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Modulation of Cannabinoid Receptor Activity by Allosteric Modulators, Inverse Agonists and Receptor Binding Partners

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Modulation of Cannabinoid Receptor Activity by Allosteric Modulators, Inverse Agonists and Receptor Binding Partners

Mariam Mohamed Mahmoud, PhD

University of Connecticut, 2015

The regulation of G protein coupled receptors (GPCRs) plays a fundamental role in physiologic homeostasis. There are two cannabinoid receptors: CB1, found on neurons of the central and peripheral nervous system and regulate neuromodulatory processes and CB2, found in peripheral tissues, particularly in immune tissues. In this research, three different modulation mechanisms of the cannabinoid receptors are studied. Chapter 2 focuses on the structure-activity relationships of novel allosteric modulators from the indole-2-carboxamide class of compounds. These novel allosteric modulators were optimized for their K_B (binding affinity to the allosteric site) and their cooperativity factor α (magnitude by which affinity of orthosteric ligand is changed). Although these positive allosteric modulators enhanced orthosteric CP55, 950 agonist affinity and decreased SR141716A inverse agonist affinity to CB1 consistent with an active receptor conformation, they antagonized basal and agonist-induced G protein coupling. Chapter 3 focuses on the characterization of a group of new inverse agonists from the class of benzhydryl piperazine analogs. These compounds exhibit high nanomolar binding affinities to CB1, antagonize basal as well as agonist-induced G protein coupling and increase receptor cell surface localization, consistent with inverse agonist behavior. Docking and mutational analyses revealed SR141716A-like interactions in the CB1 binding pocket. However, this benzhydryl piperazine

scaffold is structurally distinct from first generation CB1 inverse agonists and holds promise for developing peripherally active CB1 inverse agonists with fewer psychiatric side effects. Chapter 4 focuses on the development and optimization of a novel technique using the photo-cross-linking unnatural amino acid *p*-benzoyl-L-phenylalanine (*p*Bpa) in live cells. This will elucidate the binding partners of the CB2 receptor at defined locations and time points in real-time using mass spectrometry. To this end, I determined the best detergent to solubilize CB2, the ratios of DNA to tRNA and *p*Bpa-synthetase needed, the optimal concentration of *p*Bpa in the media and the ideal UV exposure times needed for the efficient incorporation of *p*Bpa into the full-length CB2 receptor. Additionally, pilot experiments using different CB2 ligands and varying treatment times were performed and possible CB2-binding partner complexes were observed. These results will aid in the discovery of more effective and selective GPCR ligands.

**Modulation of Cannabinoid Receptor Activity by Allosteric Modulators,
Inverse Agonists and Receptor Binding Partners**

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at the

University of Connecticut

2015

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Mariam Mohammed Mahmoud

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APPROVAL PAGE

Doctor of Philosophy Dissertation

Modulation of Cannabinoid Receptor Activity by Allosteric Modulators,
Inverse Agonists and Receptor Binding Partners

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CHAPTER 1

INTRODUCTION

1.1 The G protein-coupled receptor (GPCR) superfamily

G protein-coupled receptors (GPCRs) constitute the largest family of cell surface receptors (about 900 members) (Jalink and Moolenaar, 2010). Among 35,000 human genes in the human genome, approximately 750 genes encode for GPCRs (Foord et al., 2005; Kroeze et al., 2003; Vassilatis et al., 2003; Wise et al., 2004). GPCRs facilitate a host of cellular responses and transduce signals across the biological membrane to mediate the senses of smell, sight and taste (Davies et al., 2007; Gether, 2000). They are involved in many diseases and are currently the target of about 40% of therapeutic drugs (Overington et al., 2006).

1.1.1 GPCR classification system

GPCRs are divided into 6 classes (A-F) based mainly on sequence homology and functional similarity (Figure 1.1). The largest and most studied subfamily is the rhodopsin-like class A superfamily. This subclass, which includes rhodopsins, adrenergic receptors, and cannabinoid receptors, shares highly conserved regions such as an Glu/Asp-Arg-Tyr (E/DRY) motif on the intracellular side of TM3, and an Asn-Pro-X-X-Tyr (NPXXY) motif on the intracellular side of TM7. Often within this family a cysteine residue in the carboxyl terminus is palmitoylated resulting in membrane association and causing the membrane proximal region of the carboxyl terminus to form a fourth cytoplasmic loop. It is also common for an α -helix, typically referred to as helix 8 (H8), to form at the start of the carboxyl terminus. The class B superfamily is composed of members such as the secretin receptor, calcitonin receptor, parathyroid receptor, and the glucagon receptor. This class is characterized by ligands that are mostly peptide

hormones. These receptors have large extracellular loops and a high degree of diversification in the relatively long amino terminus that often contains many well-characterized protein domains such as EGF domains (McKnight and Gordon, 1996; Otaki and Firestein, 2001; Segre and Goldring, 1993; Zendman et al., 1999). The amino terminus is also thought to directly modulate ligand binding as well as interact with chaperones and or accessory proteins such as receptor activity modifying proteins (RAMPs) (Bomberger et al., 2005). Another major GPCR family is class C represented by eight metabotropic glutamate receptors (GRM), two γ -aminobutyric acid (GABA) receptors, and a calcium sensing receptor (CASR). This GPCR family is characterized by long amino termini that can be as long as 600 amino acids (Bockaert, 2012) which play a primary role in ligand recognition. The remaining three GPCR families are relatively minor and include the class D fungal mating pheromone receptor, the class E cyclic AMP receptors, and the class F frizzled/smoothed-related receptors.

