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Chemical tools to study the cannabinoid receptor type 2

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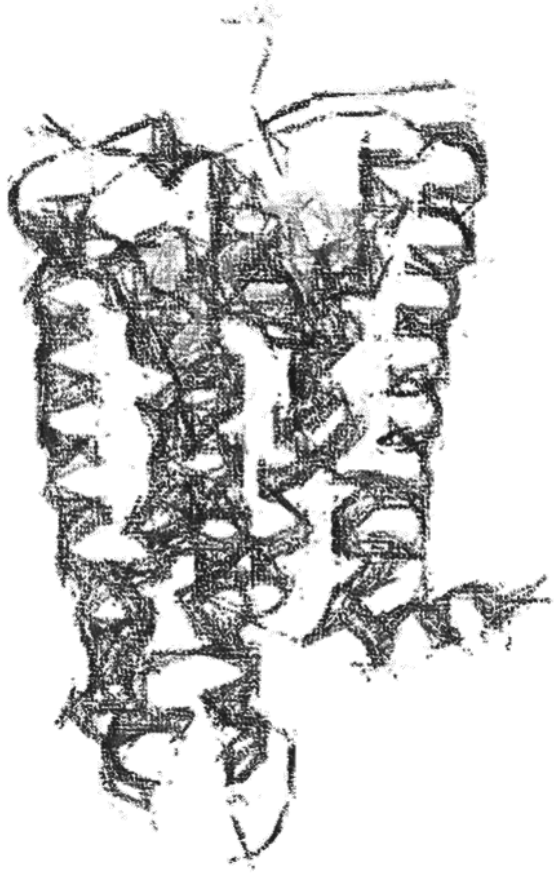
Chapter 1

General Introduction



1.1 Drug discovery approaches and challenges

Drug discovery and development comprises the whole process from the discovery of a therapeutically relevant protein to the launch of an effective and safe drug on the market (**Figure 1**). This process can take more than a decade (~13.5 years per drug on average) and the costs may rise above one billion dollars (~2.5 billion dollars per drug on average).[1] Especially the development stage is very expensive, time consuming and risky.[2, 3] Currently, market introduction rates of new drugs are low, while costs of drug development have risen substantially (the "innovation gap"),[4] often due to late stage clinical failures.[5, 6] Main causes for clinical trial failures are a lack of efficacy and the occurrence of adverse side effects. Possible reasons for toxicity and the lack of efficacy include 1) the protein target has not been properly validated for the disease under investigation, 2) preclinical profiling of the drug was not sufficient, 3) the translation between preclinical models and the human situation is very poor, or 4) the drug simply does not reach the protein target at the site of action.

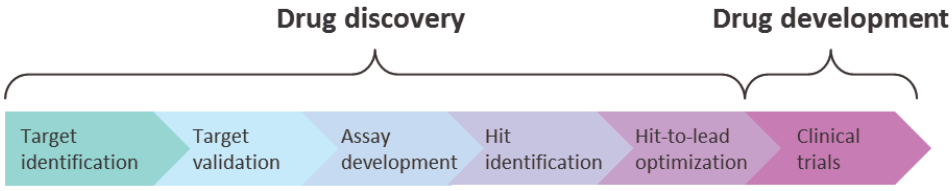


Figure 1. Schematic representation of the drug discovery and development process.

1.1.1 Target identification and validation

The goal of target discovery is to identify a protein (i.e. a target) which is therapeutically relevant for a particular disease. In case of natural product-based drugs, such as herbal mixtures, it is important to identify the protein target, because this will enable the development of synthetic and more selective lead compounds.[7] Moreover, selective modulators of a protein are required to establish efficacy in animal models of the disease (i.e. target validation).[8] Most lead compounds and drug candidates are small molecules, which are organic compounds with a low molecular weight (< 900 Da) and size in the order of 1 nm. It is essential that the small molecules used for target validation are highly selective and have a well-defined molecular mode of action.

