Development of new chemical tools to study the cannabinoid receptor type 2

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Chapter 1

General Introduction

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Cannabinoid Receptors

The endocannabinoid system (ECS) is a collection of lipid transmitters that bind receptors to elicit cellular responses, as well as the enzymes involved in their synthesis and catabolism.[1] The system is involved in a plethora of physiological processes, including cognitive and immune responses, as well as the psychoactive effects of *cannabis sativa*.[2]

The two main receptors in the ECS are cannabinoid receptor type 1 (CB_1R) and cannabinoid receptor type 2 (CB_2R). Though there are some other receptors, such as GPR55, that are also targeted by endocannabinoid-like compounds, their role in the ECS is much less understood.[3] The cannabinoid receptors are class A G protein-coupled receptors (GPCRs), which rely on dynamic changes in their structure to relay the binding of an extracellular ligand to an internal signal.[4] As their name implies, they convey the main portion of their signalling through the recruitment of G proteins. Various G proteins exist, generally consisting of an α and βγ subunit.[5] CBRs recruit $G_{i/o}$ proteins for their signalling, although CB_1R has also been shown to recruit $G_{q/11}$ proteins in astrocytes.[6]

CB_1R and CB_2R have some similarities in their mode of action. Both inhibit adenylyl cyclase, promote PI3K, MAPK, ceramide production and gene transcription. While CB_1R is also adept in modulation of voltage-gated calcium channels (VDCCs) and G protein inwardly-rectifying potassium channels (GIRKs), CB_2R is a very poor modulator of these channels.[7] Additional differences lie in their cell type distribution, leading to different effects. For example, the specific neuronal distribution of CB_1R leads to the psychoactive effects of cannabinoids, while CB_2R does not elicit such responses.[7]

The best studied endogenous ligands of CB_1R and CB_2R are anandamide (AEA, Figure 1.1) and 2-arachidonoylglycerol (2-AG), though many more have been postulated, including noladin ether, *N*-arachidonoyldopamine (NADA), and docosatetraenylethanolamide (DEA).[1] Δ^9-Tetrahydrocannabinol (THC) is considered the primary phytocannabinoid in *cannabis sativa* and similarly binds to both CB_1R or CB_2R.[8,9]

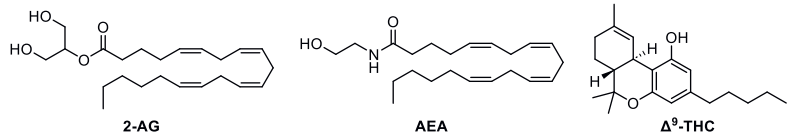


Figure 1.1 The chemical structures of the major two endocannabinoids (2-AG and AEA) that act as ligands on CB_1R and CB_2R, as well as the structure of the primary constituent of cannabis, Δ^9-THC.

CB_1R was first cloned in 1990; which proved that cannabinoids act by interaction with a specific receptor to elicit central nervous system (CNS) effects, rather than through membrane disruption.[10] CB_1R is the most abundantly expressed GPCR in the brain.[11] It has been found in high levels in the basal ganglia, cerebellum, prefrontal cortex, hippocampus, nucleus accumbens, hypothalamus, paraventricular nucleus and the central amygdala, mainly in the terminals of neurons and glial cells.[12,13] Here it plays a role in many neurological processes, including appetite, learning and memory, anxiety, depression and addiction. Consequently, CB_1R is reported to play a role in many neurological disorders, such as stroke, multiple sclerosis, epilepsy and neurodegenerative diseases.[14–17] CB_1R expression is not restricted to the nervous system, as it is also present in peripheral tissues including the muscles, liver, pancreas, GI tract and fat tissue.[18] The peripheral roles of CB_1R include nociception, inflammation,

energy metabolism, functioning of the gastrointestinal tract and the musculoskeletal system, intraocular pressure, cardiovascular, glandular and reproductive functioning and cancer.[13,19,20]

CB_2R is thought to be exclusively expressed in cells and organs of the immune system, including spleen, tonsils, thymus and lymphocytes[21], where its major role is the regulation of (neuro)inflammatory processes. During inflammation CB_2R inhibits cytokine production, lowers antigen presentation, and modulates microglia M1/M2 differentiation.[22–24] Disruption of the inflammation modulation of CB_2R may play a role in various autoimmune and neurodegenerative diseases, such as Alzheimer's, Parkinson's, Huntington's, dementia and others.[25] The presence and activity of CB_2R in neurons is highly debated.[26]