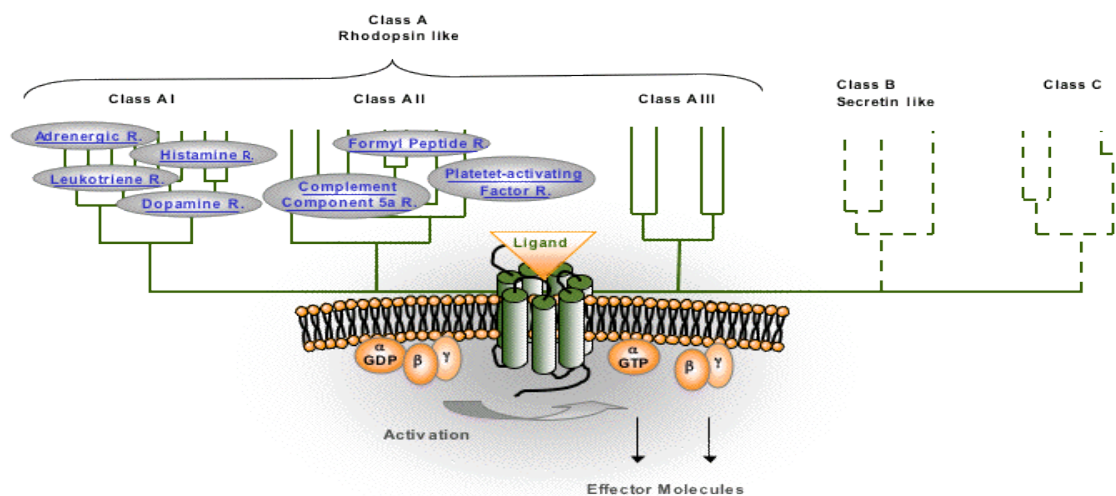


Figure 1.1 Classification of major GPCR families. The superfamily of G-protein-coupled receptors (GPCRs) is the largest single class of receptors. Despite extremely diverse roles in the

cell, all GPCRs share the structural hallmark of seven helical transmembrane domains. (modified from jenabioscience.com)

The most recent and commonly used system is the GRAFS classification system (Schioth and Fredriksson, 2005) which has been developed using phylogenetic analyses. Within this system, the GPCR superfamily is subdivided into five main families based on several common structural features indicating that the human GPCRs in the GRAFS families share a common ancestor. The GPCR subfamilies are as follows: rhodopsin (former class A), glutamate (former class C), adhesion (part of former class B), frizzled/taste2 (former class F), and secretin (part of former class B). Other former classes, D (fungal mating pheromone receptors) and E (cyclic AMP receptors), do not contain human receptors and are therefore not included in the GRAFS classification.

The older A–F class system is designed to cover all GPCRs, in both vertebrates and invertebrates. Some families in the A–F system do not exist in humans. Examples of this are classes D and E, which represent fungal pheromone receptors and cAMP receptors, family IV in class A, which is composed of invertebrate opsin receptors, and class F, which contains archaeobacterial opsins. The overall classification of the GPCRs has been hampered by the large sequence differences between mammalian and invertebrate GPCRs. The GRAFS system classifies GPCRs based on chromosomal localization and positioning in paralogous groups of the genes and this gave insight into the mechanism involved in creating the receptor genes. The different families were also analyzed for common sequence motifs and showed common descent between GPCRs of the same family (Fredriksson et al., 2003).

1.1.2 General structural features of GPCRs

Generally, GPCRs comprise seven membrane spanning α -helices (7TM) composed of predominantly hydrophobic amino acids that are connected by three extracellular loops (EC1-EC3) and three intracellular loops (IC1-IC3). The extracellular region (EC), which may be involved in ligand binding, also includes the amino terminus that can range from relatively short sequences in rhodopsin-like receptors to large extracellular domains in other classes of GPCRs, such as the hormone-binding domain in adhesion receptors (Katritch et al., 2012). The intracellular (IC) region interacts with G proteins, arrestins and other downstream effectors. It includes cytoplasmic H8 and a carboxyl terminus that may provide sites for palmitoylation (Figure 1.2a). Within the membrane bilayer, the 7 TM GPCR is arranged as a helical bundle and is stabilized by non-covalent interactions (reviewed in (Rosenbaum et al., 2009); Figure 1.2b). The bundle also contains a number of kinks, mostly induced by Pro residues. In general, sequence diversity among GPCRs is highest within the EC region which can be involved in ligand binding and undergoes small movements during activation. In contrast, the IC region, which is usually involved in binding downstream proteins including G proteins and arrestins, is more conserved within each GPCR family but is subjected to much larger conformational changes upon receptor activation than the EC regions ((Katritch et al., 2012); Figure 1.3).

The 7TM core also contains well conserved motifs that are characteristic of GPCRs. These include the E/DRY motif in TM3, the CWXP in TM6 and the NPXXY motif in TM7 (Mirzadegan et al., 2003) (Figure 1.2). Even for members of the same subfamily, such as rhodopsin and the β 2-adrenergic receptor (β 2AR) (Figure 1.3), their structural diversity still makes homology modeling challenging.