1.1.2 Hit discovery and optimization

Target discovery and validation are followed by hit identification, which may involve high throughput screening of chemical libraries, identification of active ingredients from a natural product, and structure- or ligand-based design (e.g. using previously published data and three dimensional models of the protein structure).[9]

In the hit-to-lead optimization phase, the interaction of the compound with the protein target is optimized, while reducing off-target activities, thereby enhancing the selectivity and safety of the lead.[10] During the hit-to-lead optimization a strict control of the physicochemical properties of a small molecule is exerted to improve the pharmacokinetic and toxicity profile of a drug. Binding kinetics, i.e. the association and dissociation rate of a drug to a protein target and off-target proteins, is increasingly investigated to improve the efficacy and safety profile of a lead.[11-14]

1.1.3 Target engagement in drug development

After optimization of a lead compound, it is 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. Several chemical tools and strategies have been reported to determine target engagement in living systems,[15] such as radioligands,[16] positron emission tomography (PET),[17-19] fluorescent or biotinylated small molecules,[20] fluorescence or bioluminescence resonance energy transfer (FRET and BRET, respectively),[21] fluorescence polarization microscopy,[22] or activity/photoaffinity-based protein profiling (ABPP/pA$_f$BPP, respectively).[15, 23]

1.2 G protein-coupled receptors

An important class of drug targets is the 700-membered superfamily of G protein-coupled receptors (GPCRs).[24] Currently, more than 30% of the available drugs on the market act on GPCRs,[25] which are involved in the regulation of many physiological processes, including vision, behavior, mood, energy balance, immunity and inflammation.[26-28]

GPCRs are cell surface receptors consisting of seven helices spanning the cell membrane.[26, 27] They convey extracellular signals from different types of stimuli, such as light, (peptide) hormones and neurotransmitters, to intracellular second messenger systems, thereby allowing cells to respond to their environment. This signaling is mediated by a G protein, which consists of three subunits α, β and γ (**Figure 2**). Activation of the receptor results in a conformational change, causing the G protein-bound guanosine diphosphate (GDP) to be exchanged for guanosine triphosphate (GTP), leading to dissociation of the βγ-dimer. The GTP-bound Gα-subunit activates or inhibits (depending on Gα-subunit subtype) several different second messenger pathways, such as cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP_3) and Ca^{2+} ions. These second messengers in turn regulate several cell signaling pathways.[29] To date, 21 Gα-subunits, 6 Gβ-subunits and 12 Gγ-subunits have been identified,[30] which constitute four main classes of G proteins: G_s, G_i, $G_{12/13}$ and G_q. G protein signaling is terminated when the Gα-subunit hydrolyzes its bound GTP molecule to GDP, followed by the reassociation of the Gα-subunit with the βγ-dimer. In addition to G protein-dependent signaling, GPCRs can also signal through the binding and subsequent activation by G protein-coupled receptor kinases (GRKs) and β-arrestins.[31]

Binding of these proteins to the GPCR can result in receptor desensitization and trafficking, resulting to internalization, resensitization and/or degradation, or in the activation or inhibition of G protein-independent signaling pathways, such as mitogen-activated protein kinases (MAPKs), tyrosine kinases or E3 ubiquitin ligases.[32]

