induces the complementation of both enzyme fragments, resulting in the formation of an active β-galactosidase enzyme.[37] The active enzyme is able to convert a substrate into a chemiluminescent product.[38] Therefore, the light emission by the product is directly related to the activity of the β-galactosidase, and thus the level of β-arrestin recruited to the receptor after ligand binding.[35] The Pathhunter® β-arrestin recruitment assay is an easy-to-use assay that has shown to deliver reproducible results.[36, 39, 40] In addition, its 384-well format enables high-throughput screening, a useful feature for lead optimization in early drug discovery research. This assay was successfully validated and applied to characterize a set of cannabinoid receptor reference ligands (**Chapter 4**), to identify a suitable ligand for $CB_2R$ target validation, and novel $CB_2R$ chemical tools (**Chapters 5** and **6**).

**Chapter 4** describes a multi-laboratory, comprehensive profiling of cannabinoid ligands to identify the most suitable ligand for $CB_2R$ target validation.[12] To study therapeutic relevance of $CB_2R$ in early stage drug discovery,[41] it is essential to use selective ligands with a well-defined molecular mode of action. In this chapter, the 18 most widely used $CB_2R$ ligands were profiled. This 'cannabinoid reference library' consisted of widely used ligands to explore CBR biology, such as dual CBR agonists $\Delta^9$-THC, CP55940, WIN55212-2, HU210, 2-AG and AEA, $CB_1R$-selective antagonists SR141716A (rimonabant) and AM251, $CB_2R$-selective agonists HU308, HU910, Gp-1a, JWH015, JWH133 and AM1241 and $CB_2R$-selective antagonists AM630 and SR144528.[18, 42, 43] These ligands were profiled on receptor binding of human and mouse $CB_2R$, and on multiple signal transduction pathways (GTPγS, cAMP, β-AR, pERK and GIRK). Their physico-chemical, *in vitro* ADME, pharmacokinetic parameters and cross-reactivity in the CEREP panel of 64 common off-targets were also determined. The 'best' three $CB_2R$ agonists (good selectivity over $CB_1R$, mininal off-target activity, reasonable pharmacokinetics) were further investigated *in vivo* to study potential $CB_1R$-mediated effects by their metabolites.

Interspecies differences in $CB_2R$ selectivity over $CB_1R$ were identified for several ligands as well as marked differences in signal transduction preference (i.e. biased signaling). For example, HU308 and HU910 were balanced agonists on human $CB_2R$, but showed significant biased agonism on mouse $CB_2R$. Most ligands were found to display a rich poly-pharmacology, but $CB_2R$-selective agonists HU308, HU910 and JWH133 and $CB_2R$-selective inverse agonist SR144528 had the least off-targets. Although the pharmacokinetic profile of these ligands was far from optimal,[44] *in vivo* experiments showed that effective drug concentrations can be achieved after intravenous as well as oral administration. Finally, HU308, HU910 and JWH133 were found to have no $CB_1R$ activity *in vivo* when tested in the mouse cannabinoid triad (anti-nociception, catalepsy and hypothermia). All together, HU910, HU308 and JWH133 (**Figure 1**) were identified as the most suitable $CB_2R$ agonists to study $CB_2R$ biology for target validation purposes. SR144528 was selected as the most suitable antagonist, because of its high selectivity profile for $CB_2R$ in both humans and mice.