The Structure of CB_1R and CB_2R

CB_1R and CB_2R share a sequence homology of 44%, which is increased to 68% in the transmembrane domain.[2728] They exhibit the general features of the Class A GPCRs. The receptors both consist of seven transmembrane helices (TMH), three intracellular (IC) and extracellular (EC) loops, an extracellular N-terminus and intracellular C-terminus which includes a short helix (Hx8).[29] The ligand binding site is located in the interior of the seven (TMH) bundle. The biggest difference between CB_1R and CB_2R is the ECL2. The ECL2 from CB_1R is much longer and can dip into the binding pocket in the inactive state.[30–32] The shorter ECL2 of CB_2R acts more like a lid on the binding pocket in active and inactive CB_2R, akin to active CB_1R.[33]

The first 3D structure of a GPCR was elucidated in 2000 with X-ray crystallography, which was rhodopsin.[34] The first CB_1R crystal structure was elucidated in 2016, in complex with antagonist AM6538 (Figure 1.2A).[31] The receptor required several modifications to increase stability and limit flexibility during the crystallization process. The protein was crystallized in a lipidic cubic phase with additional cholesterol to mimic the CB_1R membrane environment.[31] In 2019 the same group was able to crystallize CB_2R to elucidate the 3D protein structure (Figure 1.2B). By using the antagonist AM10257 and similar modifications as for CB_1R, they were able to form a stable complex.[33]

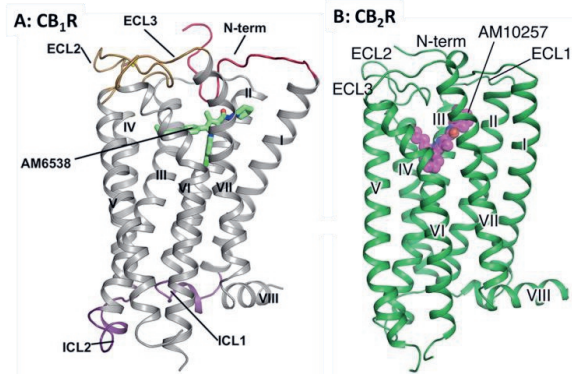


Figure 1.2 Structure of the Cannabinoid Receptors. (A) A side view of CB_1R (grey) in complex with antagonist AM6538 (light green). The N-terminus is shown in red, while the extracellular loops are shown in brown and the intracellular loops in purple. (B) A side view of CB_2R (green) in complex with antagonist AM10257 (purple). Picture adopted from Hua *et al.* Cell (2016)[31] and Hua *et al.* Cell (2019).[33]

Since then, several other CBR crystal structures with other ligands have been published.[35–39] Of note, the crystal structures are limited to the inactive state as they are all bound to antagonists. Information about the respective active states of the receptors was obtained using cryogenic electron microscopy (Cryo-EM). This new technique does not rely on X-ray scattering from very uniform protein crystals, but

rather uses electrons to visualize the surface of frozen protein particles. Cryo-EM does not require the growth of crystals, which can take months up to years to find the right receptor constructs and crystallization conditions. Instead the proteins are frozen at liquid nitrogen or helium temperatures. Thereupon a surge of structures obtained from cryo-EM has been observed in the RCSB database. In 2017 the first Cryo-EM structure of a GPCR, calcitonin[40], was published, followed in 2019 by the first Cryo-EM structure of an active state CB_1R in complex with both the G protein and an agonist (Figure 1.3A).[41] One year later, a CB_2R-G protein in complex with agonist WIN55,212-2 (Figure 1.3B) was published.[42]

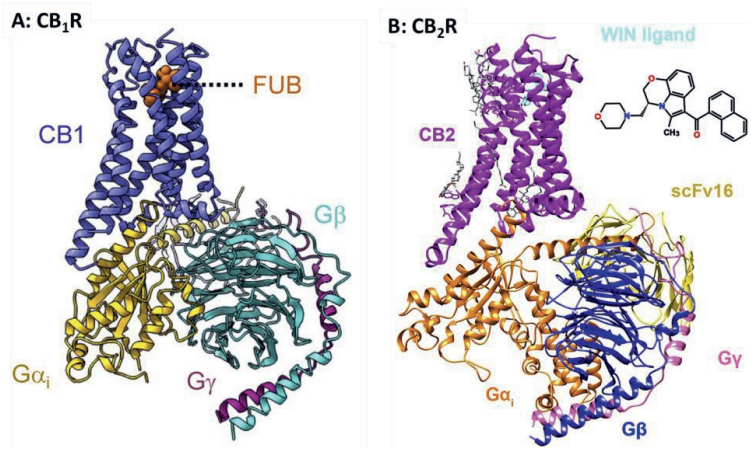


Figure 1.3 The three-dimensional protein structures of the CBR-G protein complexes bound to an agonist, obtained through cryo-EM. (A) Side view of CB_1R (blue) with agonist MDMB-Fubinaca (orange) and $G_{\alpha i}$, G_β and G_γ (yellow, cyan and purple respectively). (B) Side view of CB_2R (magenta) with agonist WIN55,212-2 (cyan), $G_{\alpha i}$, G_β and G_γ (orange, blue and pink respectively) and scFv16 (gold; additive to stabilize complex). Pictures adopted from Kumar K, Cell (2019) and Xing C, Cell (2020).[41,42]