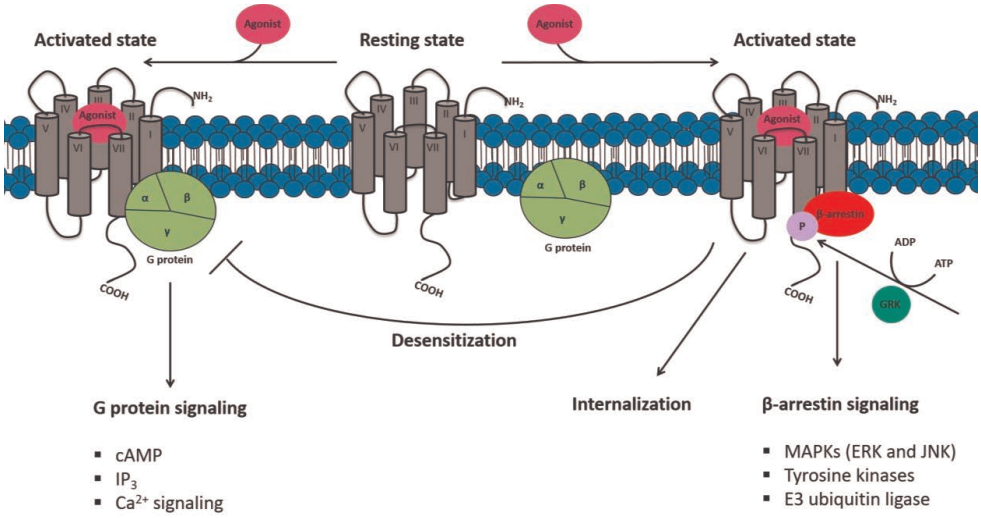


Figure 2. Schematic representation of GPCR signaling. Activation of a G protein-coupled receptor (GPCR) is mediated by a conformational change induced by binding of an agonist. Activated receptors couple to heterotrimeric G proteins, which consist of Gα, Gβ and Gγ subunits. In case of Gα signaling, the Gα subunit activates or inhibits (after dissociation of the Gβγ subunit) the generation of second messengers, such as cyclic AMP (cAMP), inositol triphosphate (IP_3) and Ca^{2+} ions. Activated receptors are phosphorylated by GPCR kinases (GRKs), which then recruit β-arrestins. β-Arrestins can block G protein – GPCR coupling (i.e. desensitization), or mediate clathrin-dependent endocytosis of activated GPCRs (i.e. receptor trafficking, internalization, resensitization or degradation). Finally, β-arrestins may mediate G protein-independent signaling pathways, such as mitogen activated protein kinases (MAPKs, e.g. extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs)), tyrosine kinases or E3 ubiquitin ligases.

The activity of a GPCR is dependent on its conformational state, which varies from completely inactive to several active states.[33, 34] Some GPCRs are active when no ligand is bound, a phenomenon that is called “constitutive” or “basal” activity.[35] A ligand that reduces this basal activity by inducing an inactive state of the GPCR is termed an inverse agonist,[36] whereas a ligand that merely binds the receptor (thereby blocking the binding of other ligands), without inducing a conformational change, is defined as an antagonist.[36] A ligand that stabilizes an active conformation, which leads to modulation of cell signaling pathways, is called an agonist. Dependent on the structure of an agonist or inverse agonist, they may induce different active or inactive conformations, respectively.[37] Ligands that do not modulate the signal transduction pathway to the full extent compared to a reference ligand are classified as partial (inverse) agonists, while ligands that fully (in)activate a GPCR are classified as full (inverse) agonists, respectively.[38-41]

Of note, a ligand may also preferentially activate or inactivate one or more specific signaling pathways over others. This concept is known as "biased signaling" or "functional selectivity".[42] Whereas biased agonism is well characterized,[43, 44] biased inverse agonism is a relatively new concept.[45, 46]

The three dimensional structure of GPCRs (consisting of seven transmembrane α-helices, an extracellular N-terminus, an intracellular C-terminus, and three extra- and –intracellular loops (ECLs and ICLs, respectively)) is highly conserved (**Figure 3**).[47] Nonetheless, the GPCR superfamily can be divided into six classes based on their functional similarity and sequence homology:[48] class A rhodopsin-like receptors, class B secretin receptor family, class C metabotropic glutamate/pheromone receptors, class D fungal mating pheromone receptors, class E cyclic AMP receptors and class F frizzled/ smoothened like receptors. Class A is the largest and most studied. It is characterized by the conservation of specific amino acids (especially the DRY motif in TM3[49]) and have relatively short extracellular domains.[50, 51] An example of class A receptor GPCRs is the family of cannabinoid receptors, which is the subject of this thesis.

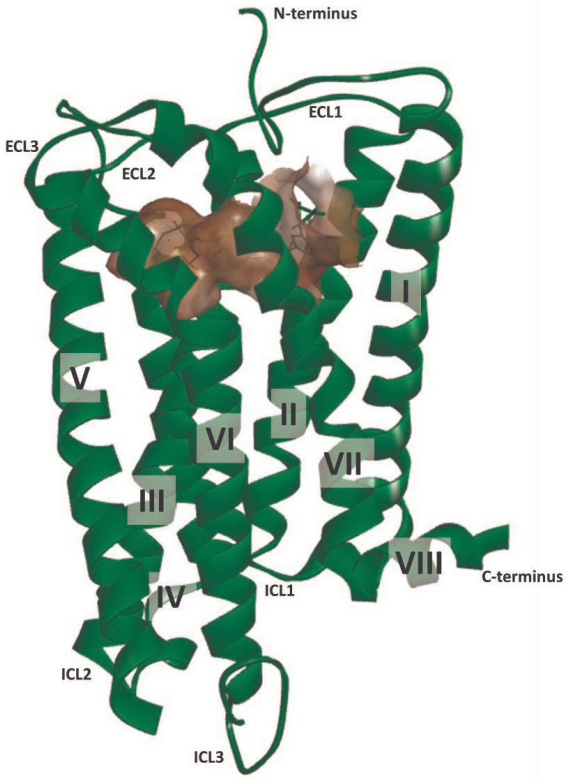


Figure 3. 3D structure of a GPCR. The 3D structure shown is from the cannabinoid receptor type 2 (CB_2R), a representative class A GPCR. The 3D structure was derived using a homology model based on a crystal structure of the cannabinoid receptor type 1 (CB_1R).[52] which was co-crystallized with AM6538. The orthosteric binding site is visualized by docking of a CB_2R-selective agonist, LEI101.[53]