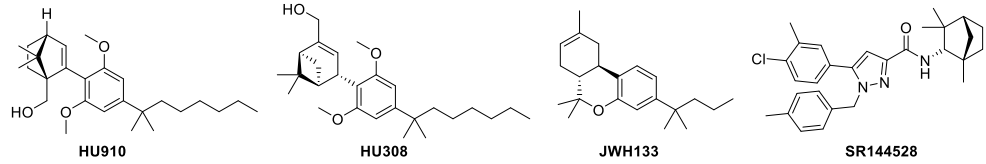


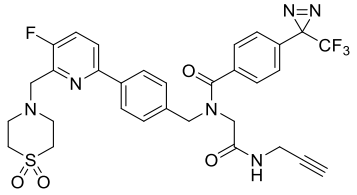

**Figure 1. Structures of HU910, HU308, JWH133 and SR144528.**

**Chapter 5** describes the synthesis and molecular pharmacology of an agonist library, based on the recently reported *in vivo* active $CB_2R$-selective agonist, LEI101.[45] The aim of this chapter was to identify relationships between physicochemical properties, ligand binding kinetics and functional activity on $CB_2R$. To this end, a series of 24 agonists, for which the lipophilicity and basicity was systematically varied, was synthesized. The equilibrium binding affinity and Kinetic Rate Index (KRI), a high-throughput measure as an indication for ligand-receptor (dissociation) kinetics,[46] was measured for all compounds. In addition, the full kinetic profile, as well as functional potency and efficacy in G protein activation and β-arrestin recruitment, was measured for 14 of these ligands. This led to the finding that increasing lipophilicity, specifically on the $R^2$ position, led to increased receptor residence time, which was correlated with increased potency, but not with efficacy, in the two signaling pathways without biased agonism. In contrast, basicity of the agonists did not show a relationship with affinity, residence time or functional activity. These findings provide important insights how $CB_2R$ agonists can be designed to have optimal kinetic profiles. This may aid the lead optimization process to study or treat inflammatory diseases.

**Chapter 6** reports on the design, synthesis and application of a LEI101-based photoaffinity probe as a tool to detect endogenous $CB_2R$ expression and target engagement in primary human immune cells.

Previously reported structure-activity relationships (SAR) of LEI101[47] combined with a docking study in a homology model of the human $CB_2R$ ($hCB_2R$)[48, 49] were used for the rational design of the two-step photoaffinity probe LEI121 (**Figure 2**). The probe LEI121 was applied in a photoaffinity-based labeling workflow as introduced in **Chapter 2**. The probe possesses a diazirine as photoreactive group to capture $CB_2R$ and an alkyne as ligation handle to enable visualization or isolation of the protein by conjugation to fluorophores or biotin, respectively. LEI121 was identified as a potent $CB_2R$ inverse agonist and shown to be selective over $CB_1R$. The ability of LEI121 to target $CB_2R$ was validated in $CB_2R$-overexpressing CHO cells using gel-based imaging. Two main fluorescent bands, probably corresponding to different glycosylated forms of the receptor, were visualized. The labeling was dependent on UV-irradiation and copper-catalyzed conjugation of a fluorophore and could be prevented by pre-incubation with various, structurally diverse $CB_2R$ ligands. In addition, LEI121 could be used to identify the $CB_2R$ via chemical proteomics using live cells by bioorthogonal ligation with biotin, followed by streptavidin enrichment, tryptic digestion and mass spectrometry analysis. Finally, LEI121 was successfully applied to determine endogenous $CB_2R$ expression and target engagement in HL-60 cells and in a specific subset of human immune cells using flow cytometry.

The development of LEI121 as a two-step photoaffinity probe provides new opportunities to study $CB_2R$ biology. For example, it is envisioned that LEI121 may serve as an alternative to the highly unselective $CB_2R$ antibodies to profile $CB_2R$ expression levels in PBMCs of patients suffering from inflammatory pain and/or other diseases, such as rheumatoid arthritis, multiple sclerosis and Crohn's disease. Additionally, isolation of $CB_2R$ using primary cells and tissues may facilitate the identification of potential protein interaction partners of the receptor to further elucidate the cellular processes of $CB_2R$ and to enable drug discovery efforts for e.g. dual inhibitors. The photoreactive probe may also help stabilizing $CB_2R$ to facilitate crystallization studies of the protein.




**LEI121**

**Figure 2. Structure of LEI121**

Finally, to study the THC protein interaction landscape, the development of a THC-based photoaffinity probe, is described in **Chapter 7**. In this chapter, a $\Delta^8$-THC-based photoaffinity probe ("probe **1**"), carrying a diazirine as the photoreactive moiety and a terminal alkyne as the ligation handle, was designed and synthesized in 14 steps. While probe **1** had high affinity for both CBRs it was unable to covalently label the CBRs.

Probe **1** enriched ~150 proteins in mouse Neuro2A cells. The abundance of four of these proteins (i.e. Cox4i1, Reep5, Mtch2 and Gnb1) was significantly lowered by pretreatment with either $\Delta^9$-THC or $\Delta^8$-THC, indicating these proteins are putative protein targets of THC. Follow up studies should validate the interaction of THC with these targets and whether modulation of these proteins by THC can be attributed to the beneficial effects of THC observed in several mouse models.