When comparing active and inactive structures, both CB_1R and CB_2R feature an outward movement of TM6 upon activation, as well as movement of $Y^{7.53}$ (Ballesteros-Weinstein numbering in superscript) towards TM5. There are additional changes in conformation that are specific to either CB_1R or CB_2R, and may explain ligand selectivity and their differences in signalling.[43] In CB_1R the N-terminus moves away from the orthosteric binding site upon activation, while the position of the N-terminus in CB_2R does not move substantially between the inactive and active state. Furthermore, the TM1 in the CB_1R is positioned more outward in the antagonist bound structure than in the agonist complex. Again CB_2R shows no significant movement of TM1. The best known difference is probably the (twin-)toggle switch. In CB_1R $W356^{6.48}$ and $F200^{3.36}$ form a twin-toggle switch, where $F200^{3.36}$ locks $W356^{6.48}$ into the inactive state until an agonist disrupts the pi-bond, leading to a translational change for both residues.[43] In CB_2R only $W258^{6.48}$ acts as a toggle, and no transitional change is observed. Instead, $W258^{6.48}$ undergoes a 64° clockwise rotational change and $F117^{3.36}$ has a small 10° counter clockwise rotation upon CB_2R activation.[42] Note that these observations were made by comparison of the inactive receptor states and the G protein bound active receptor states. There may be differences in activation mechanism for β-arrestin bound active states.[43] The three-dimensional protein structures showed the difference between the agonist binding pocket of CB_1R an CB_2R is minimal, and it remains unclear how selective CB_2R agonists selectively activate CB_2R.[44]

CBR Therapeutics

Starting from the chemical structure of the psychoactive CB_1R/CB_2R agonist tetrahydrocannabinol (Δ^9-THC) isolated from *Cannabis Sativa*, many small molecules have been designed that have affinity for CB_1R and/or CB_2R. The first generation were THC-derived classical cannabinoids such as L-759,656, CP-55,940, JWH-133 and HU210 (Figure 1.4). Many of these were non-selective or only moderately selective for CB_2R over CB_1R. A next generation included analogues of natural products (*e.g.* hexahydrocannabinol) and endocannabinoid derivatives (*e.g.* AM356).[45,46] The pool of small molecules was then expanded to include compounds with a wide variety of scaffolds and chemical groups (*e.g.* JWH-018, WIN55,212-2, APD371).[47,48]

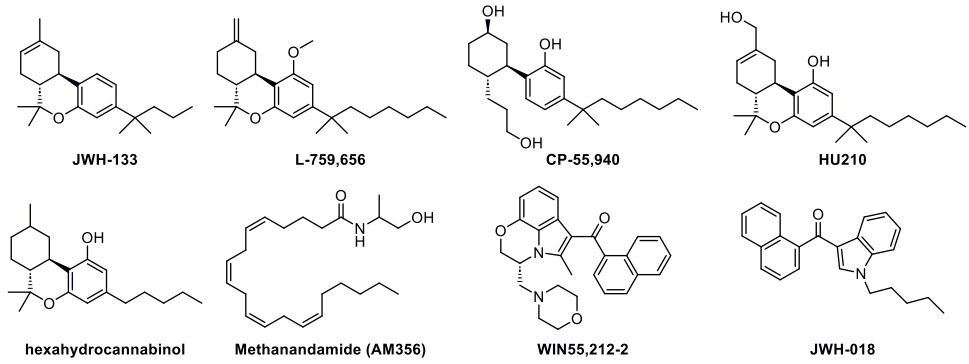


Figure 1.4 The chemical structures of several classical and non-classical CB_2R agonists.