1.3 Cannabinoid receptors and the endocannabinoid system

The cannabinoid receptor family consists of two subtypes: the cannabinoid receptor type 1 (CB_1R) and cannabinoid receptor type 2 (CB_2R). The receptors share an overall sequence homology of 44%, and 68% homology in the ligand-binding domain.[54] The CB_1R is mostly present in the central nervous system,[55] whereas the CB_2R is predominantly found in cells of the immune system, such as macrophages,[56] B lymphocytes,[57] and polymorphonuclear neutrophils.[58] Both cannabinoid receptors (CBRs) are activated by Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive constituent of the plant *Cannabis sativa*,[59] more commonly known as marijuana. The CBRs are also activated by endogenous ligands called endocannabinoids, of which *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are the most well-known. 2-AG is predominantly biosynthesized by diacylglycerol lipases and phospholipase C,[60, 61] and degraded by monoacylglycerol lipase,[62] whereas AEA is produced by *N*-acylphosphatidylethanolamine phospholipase D (NAPE-PLD),[63] and metabolized by fatty acid amide hydrolase (FAAH).[64] The CBRs, the endocannabinoids and their biosynthetic and metabolic enzymes constitute the endocannabinoid system (ECS).

Both CBRs are coupled to $G_{i/o}$ proteins and modulate various intracellular signal transduction pathways, such as inhibition of cAMP-production,[65] activation of mitogen-activated protein kinases (p38, p42/44 MAPKs, also called extracellular signal-regulated kinases (ERKs)),[66-68] phospholipase C,[69] and G protein-coupled inwardly rectifying K^+-channels (GIRKs),[69] and recruit β-arrestins to the receptor.[70-72] CBRs display ligand-dependent biased signaling as well as interspecies differences.[73-76] Different chemical classes have been described as CBR ligands, such as endocannabinoids and 'classical cannabinoids'.[77] This latter group is based on THC and is characterized by its tricyclic core scaffold. Other classes include aminoalkylindoles, diaryl pyrazoles and bicyclic ligands. Most of them are 'mixed' CBR ligands (i.e. display similar affinity for both CBRs), but also CB_1R- or CB_2R-selective ligands have been developed, [78] such as pyridines, pyrimidines, quinolones, oxoquinolines, triazines, biaryl hydantoins,[53, 79] and others (for reviews see Thakur *et al*.[77] and Morales *et al*.[78]).

1.4 The CB_2 receptor as therapeutic target

The CB_1R is associated with many physiological processes, such as food intake[80-82] and locomotor activity,[83, 84] but also with undesirable effects including addiction,[85] anxiety,[86, 87] and psychoactive effects.[55] The CB_2R plays an important role in cell migration, immunosuppression[88] and pain sensation,[89] and its cellular expression is upregulated under pathophysiological conditions,[90] such as neuroinflammation and neurodegenerative diseases[28] (e.g. Alzheimer's disease, Parkinson's disease,[91] neuropathic pain, amyotropic lateral sclerosis and multiple sclerosis[92]).

The CB_2R is considered a therapeutic target for a variety of diseases, such as cardiovascular,[93] liver,[94] kidney[95] and neurodegenerative diseases (mentioned above),[96] as well as for the treatment of inflammatory or neuropathic pain,[97] cancer,[98] osteoporosis,[99] and inflammatory diseases, such as rheumatoid arthritis,[100] Crohn's disease[101] and multiple sclerosis.[92]

Currently, the only drugs on the market targeting CB_2R are medical cannabis, Sativex (a mixture of Δ^9-THC and cannabidiol (CBD)) and Nabilone (a THC analog).[102] However, these are non-selective cannabinoid ligands that also activate the CB_1R, leading to unwanted psychoactive side effects. Therefore, drug discovery efforts have focused on the development of CB_2R-selective ligands,[75, 103, 104] because it is anticipated that these drugs will lack the CB_1R-mediated side effects.[55, 85-87] Since it has been reported that 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.[84] Both the identification of additional protein targets of THC as well as the development of selective drugs targeting CB_2R could ultimately lead to the development of novel therapeutics with fewer side effects.[105]