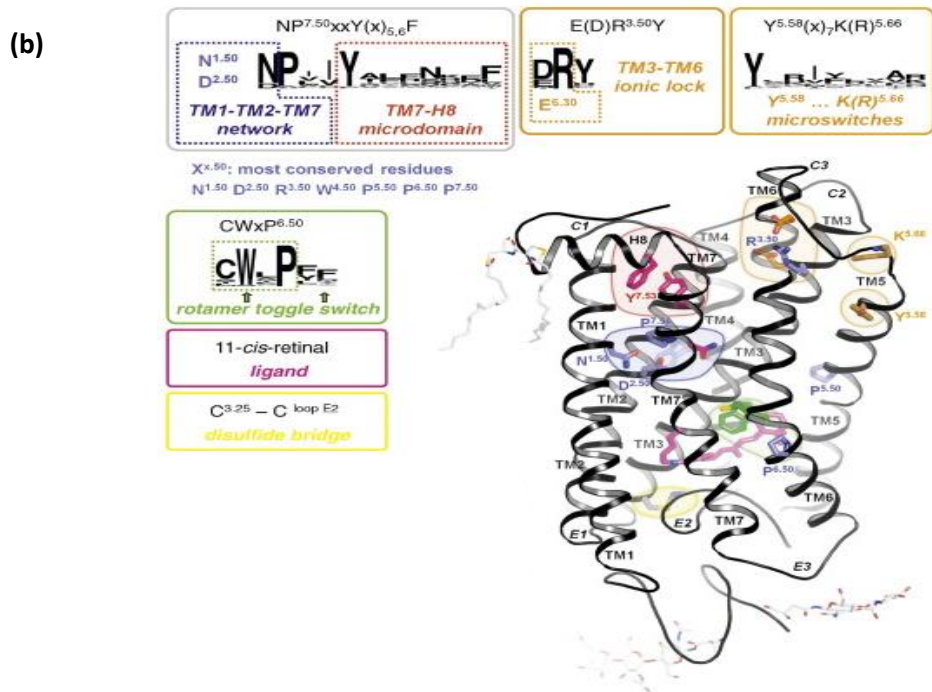
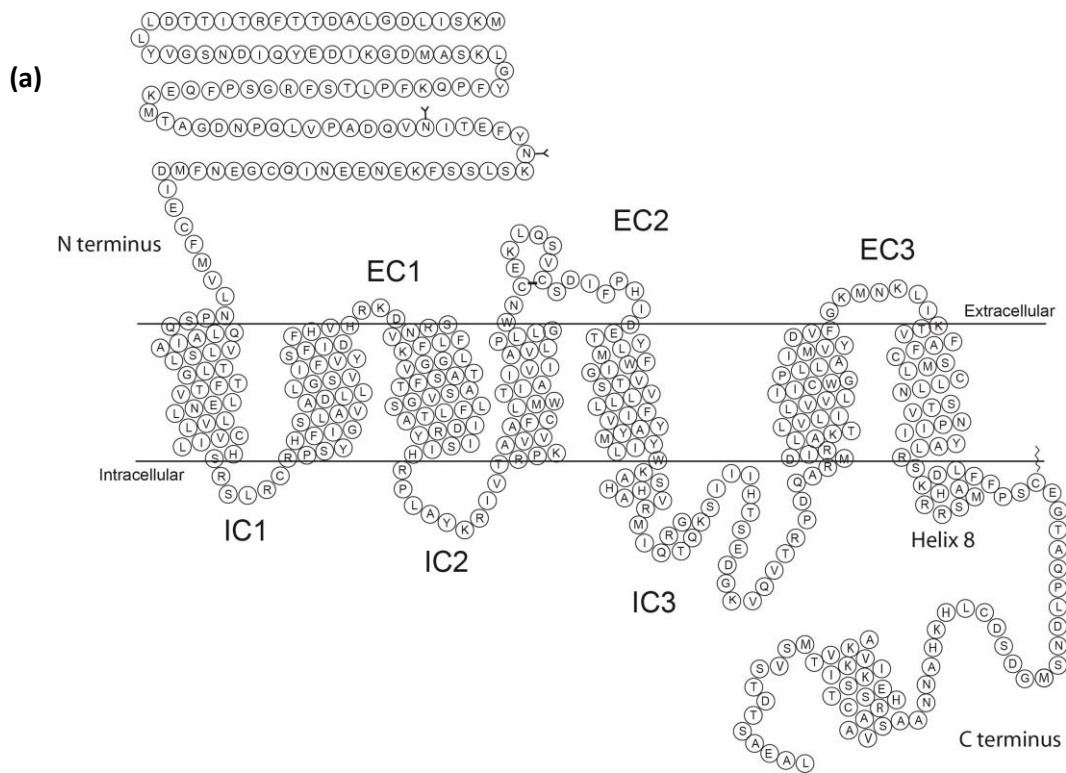


Figure 1.2 Conserved residues and functional microdomains in GPCRs. (a) Schematic 2-D diagram of the human CB1 receptor showing its amino terminus with a Y to mark glycosylated residues, extracellular (EC) loops with a disulfide bond between 2 Cys residues, heptahelical transmembrane (TM) domains, intracellular (IC) loops, helix H8 and the carboxyl terminus with a membrane palmitoylation anchor on a Cys residue. (b) Rhodopsin (PDB accession 1GZM) with bound inverse agonist 11-cis-retinal is shown as a representative GPCR. TM domains are helical in structure and exist in a bundle stabilized by various inter- and intra-molecular interactions. The cytoplasmic helix H8 follows directly after TM7 and is frequently terminated by one or two palmitoylated Cys residues (Cys 322 and Cys 323 in rhodopsin). Oligosaccharide chains are bonded to residues at the amino terminus (Asn2 and Asn15 in rhodopsin). A conserved disulfide bridge constrains the EC end of TM3 and the middle of loop EC2 (Cys110 and Cys187 in rhodopsin) is shown. The most conserved residue in each TM helix is shown in blue. According to Ballesteros-Weinstein numbering, these residues are designated x.50, where x is the TM helix number, and other residues are designated relative to the reference residue on each helix (Ballesteros and Weinstein, 1992). (Figure 1.2b and conserved motifs are modified from (Hofmann et al., 2009)).