CB_2R as Therapeutic Target

CB_2R plays an important role in immunoregulation, but does not induce the psychoactive effects that plagues CB_1R activation. It has been shown that CB_2R is upregulated under several pathological conditions.[49] Inflammation is reduced by CB_2R as it modulates macrophages to adopt the anti-inflammatory M2 subtype.[50] As such, CB_2R therapeutics could be beneficial for arthritis[51], cardiovascular[52] and inflammatory bowel disease[53], fibrosis[54], inflammation-induced pain[55] and neurodegenerative diseases.[56] Similarly to CB_1R, it also shows beneficial effects in other peripheral areas including skeletal and metabolic disorders[57,58], and cancer.[59]

CB_2R ligands will require high selectivity over CB_1R to avoid side effects, but when achieved will have therapeutic potential.[60] An advantage is that CB_2R agonists promote similar analgesic effects as CB_1R agonists, yet do not create tolerance over time, physical dependence or autonomic withdrawal.[61] While no selective CB_2R ligand has yet reached the market, many are currently under evaluation in animal studies and clinical trials.[62] AM1241 (Figure 1.5) was one of the first compounds used to investigate the analgesic properties of CB_2R agonists. However, AM1241 did not reach clinical trials due to its engagement with an endogenous opioid receptor, low efficacy and complex molecular pharmacology which differed for each enantiomer.[63] Cannabinor (PRS-211,375) was introduced in phase I and II clinical trials after beneficial anti-nociceptive effects in osteoarthritis in rat models. However, the promising pre-clinical results could not be replicated in humans.[64] GW842166 from GlaxoSmithKline showed the same problem with translation to humans.[60] S-777469 completed a Phase II clinical trial for atopic dermatitis in 2009, but no follow-up clinical research has been reported or planned since.[65–67] Lenabasum (HU239) was shown to have anti-inflammatory effects in a Phase II clinical trial, and has been given the FDA Orphan drug designation for treatment of diabetes mellitus.[68] It is currently in consideration as treatment for cystic fibrosis[69], Systemic Lupus Erythematosus[70], and Dermatomyositis[71], though it showed no significant effect in Phase III trials for Diffuse Cutaneous

Systemic Sclerosis.[72] HU308 is a synthetic cannabinoid with 440-fold selectivity for CB_2R over CB_1R, and was used as a case study to show anti-inflammatory and analgesic properties stemmed from CB_2R activation by showing absence of CNS-side effects in mouse models.[73] However, its lipophilic structure resulted in poor solubility, which prevented success in clinical trials.[74] In contrast, APD371 is one of the most polar CB_2R-selective agonists, which gives it excellent pharmacokinetics, peripheral restriction and oral availability.[60] It showed promising results in a Phase 2a trial for gastrointestinal pain in Crohn's disease, though a Phase 2b trial did not reach its primary endpoint.[75–77] Similarly Vicasinabin (RG-7774) was pulled by Roche after a Phase 2 trial in diabetic retinopathy showed underwhelming efficacy.[78]

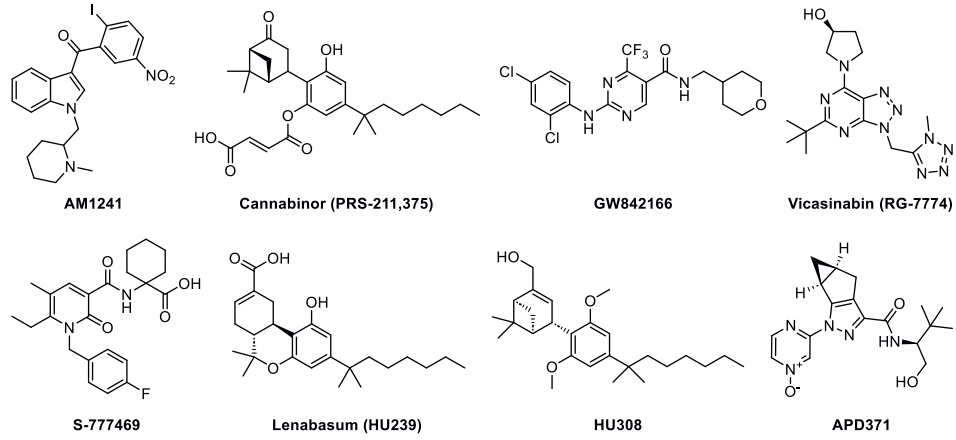


Figure 1.5 The chemical structures of several CB_2R agonists that have previously participated in clinical trials or have a clinical trial ongoing.