Several selective CB_2R ligands were active in various animal models of chronic and inflammatory pain, diabetic neuro- and nephropathy, liver cirrhosis, and ischemic-reperfusion injury,[79, 88, 106] but only a few were tested in clinical trials (e.g. GW842166X (**1**),[107] PRS-211375 (Cannabinor, **2**),[108] LY2828360 (**3**),[104] Tedalinab (**4**)[109] and S-777469 (**5**)[110] (**Figure 4**)). These ligands have been mostly explored for their analgesic (GW842166X and Cannabinor) and anti-inflammatory (GW842166X, LY2828360, Tedalinab and S-777469) properties. Specifically, GW842166X and Cannabinor were tested as analgesics in a third molar tooth extraction model,[103] while GW842166X, LY2828360 and Tedalinab were developed for the treatment of osteoarthritis[104] and S-777469 was tested for the treatment of atopic dermatitis.[110]

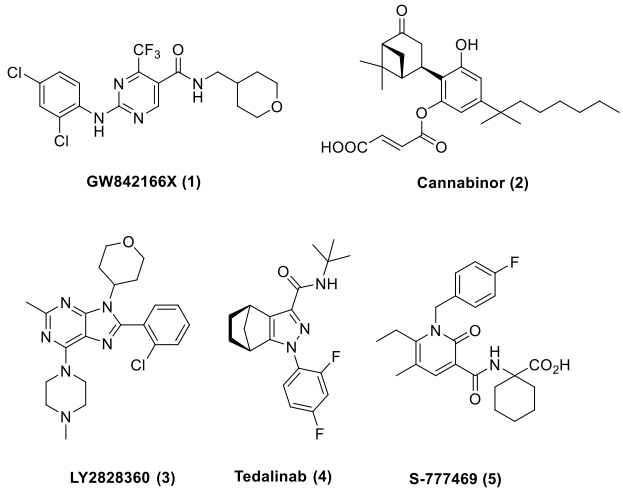


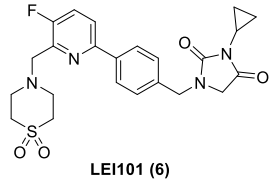
Figure 4. Structures of CB_2R-selective clinical trial candidates.

Despite compelling evidence of efficacy in preclinical settings, these ligands failed in phase II clinical trials due to a lack of efficacy (Tedalinab has likely been discontinued, because no reports from this drug candidate have appeared since 2009).[71, 111] Although the reasons for these failures are yet unclear, possible explanations may be 1) a lack of translational animal models for therapeutic efficacy, possibly due to interspecies signaling differences,[73] 2) no proof of target engagement, and/or 3) the role of CB_2R was not properly validated for the patient population studied. Hence, novel chemical tools and strategies are required to aid CB_2R target validation, engagement or modulation. Ideally, these chemical tools should have high selectivity and a well-defined mode of action.[15, 20, 75] This would then enable the translation of information from preclinical studies on non-human species to the patient.

1.5 Aims and outline of this thesis

This thesis describes the development and application of chemical tools and strategies to study the cannabinoid receptor type 2 (CB_2R). The research described in this thesis may ultimately aid in the discovery and development of novel CB_2R-based therapeutics that lack the unwanted side effects associated with activation of the CB_1R.[105]

Chapter 2 provides a comprehensive overview of the chemical tools that have been reported to study CB_2R distribution, expression levels, occupancy, internalization and pharmacology both *in vitro* and *in vivo* models. **Chapter 3** describes the development of a functional assay to study β-arrestin recruitment of CBR ligands and a step-by-step protocol.[112] This high-througput screening assay is used for compound characterization in **Chapters 4**, **5** and **6**. **Chapter 4** describes a comprehensive and extensive profiling of most widely used cannabinoid reference ligands to identify the most suitable ligand for CB_2R target validation.[75] In **Chapter 5**, a compound library based on LEI101 (**6**) (**Figure 5**),[79] an *in vivo* active CB_2R-selective agonist, is described in order to identify structure-kinetic relationships. **Chapter 6** reports on the design, synthesis and application of a LEI101-based two-step photoaffinity probe as a tool to detect endogenous CB_2R expression and target engagement in primary human immune cells. Finally, two-step photoaffinity labeling with a THC-based probe for THC protein target identification is described in **Chapter 7**. **Chapter 8** summarizes the work described in this thesis and presents future prospects and challenges in the field.



LEI101 (6)

Figure 5. Structure of LEI101

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