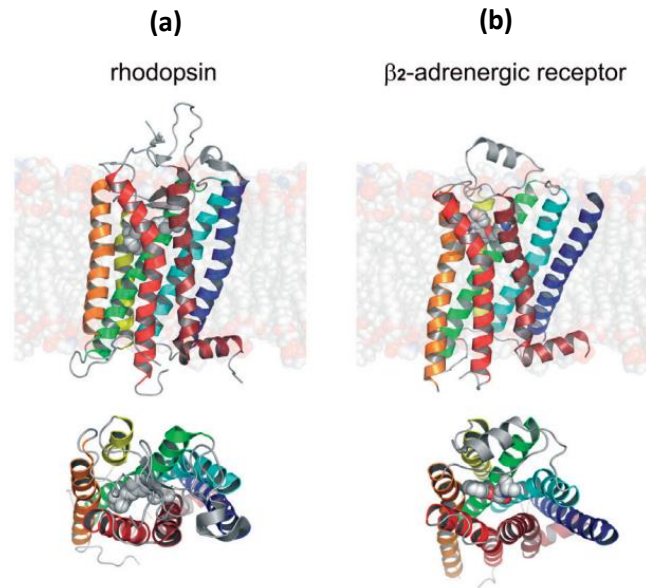


Figure 1.3 Crystal structures of (a) rhodopsin (PDB id:1F88) and (b) β 2-adrenergic receptor (PDB id: 2RH1). Top, view along the membrane plane; bottom, from the extracellular side. (modified from (Latek et al., 2012)).

1.1.3 GPCR ligands and receptor states

The molecules which bind to and regulate GPCR activation are called ligands. These ligands bind the receptor at an orthosteric site typically within the transmembrane (TM) domain. There are more than 250 “known” GPCRs for which the endogenous ligand has been identified, while receptors for which the endogenous ligand has not been identified are referred to as "orphan" receptors. The ligands that bind and activate these receptors include light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters, and vary in size from small molecules to peptides to large proteins. There are different binding domains, depending upon each receptor and its ligands, that are located in the amino terminal end or within the TM domain.

Some GPCRs are also known to have constitutive activity, i.e. activity in the absence of endogenous ligand or agonist. This observation of ligand-independent constitutive activity (R') led to the classical two-state model for receptor-G protein activation (Leff, 1995). Constitutive activity may be considered to be an equilibrium of a GPCR population including receptors in the ground state with no activity (R) and receptors in their fully activated state (R*). Agonists can still bind to constitutively active GPCRs to produce further activity. Moreover, some compounds can bind to constitutively active receptors and produce opposite responses to agonists. For example, if an agonist of a particular GPCR stimulates an increase in Ca^{++} mobilization and the constitutively active receptor has high basal Ca^{++} mobilization, some ligands are able to bind to this receptor to reduce basal Ca^{++} mobilization towards the ground state. These ligands are referred to as 'inverse agonists'. In many cases, these ligands were originally characterized as antagonists on non-constitutively active GPCRs and their inverse agonism is primarily observed when they interact with constitutively active GPCRs. The existence of constitutive activity has required a reconsideration of how we think about GPCR antagonists. Antagonists are classically considered compounds that bind to GPCRs to block binding and activation by agonists, but do not change the distribution of receptor in the active and inactive states. In fact, compounds that block both agonist and inverse agonist binding but produce no response by GPCRs are now referred to as 'neutral antagonists'. The model of GPCR activation has therefore been adapted to account for multiple activation states (De Lean et al., 1980) with distinguishing biochemical characteristics, including extent and selectivity of promiscuous G protein coupling and arrestins for signaling (Figure 1.4).

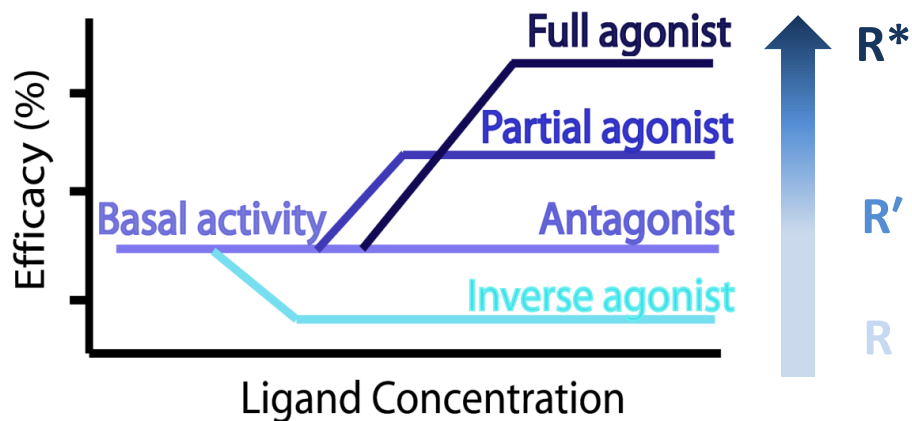


Figure 1.4 GPCR activation states. Agonists promote the active R^* state of the receptor and enhance the biological activity and signal transduction of the receptor while inverse agonists promote the inactive R form of the receptor reducing basal R' levels of signal transduction. Antagonists bind the orthosteric site competitively but do not alter the equilibrium of R and R^* and do not directly modulate signal transduction levels.