CB_1R as Therapeutic Target

Despite the wide distribution of CB_1R, its role in many pathologies makes it a popular target. Indeed, together with the serotonin receptor 2A it is the joined most popular target for CNS diseases in current preclinical studies.[62] Nonetheless, the development of drugs targeting CB_1R is difficult due to the numerous roles of CB_1R in a variety of peripheral and central nervous system processes, as well as the difficulty of observing neuropathologic symptoms. Additionally, targeting CB_1R can result in undesirable effects such as addiction, anxiety and psychoactive effects that limit its possibilities as a therapeutic target.[14] Regardless, many beneficial effects have been noted for CB_1R agonists. Foremost, both synthetic analogues of THC and cannabis extracts have been approved for treatment of neuropathic pain.[79] Furthermore, CB_1R agonists have been suggested as potential treatment of post-traumatic stress disorder (PTSD) due to the memory impairment effect.[80] Other avenues currently under investigation include their ability to reduce anxiety[81] and depressive tendencies[82], as an anticonvulsant for epilepsy[83] and to ensure neuroprotection during cerebral ischemia events.[84] Additionally, the effect of CB_1R agonists in neurodegenerative diseases such as Alzheimer's, Huntington's[85], and Parkinson's[86] is being investigated. CB_1R antagonists have had a set-back with the withdrawal of the first antagonist Rimonabant from therapeutic use due to CNS-mediated psychological side effects.[87] Nevertheless a new generation of peripherally restricted antagonists is showing promise in treatment of obesity, several forms of tissue fibrosis and improvement of cardiometabolic profile while preventing neurological side effects.[88,89]

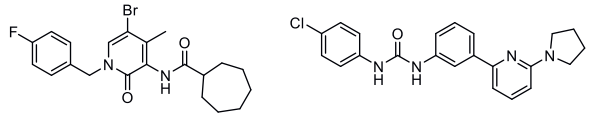
Strategies to Limit Side Effects

As mentioned above, CB_1R and CB_2R have a plethora of roles in different systems and diseases. Many promising drug candidates fail in clinical trials because tissue exposure/selectivity is not considered

during development.[90] Therefore several strategies have been devised to limit side effects while preserving the therapeutic effect, such as allosteric modulation, functional selectivity and controlled delivery through localized delivery or activation, or target-directing modification.

Allosteric Modulation

Allosteric modulators work differently from orthosteric ligands in the sense that they do not bind in the same binding pocket as the endogenous ligand, and thus result in a non-competitive mode of action. As such, positive allosteric modulators (PAM) can enhance the response of CB_2R to endogenous ligand binding, while negative allosteric modulators (NAM) decrease the response.[91] The idea is that exacerbation of the signal rather than creating a *de novo* signal retains the native signalling pattern of the receptor. Additionally, allosteric binding sites are not conserved and may provide selectivity between highly homologous subtypes (which is relevant for CB_1R and CB_2R).[92] An example of CB_2R PAM is Ec2la (C-2)[93], while PSNCBAM-1 is a CB_1R NAM (Figure 1.6).[94]



Ec2la (C-2) (PAM) **PSNCBAM-1**

Figure 1.6 An Example of a CB_2R positive allosteric modulator (Ec2la (C-2) and a CB_1R negative allosteric modulator (PSNCBAM-1).

Functional Selectivity

Like many GPCRs, CB_2R conveys its signals through both G protein activation and β-arrestin recruitment. However, not all agonists activate these two signalling pathway equally well. Some ligands are functionally selective, *i.e.* they do not result in equal signal strength via the G protein or β-arrestin. Certain synthetic agonists may initiate only a slight activation of G protein signalling, while a significant amount of β-arrestin is recruited upon agonist binding, or *vice versa*. Endocannabinoids can also show bias, *e.g.* 2-AG has significant bias for the β-arrestin pathway on CB_2R.[74] Contrarily, Δ^9-THC shows prominent G protein activation but barely any β-arrestin recruitment.[74] A functionally selective drug may perhaps discriminate between beneficial and adverse effects, thus minimizing side effects without compromise on the therapeutic effect.[95] Interestingly, HU308 is a balanced agonist on human CB_2R, but does show bias towards G protein activation on mouse CB_2R.[74] This further highlights the difficulties of developing CB_2R agonists as there are high interspecies differences that may account for the high failure rate of ligands in clinical trials after promising results in animal models.

Controlled delivery

Through controlled delivery accumulation of drugs in healthy tissue can be minimized through control over the transport of the drug. Nanocarriers can optimize drug administration, site-specific accumulation and (timed) drug release due to their (tuneable) physicochemical surface properties. Additionally, capsulation of the drug avoids off-target toxicity and prevents premature enzymatic breakdown.[96] Liposomes, micelles and polymeric nanoparticles have been proposed as possible nanocarriers.[97] Currently no nanocarriers with CBR (ant)agonists have yet reached clinical trial,[98] however, there are some promising applications. For example, recently the capsulation of CB_2R agonist β-caryophyllene (BCP) was described with a polymer of ethylene glycol (PEG) which allowed for administation through the intranasal route, circumventing the first-pass metabolism.[99] Additionally, a protein-based nanoparticle for cannabidiol (PNP-CBD) was designed to improve water solubility and increase CB_2R targeting.[100] For CB_1R efficient liver-targeting was shown with a ((poly(lactic-co-glycolic

acid)) (PLGA) encapsulated rimonabant nanoparticle.[101] There are currently over 90 marketed nanoformulations (non-CBR) and such an application is also being investigated for the CBRs.[102]

Peripheral restriction is also a form of controlled delivery.[103] By altering the physicochemical properties, such as topological polar surface area (tPSA) and H bonding capacity of known compounds[103], the blood brain barrier (BBB) penetration can be limited, preventing neurological side effects.