## 8.2 Towards $CB_2R$-based therapies

The work described in this thesis provides important insights in $CB_2R$ ligand pharmacology for target validation (**Chapter 4**) and binding kinetics in lead optimization (**Chapter 5**), and has resulted in the development of a two-step photoaffinity-based strategy to study $CB_2R$ expression and target engagement (**Chapter 6**) and to identify novel protein targets of THC (**Chapter 7**, **Figure 3** upper right). It is anticipated that this work may help in the discovery and development of $CB_2R$-based therapeutics that have fewer side effects than medicinal cannabis or purified THC (**Figure 3**).

$CB_2R$ expression is highly upregulated in several conditions that have an unmet medical need, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, Crohn's disease, neuropathic pain and rheumatoid arthritis.[6, 7, 50-61] Development of $CB_2R$-selective drugs for these type of diseases would be highly valuable, but clinical trials to evaluate the efficacy of $CB_2R$ agonists in these diseases have only recently started (Alzheimer's disease (NTRX-07/MDA7),[62] dermatomyositis, cystic fibrosis, systemic lupus erythematosus and diffuse cutaneous systemic sclerosis (Resunab/JBT-101, NCT02466243, NCT02465450, NCT03093402 and NCT02465437, respectively) and an analgesic for visceral pain associated with Crohn's disease (APD371, NCT03155945). Previously, $CB_2R$-selective ligands have only been tested in clinical trials for their analgesic properties in a third molar tooth extraction model, osteoarthritis or atopic dermatitis.[13,63-66] The development of these ligands as drugs was discontinued due to a lack of *in vivo* efficacy (or unspecified reasons), despite compelling evidence of efficacy in preclinical settings. Although the reasons for this non-translatability are unknown and could be related to the predictive quality of the preclinical animal models, it is hypothesized that the therapeutic role of $CB_2R$ is not properly validated for these applications.[67] Furthermore, a lack of target engagement might also explain the lack of efficacy observed in the clinical trials.

For future development of $CB_2R$ drug candidates, it is essential that the therapeutic relevance of $CB_2R$ in a particular disease is properly validated, using highly selective, well characterized molecules (**Figure 3**, upper left). Ligands used for target validation should lack inter-species differences in $CB_2R$ selectivity (both affinity and activity) as well as in signal transduction preference (i.e. biased signaling).

Differences in selectivity and signaling profile of ligands between rodents and humans may have important consequences in the translation of preclinical models to the clinic when testing novel drug candidates (reviewed in Kenakin and Miller[68] and Violin *et al*[69]). For $CB_2R$, it is currently unknown whether different signaling pathways may lead to different (patho)physiological outcomes, and it is therefore not clear which signal transduction pathways (or combinations thereof) are relevant for therapeutical purposes.