1.1.4 GPCR crystal structures: inactive and active receptor forms

The structural determination of GPCRs has been remarkably accelerated in recent years due to innovative protein engineering such as fusion proteins and chimeric proteins (Chun et al., 2012; Kobilka and Schertler, 2008; Tate, 2012), novel crystallization techniques (Caffrey et al., 2012), and crystallography methods (Moukhametzianov et al., 2008; Riek et al., 2005). Despite these advances, only a few GPCRs are crystallized in the active conformation (Choe et al., 2011; Deupi et al., 2012; Singhal et al., 2013; Standfuss et al., 2011; White et al., 2012) and only one structure of an active GPCR in complex with a G protein exists (Rasmussen et al., 2011).

The structures of the different GPCRs crystallized to date exhibit limited differences even though they were crystallized differently, i.e. in different crystal packing orientations, with different antagonists and inverse agonists bound and also in two different activation states, either activated (agonist bound) or inactivated (antagonist or inverse-agonist bound) (Rasmussen et al., 2011). The first X-ray crystal structure of any GPCR was that of ground-state rhodopsin (Palczewski et al., 2000). Refinement of crystallization conditions yielded higher resolution data, extending the rhodopsin model to 2.2 Å, the highest resolution of all rhodopsin structures determined to date (Okada et al., 2004). The first structure of a diffusible ligand-responsive GPCR resulted from the work of Kobilka and Stevens, who reported the crystal structure of a human β 2AR-T4 lysozyme fusion protein bound to its partial inverse agonist carazolol at 2.4 Å resolution (Cherezov et al., 2007; Rosenbaum et al., 2007). The highest resolution structure of any GPCR with well-defined Na^+ ions and water molecules is the 1.8 Å resolution structure of the engineered human A2A adenosine receptor with its third intracellular loop replaced with apocytochrome b(562)RIL (Liu et al., 2012). Water molecules play critical roles in the GPCR activation process by stabilizing intramolecular interactions (Garczarek and Gerwert, 2006; Osyczka et al., 2005; Wikstrom et al., 2003). Along with amino acid side chains, water molecules have been observed to form a signal transmission network extending from the ligand binding site to the cytoplasmic surface (Angel et al., 2009; Orban et al., 2010).

Subsequent improvements in resolution of the initial rhodopsin and β 2AR structures have provided templates for the creation of homology models (Filipek et al., 2003; Zhang et al., 2006). To date, there are 15 unique GPCR structures deposited in the Protein Data Bank (PDB) involving a variety of ligands, activation states and point mutations. These structures all belong to the rhodopsin-like family of GPCRs and include the amine receptors (histamine H1R,

dopamine D3R, muscarinic M2R and M3R, β 1- and β 2-adrenergic receptors), opsins (rhodopsin), adenosine A2AR and lipid S1P1R receptors. In addition, the neurotensin receptor has also been crystallized.

Rhodopsin is one of the most extensively studied members of the GPCR family. It works as a photoreceptor pigment protein in retinal rod cells where it senses light via covalently bound 11-*cis*-retinal that in the dark acts as a potent inverse agonist and suppresses the activity of the receptor (Li et al., 2004; Okada et al., 2004; Palczewski et al., 2000) (Figure 1.2b). Upon the absorption of light, the inverse agonist 11-*cis*-retinal isomerizes to the full agonist all-*trans*-retinal which in turn activates the receptor. The two highly conserved E/DRY and NPXXY motifs (Mirzadegan et al., 2003) are part of functional microdomains that constrain the 7TM helix bundle in this compact inactive conformation. In rhodopsin, Arg135 of the E/DRY motif on TM3 plays a key role in tethering TM3 to TM6. It forms a hydrogen bonding network with Glu134 on TM3 and Glu247 on TM6; in rhodopsin the network also includes Thr251 (Figure 1.2b). This TM3-TM6 ionic lock revolving around Arg135 is also proposed for other class A GPCRs (Ballesteros et al., 2001; Dror et al., 2009). The NPXXY motif on TM7-H8 constrains both TM7 with TM1 and TM2 and also TM7 with H8. The latter is enabled by an electrostatic interaction between the aromatic side chains of Tyr306 on TM7 and Phe313 on H8. Asn302 at the N-terminal part of the NPXXY motif and the two residues Ala299 and Ser298 in the preceding helix turn are involved in a hydrogen bonding network with Asn55 on TM1 and Asp83 on TM2 and form the TM1-TM2-TM7 network (Figure 1.2b). TM6 is interrupted due to a proline kink induced by Pro267, which is part of the conserved CWXXP motif, which is the basis of the rotamer toggle switch hypothesis (Shi et al., 2002). In GPCR activation, the rotamer states of Trp265 and Phe269 are predicted to change at the same time during receptor activation,

thus providing a link between the CWXXP motif and motion of the cytoplasmic part of TM6 (Shi et al., 2002). In the case of rhodopsin, the phenyl side chain of Phe269 is missing, but the β -ionone ring of the inverse agonist 11-*cis*-retinal is in direct contact with Trp265. It is assumed that this β -ionone ring of retinal, together with Ala269, plays the role of the missing Phe269 (Crocker et al., 2006).