Phototherapy may also be considered controlled delivery. The inactive compound only activates with the light source, thus only activating receptors at the location with the light.[101,104] While this has not yet been translated to therapeutic CBR drugs, for research purposes a photoswitchable Δ^9-tetrahydrocannabinol (*azo*-THC) has been designed[105], as well as a 'caged' 2-AG with photoactivated release.[106]

A new type of controlled delivery instead looks at the drugs cellular distribution. In 2013 it became evident that immune cells have CB_2Rs present both on the plasma membrane and intracellularly.[107] This intracellular fraction of (active) CB_2R is present in the endoplasmic reticulum (ER) and endolysosomes.[108–110] The different roles of extracellular and intracellular CB_2R have yet to be determined. Nevertheless, evidence of a functional role for internal CB_2R is the time delay in CB_2R-dependent Ca^{2+}-response observed when administering CB_2R agonists extracellularly via bath application versus microinjection.[109] (a.k.a. the agonist first had to internalize when applied extracellularly.) Similarly CB_1R is distributed to both the plasma membrane and intracellularly in mitochondria[111] and lysosomes.[112,113]

Neutral Antagonism

Decrease of CB_1R activity by Rimonabant and other inverse agonists has been shown to lead to side effects of anxiety, depression and sometimes suicidal behaviour.[114] Hence, neutral antagonists for CB_1R have been in focus recently, for example in relation to substance abuse disorder (SAD) and weight loss.[115] In theory, neutral antagonism can limit side effects, when these are the result of decreased basal or constitutive activity of the receptor. For example, CB_1R antagonist AM4113 (Figure 1.7) showed great results in preclinical mice studies for SAD without inducing depression like behaviour, however, its poor oral availability and outcome of a recent study that did show anxiety-like behaviour do not make it the best candidate.[116,117] AM6527 does show promise with increased oral availability.[116] PIMSR is a neutral antagonist based on Rimonabant that exhibits the same depression of cocaine self-administration in mice, but without the detrimental effects.[114] Current results are promising, but, more research will need to be done on these types of drug candidates. Of note, whether CBRs are constitutionally active or are activated by a constitutive agonist tone, *i.e.* constitutive endocannabinoid levels, is difficult to determine.[118] Moreover, constitutive activity of receptors *in vitro* may be an artefact of the overexpression systems used, which could result in an inverse agonist *in vitro* behaving as a neutral antagonist *in vivo*.[119] This is an important consideration when pursuing neutral antagonists in drug discovery.

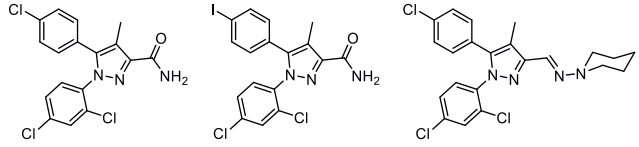


Figure 1.7 Chemical structures of several CB_1R neutral antagonists currently in development.

The Current Tools for CBR Research: Small Molecules

To determine target validation, CB_2R engagement and mode of action, tools are needed.[120,121] Small molecules can be turned into probes by introducing the right chemical groups. Probes can serve as a tool to image ligand-protein interaction and early identify potential problems such as off-target engagement. Many early drugs failed in clinical trials because it was not ascertained if the ligand had sufficient access to the target site and exhibited actual target engagement prior to entry.[120]

Positron Emission Tomography (PET) probes can image proteins and identify variations in activity and distribution with a radioisotope. They are widely used in medical imaging. However, drawbacks are short lifetime, exposure to radiation and nanomolar affinity requirements and possibility of false-positives.[122,123] The first CBR PET tracer was based on THC: [^{18}F]-Δ^8-THC (Figure 1.8), but it proved unstable and was unable to show specific binding *in vivo*.[124] [^{11}C]NE40 was used in human subjects to perform *in vivo* PET imaging, but CB_2R proved a bad biomarker for Alzheimer's Disease.[125] Many other designed PET tracers were abandoned due to high non-specific binding.[126] A recent CB_2R specific PET tracer is [^{18}F]RoSMA-18-d_6, which has nanomolar affinity and has successfully mapped CB_2R in nonhuman primates.[127,128]

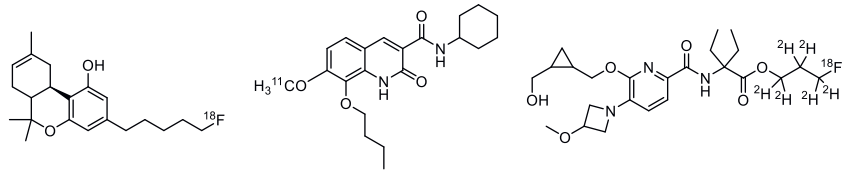


Figure 1.8 The chemical structures of several CBR PET tracers.