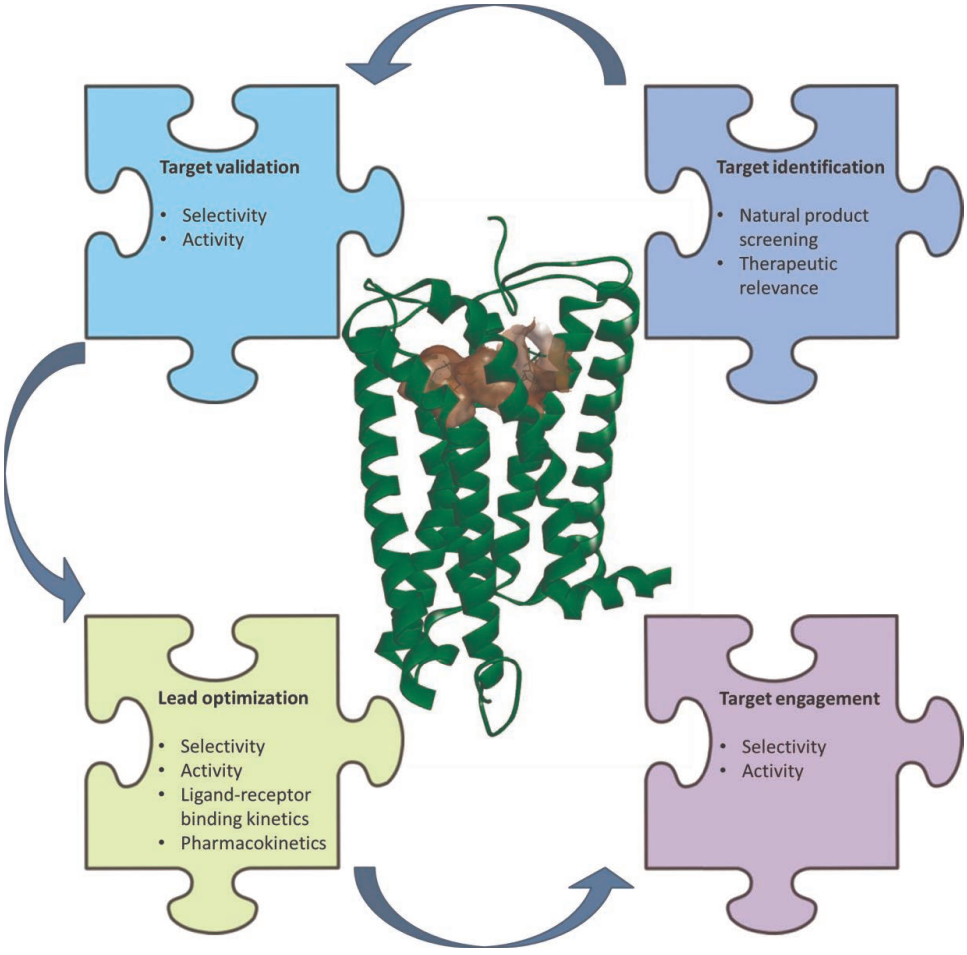



**Figure 3. Drug discovery challenges for $CB_2R$-based therapies with fewer side effects.** The puzzle pieces represent the different stages of drug discovery, in which the parameters are listed that need to be taken into account in these stages. After target identification from therapeutic relevant molecules (e.g. THC, **Chapter 7**), the therapeutic relevance of the target need to be validated per disease type and progession, using selective ligands with a well-defined molecular mode of action (**Chapter 4**). In the hit-to-lead optimization stage, selectivity, activity and drug-target binding kinetics (**Chapter 5**) as well as pharmacokinetics need to be optimized. Finally, selective small molecules are essential to verify that the compound engages with its target at the site-of-action in a dose-dependent manner (**Chapter 6**).

In the hit-to-lead optimization stage, it is equally important to take biased signaling into account, because small structural changes have been associated with changes in signaling preference (**Figure 3**, lower left).[70] In addition, structural changes may lead to significant differences in binding kinetics, which in turn may have important implications for the functional activity of ligands, in potency or efficacy, but also in signaling preference.[71] For example, biased signaling was observed for the endocannabinoids 2-AG and AEA (**Chapter 4**), but not for the LEI101-based library reported in **Chapter 5**. This indicates that the key determinants for ligand binding kinetics as well as biased signaling is dependent on the scaffold of the ligand. Previously, it was reported that JWH133 and HU308, both *in vivo* active cannabinoid ligands have different kinetic profiles.[72] This suggests that the optimal kinetic profile of $CB_2R$ ligands for *in vivo* activity is not a determinant factor and can be flexible, or may be dependent on disease type and its progression. The binding kinetics of endogenous ligands has been hypothesized to be an indication of the kinetics required to maintain homeostasis.[73,74] The divergent binding kinetics of three different endocannabinoids[72] may, therefore, support the notion that the $CB_2R$ accepts flexible kinetic profiles.

Next to the determination of the efficacy and safety profile of a drug candidate, it is also essential to verify that the compound engages with its target at the site-of-action in a dose-dependent manner in preclinical animal models as well as in humans (**Figure 3**, lower right). Proof of target engagement has not been possible for $CB_2R$ with the current reported chemical tools (as summarized in **Chapter 2**). The development of LEI121 (**Chapter 6**) enables, for the first time, proof of *in vitro* $CB_2R$ engagement in human cells using FACS analysis and may enable target engagement *ex vivo* in clinical trials, for example to determine whether a clinical candidate targets $CB_2R$ on these cell types.

## 8.3 Conclusions

This thesis has shown that comprehensive profiling of ligands on protein binding, kinetics, functional activity on both human and rodent $CB_2R$ as well as off-target activity may reveal important consequences for the interpretation of biological results and the drug discovery process. In addition, this thesis described the design, synthesis and application of the first two-step photoactivatable probe that allows the visualization of $CB_2R$ expression on human cells and its engagement by various ligands. It is anticipated that these new chemical tools and insights will help to improve the drug discovery and development of $CB_2R$ ligands.

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