Upon the absorption of light, the inverse agonist 11-*cis*-retinal isomerizes to the full agonist, all-*trans*-retinal, which in turn activates the receptor. The crystal structure of the active ligand-free opsin (Park et al., 2008) alone and in complex with a short G protein peptide fragment (Scheerer et al., 2008b), were solved at 2.9 Å and 3.2 Å, respectively, in 2008. In Figure 1.5 (Hofmann et al., 2009), the structures of rhodopsin, opsin and opsin in complex with a G α -derived carboxyl-terminal peptide directly show that inactive and active GPCR conformations differ at their cytoplasmic side (Park et al., 2008; Scheerer et al., 2008b). The cytoplasmic part of TM6 is tilted outwards from the helix bundle by 6-7 Å. TM5 is extended by 1.5-2.5 helix turns and moves 2-3 Å towards TM6. This helix rearrangement results in the formation of an opening/deep crevice at the cytoplasmic side of the receptor, which contains the conserved Arg135 of the E/DRY motif that is involved in the TM3-TM6 ionic lock (Park et al., 2008; Scheerer et al., 2008b). In the active opsin conformation (Figure 1.5b), Tyr223 forms a hydrogen bond to Arg135 and replaces Glu134, thereby stabilizing the cytoplasmic TM3-TM5 interface. Glu247, which loses its contact with Arg135, now forms a salt bridge with Lys231. These changes in the TM3-TM6 ionic lock happen in parallel with changes in the TM7-H8 constraint of the NPXXY motif. Tyr306 is released from Phe313 and rotated into the helix bundle shown below Arg135 to keep TM6 in the outward position. The crevice that is formed in the opsin receptor allows the binding of the G α

carboxyl terminus (Figure 1.5c). Two backbone carbonyl oxygens of the $G\alpha$ -derived peptide form a hydrogen bonding network to Arg135 and Gln312, respectively (Figure 1.5c).

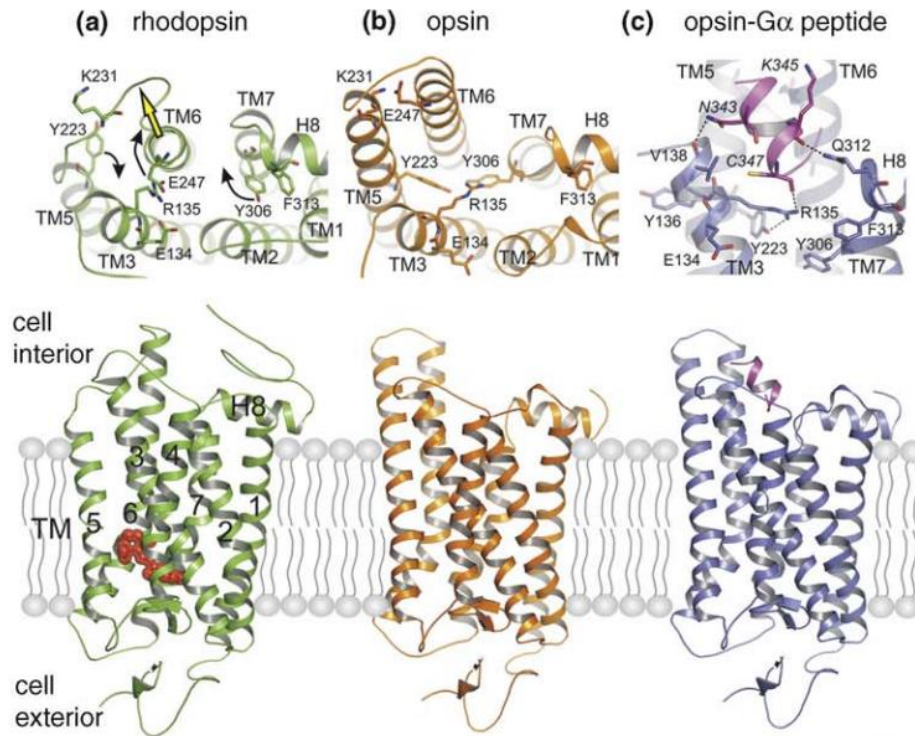


Figure 1.5 Crystal structures of inactive rhodopsin and active opsin conformations.

Cytoplasmic (top row) and lateral (bottom row) views of (a) inactive rhodopsin ground state (shown in green, PDB accession 1U19) and (b) ligand-free opsin (shown in orange, PDB accession 3CAP). Residues of the TM3-TM6 ionic lock (Glu134, Arg135, Glu247) and the TM7-H8 microdomain (Tyr306, Phe313) are shown as stick models. Side chain movements upon receptor activation are indicated by black arrows, whereas helix movement is indicated by a yellow arrow. (c) Lateral view and close-up of the cytoplasmic domain of opsin in complex with a C-terminal peptide derive from the transducin $G\alpha$ -subunit. The peptide binds into the cytoplasmic crevice of opsin opened by movement of TM5 and TM6. (modified from (Hofmann et al., 2009)).

The only crystal structure of an active GPCR/G-protein complex is that of the agonist-occupied monomeric β 2AR with a nucleotide-free Gs heterotrimer (Figure 1.6) (Rasmussen et al., 2011). The β 2AR has long been a model system for GPCR signaling and the cooperative interactions between the receptor and Gs observed in ligand binding assays were the basis and foundation of the receptor ternary complex model of GPCR activation (De Lean et al., 1980; Ross et al., 1977). Figure 1.6 compares the structures of the agonist-bound receptor in the β 2AR-Gs complex (Rasmussen et al., 2011) and the inactive carazolol-bound β 2AR (Rosenbaum et al., 2007). The largest difference between the inactive and active structures is a 14 Å outward movement of TM6 when measured at the C α carbon of Glu268. There is also a smaller outward movement and extension of the cytoplasmic end of the TM5 helix by 7 residues. Another notable difference is the second IC loop, which forms an extended loop in the inactive β 2AR structure and an α -helix in the β 2AR-Gs complex.