Fluorescence Resonance Energy Transfer (FRET) is a way to detect target engagement through a fluorescent signal that is only given when an acceptor and donor on the ligand and protein are in close proximity. However, it requires modification of the protein which may affect its structure and activity and cannot be used *in vivo*.[129] Likewise, many fluorescent small molecule probes have also been used for biological imaging. They are more accessible and stable than radioligands, do not require protein modification such as FRET, and are applicable in a wide range of biochemical assays. Introduction of a spacer chain minimizes the effect of the bulk of the fluorescent group on the scaffold's affinity for the target protein.[130] Cy5 and AF647 are commonly used due to their high quantum yield and stability under many conditions. Fluorescent probes are especially useful to image ligand-target interactions.[131,132] A Chromenopyrazole-Cy5 probe (Figure 1.9) has been shown to have moderately high affinity (hCB_2R pK_i = 7.38 ± 0.05, Table 1.1) with over 100-fold selectivity over hCB_1R.[133] Near-infrared fluorophores are popular due to their low interference with autobio-fluorescence and minimal damage to the cell during irradiation.[134,135] NIR760-mbc94 was designed and shown to effectively image tumours in murine models in a CB_2R selective manner.[136] Instead of direct ligand-fluorophore probes, sometimes two-step probes are utilized. HU210 was tagged with a biotin group, and was able to show CB_2R in microglial cells when streptavidin-AlexaFluor488 was added.[137] However, the two-step labelling and additional step to block endogenous biotin makes these probes unsuitable for staining and flow cytometry.[138] Other reported fluorescent CB_2R probes include *azo*-HU308-3, HU210-AF488, Naphthyridine-BODIPY630/650, RO6852763-SiR, HU308/AM841-chimera-NBD, HU308-AttoThio12, N-adamantyl-4-oxo-1,4-dihydroquinoline-3-carboxamide-4-DMAP, LEI-121 and more.[138–145]

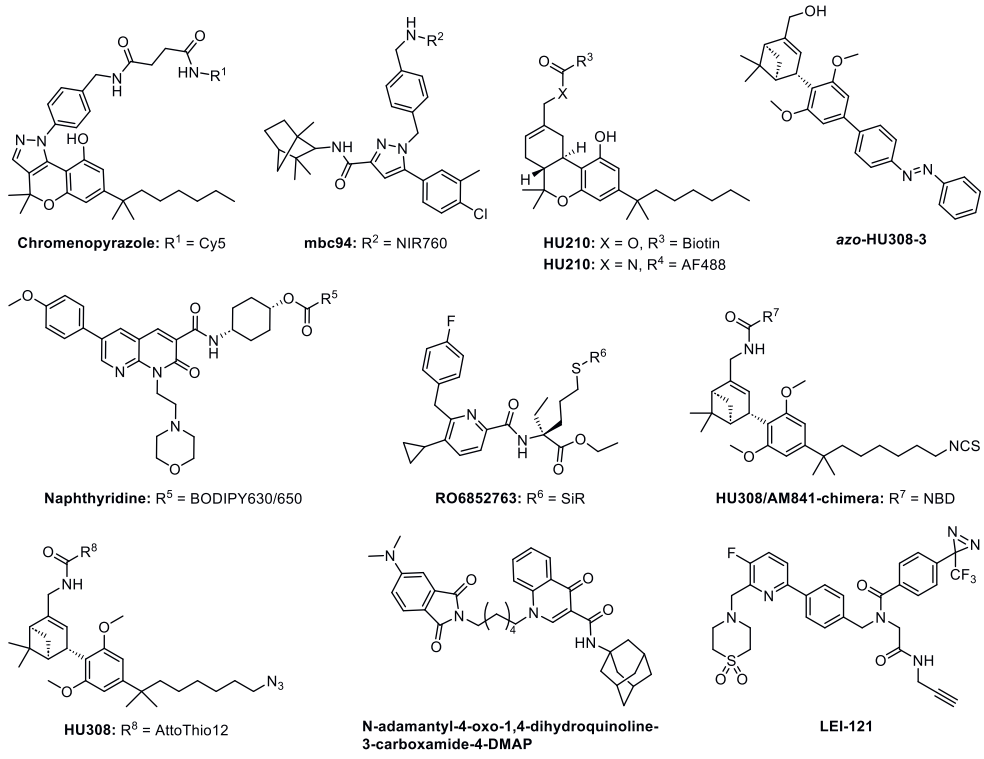


Figure 1.9 Chemical structures of reported fluorescent CB_2R probes.