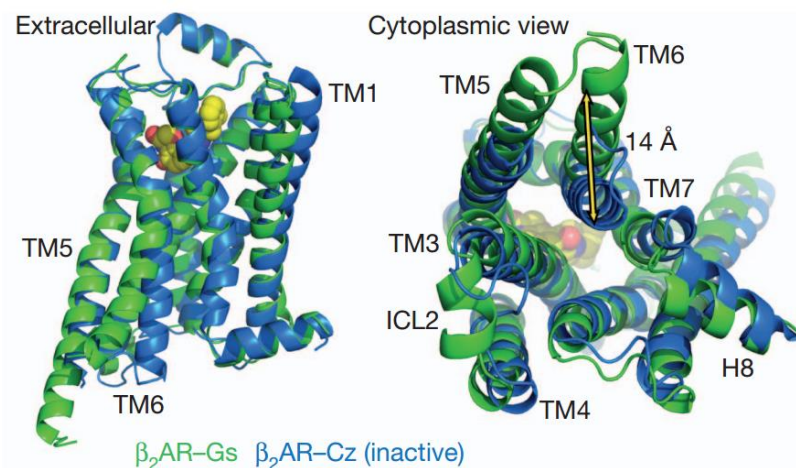


Figure 1.6 Comparison of active and inactive β 2-adrenergic receptor structures. Side and cytoplasmic views of the β 2-adrenergic-Gs structure (green) compared to the inactive carazolol-bound β 2-adrenergic receptor structure (blue). Significant structural changes are seen for the

intracellular domains of TM5 and TM6. TM5 is extended by two helical turns whereas TM6 is moved outward by 14 Å as measured at the α -carbons of Glu268 (yellow arrow) in the two structures. (modified from (Rasmussen et al., 2011)).

The principal interactions between the β 2AR and Gs involve the amino- and carboxyl-terminal α -helices of the Gs. The β 2AR is stabilized by extensive interactions with $G\alpha_s$ Ras. The α 5-helix of Gas docks into a cavity formed on the intracellular side of the receptor by the opening of TM helices 5 and 6. Within the TM core, the interactions are primarily non-polar. An exception involves packing of Tyr391 of the α 5-helix against Arg131 of the conserved E/DRY sequence in TM3. Arg131 also packs against Tyr326 of the conserved NPXXY sequence in TM7. As α 5-helix exits the receptor it forms a network of polar interactions with TM5 and TM3. Receptor residues Thr68 and Asp130 interact with IC2 of the β 2AR via Tyr141, positioning the helix so that Phe139 of the receptor docks into a hydrophobic pocket on the G protein surface, thereby structurally linking receptor-G protein interactions with the highly conserved E/DRY motif of the β 2AR (Rasmussen et al., 2011).

The structural characterization of GPCRs has been an ongoing challenge because of their highly hydrophobic transmembrane domains, their large highly flexible IC and EC domains (including the amino and carboxyl termini), their low natural abundance, and their poor structural stability in native tissues. Apart from rhodopsin, structures of active GPCRs are obtained from heavily modified proteins with truncated termini and loops, fusion domains and co-crystallized antibodies (Maeda et al., 2014). Further structures from additional receptors with minimal modifications and in complex with other types of G proteins, kinases, and arrestins are therefore

needed for a more comprehensive understanding of the molecular mechanisms of GPCR signaling.

1.1.5 GPCRs in drug discovery

The medicinal importance of GPCRs can be partially appreciated by considering their location and function within the cell. The physical location of GPCRs spanning the cell's plasma membrane connecting extra- and intracellular environments, provides a direct mechanism for the transduction of extracellular messages into intracellular responses. Emerging knowledge of GPCR structural data has provided critical information on the molecular nature of drug interaction with GPCRs, and also provides approaches that allow for crystallization of other GPCRs, revealing a more rational method for drug development. This structural picture provides information on the physical properties of the ligand binding site(s) and helps to design pharmacophore models of the ligand when docked. This lays the foundation for screening structural families of molecules with predictable chemical characteristics as well as optimal functional properties to modulate the molecular target (reviewed in (Eglen and Reisine, 2011)).

Common biological actions attributed to GPCRs include the following: modulation of neuronal firing, regulation of ion transport across the plasma membrane and within intracellular organelles, modulation of homeostasis, control of cell division/proliferation, and modification of cell morphology. When any of these fundamental processes go awry, the results can lead to acute or chronic human disease, a partial listing of which includes cardiovascular disease (β 1-adrenergic receptor) (Drake et al., 2006), asthma (β 2-adrenergic receptor) (Kawakami et al., 2004), and strokes and cerebral hypoperfusion (A2a-adenosine receptor) (Chen et al., 2007; Duan et al., 2009). Other disease states directly linked to mutations in GPCRs include retinitis

pigmentosa (rhodopsin), female infertility (follicle-stimulating hormone receptor), nephrogenic diabetes insipidus (vasopressin receptor), familial exudative vitreoretinopathy (frizzled receptors), and dominant and recessive obesity (melanocortin receptors) (reviewed in (Insel et al., 2007)).

Due to this critical role that GPCRs play in cell-cell communication and as causal agents in disease, they are a major target for the development of numerous drugs with an estimated 30 to 50% of marketed drugs acting directly on GPCRs or through GPCR-associated mechanisms (Flower, 1999; Robas et al., 2003). As of 2008, 5 of the top 15 generic drugs and 7 (Milligan, 2009) of the top 15 prescription drugs targeted GPCRs (McGrath et al., 2010). Many of the top brand name prescription drugs that target GPCRs include Claritin® (allergies), Prozac® (depression), Vasotec® (hypertension), and Ventolin® (bronchospasm).