Table 1.1 Reported fluorescent CB_2R probes.[146]

Name	CB_1R (pK_i ± SEM)	CB_2R (pK_i ± SEM)	Functionality
Chromenopyrazole-Cy5[133]	5.26 ± 0.11	7.38 ± 0.05	Inverse agonist
NIR760-mbc94[136]	N.R.	K_d = 26.9 ± 3.7 nM	N.R.
HU210-biotin[137]	8.62 ± 0.11	8.80 ± 0.11	N.R.
Azo-HU308-3[139]	N.R.	N.R.	Agonist
HU210-AF488[138]	7.57 ± 0.06	6.10 ± 0.11	Agonist
Naphthyridine-BODIPY630/650[140]	< 5	6.33 ± 0.02	Agonist
RO6852763-SiR[141]	6.94	7.21	Agonist
HU308/AM841-chimera-NBD[142]	5	8.38	N.R.
HU308-AttoThio12[143]	5.97	8.33	(Partial) agonist
N-adamantyl-4-oxo-1,4-dihydroquinoline-3-carboxamide-4-DMAP[144]	< 5	6.89	N.R.
LEI-121[145]	< 5	7.2 ± 0.4	Partial Agonist
N.R. not reported.			

Bifunctional, covalent probes can extent the applicability of the probes as they are no longer in a tenuous equilibrium where changes in environment can disengage the probe from the target. Electrophilic probes and photoaffinity probes first engage the target with high affinity through non-covalent means, after which an activatable group reacts with the protein to form a covalent bond. Electrophilic probes do this through an electrophilic group such as isothiocyanate (NCS), but are limited to reacting with nucleophilic amino acids.[147] Photoactivatable probes contain an inert group such as

diazirine that forms a reactive species upon irradiation with light, and this reactive species can insert into any bond nearby.[148] A bifunctional probe can then use a reporter tag (such as a radioisotope of fluorophore) to image the desired trait. Leiden University previously reported on the first bifunctional photoaffinity probe LEI-121 with a high selectivity for CB_2R in 2018.[145]

A new highly selective CB_2R agonist enters the stage

In 2011 LEI-101 (Figure 1.10) with its 5-fluoropyridin-2-yl-benzyl-imidazolidine-2,4-dione scaffold was reported to be an orally available, peripherally restricted, high affinity, selective CB_2R agonist.[149] It doesn't induce central nervous system (CNS) -mediated effects and protects against cisplatin induced damage.[150] LEI-101 derivatives were used to substantiate the drug-target residence time model, where compounds with increased residence time showed higher functional potency.[151] With this in mind, the improved CB_2R agonist LEI-102 was recently reported.[44]

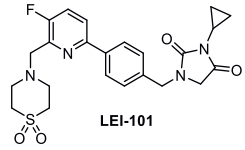


Figure 1.10 The chemical structure of LEI-101, the first in the 5-fluoropyridin-2-yl-benzyl-imidazolidine-2,4-dione series of compounds.

Thesis Outline

The endocannabinoid receptors CB_1R and CB_2R are involved in a plethora of processes, and consequently are involved in many pathological conditions. Their wide distribution makes the CBRs both an interesting therapeutic target and hard to study. Additional chemical tools are required to study and understand the function and mechanism of CB_1R and CB_2R. This thesis describes the development of several such tools to improve our insight in the (pathological) roles of the receptors in order to develop novel and improved therapeutics. **Chapter 2** describes the evaluation of three-dimensional ligand-CB_2R complexes made and analysed with Cryo-EM. Hotspots that potentially generate selectivity between CB_1R and CB_2R are evaluated with point-mutations *in vitro*. **Chapter 3** describes the development of the first tools, two-step bifunctional probes based on LEI-121 and LEI-102. Because two-step probes are not compatible with every assay, the toolbox is expanded with a one-step fluorescent probe. **Chapter 4** describes the design of a CB_2R fluorescent probe using the ligand-CB_2R complex from **Chapter 2**, and consequent synthesis. Switching to CB_1R, **Chapter 5** describes the design of CB_1R ligands with negatively charged phosphonium groups that are potentially selective for $mtCB_1R$. The thesis is concluded with a summary and outlook in **Chapter 6**.

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