1.1.6 Allosteric modulators of GPCRs

Allosteric modulators are compounds that bind to an allosteric site that is topographically distinct from the orthosteric site. There are three types of allosteric modulators (Figure 1.7). Positive allosteric modulators (PAMs) are allosteric ligands that bind to a site topographically distinct from the orthosteric site and enhance the affinity (cooperativity factor- α) and/or efficacy (modulation factor- β) of the orthosteric agonist. Negative allosteric modulators (NAMs) are allosteric ligands that decrease the affinity (cooperativity factor- α) and/or efficacy (modulation factor- β) of an orthosteric compound. Allosteric ligands that have no effect on the affinity and/or efficacy mediated by the orthosteric compounds are termed neutral or silent allosteric modulators (SAMs). The cooperativity factor- α is a value that quantifies the magnitude by which the affinity of one ligand is changed by the other ligand when both are bound to the receptor simultaneously

to form a ternary complex. If α is greater than 1.0, the modulator increases orthosteric ligand binding (PAM). If α is less than 1.0, the modulator reduces orthosteric ligand binding affinity (NAM). When α is 1.0, the modulator does not affect the binding of the orthosteric ligand (SAM) (reviewed in (Christopoulos and Kenakin, 2002)).

Allosteric modulators have several advantages over traditional orthosteric ligands. For example, they can inhibit or potentiate orthosteric ligand binding affinity and/or modulate their signaling efficacy, whereas the orthosteric ligands only bind and act competitively. In addition, allosteric modulators can be designed to achieve high selectivity and pharmacokinetic properties (Conn et al., 2009a; Gao and Jacobson, 2006; Kenakin and Miller, 2010). Because the orthosteric binding site is conserved across most subtypes of a given GPCR family, selectivity using orthosteric ligands for a particular receptor subtype within one family is hard to achieve. On the other hand, selective allosteric modulators can be developed successfully by targeting the less conserved allosteric sites. For example, several novel selective allosteric potentiators for specific muscarinic acetylcholine receptor (mAChR) subtypes have been identified. For instance, TBPB and 77-LH-28-1 have been reported as highly selective agonists of M1 relative to other mAChR subtypes. Both of these compounds are systemically active and are proving to be useful for in vivo studies of M1 activation. TBPB selectively activates M1 in cell lines and has no agonist activity at any other mAChR subtypes, while 77-LH-28-1 is also highly selective for M1 relative to other mAChR subtypes but does have weak agonist activity at M3 (reviewed in (Conn et al., 2009b)).

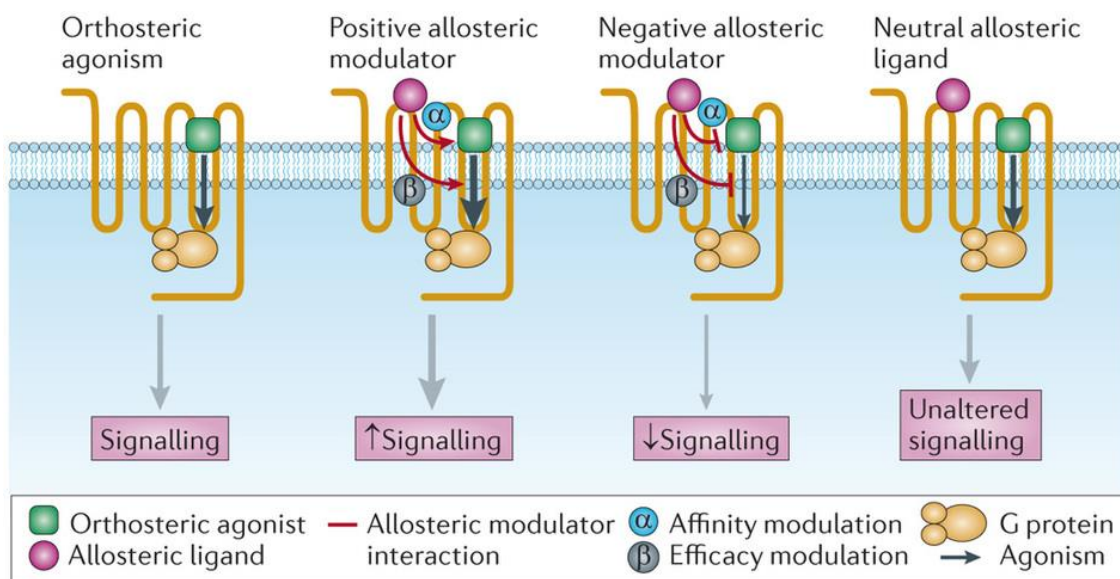


Figure 1.7 Effect of allosteric modulators on orthosteric ligands. Orthosteric agonists bind to the GPCR, which induces a conformational change that results in the activation of downstream signaling. Positive allosteric modulators (PAMs) are allosteric ligands that bind to a topographically distinct site to the orthosteric agonist and enhance the affinity (cooperativity factor- α) and/or efficacy (modulation factor- β) of the orthosteric agonist. Negative allosteric modulators (NAMs) are allosteric ligands that decrease the affinity (cooperativity factor- α) and/or efficacy (modulation factor- β) of the orthosteric agonist. Allosteric ligands that have no effect on the affinity and/or efficacy mediated by the orthosteric agonist are termed neutral allosteric ligands. The red arrows denote the allosteric interaction of the modulator with the orthosteric ligand, and the black arrows denote the allosteric interaction between the ligand binding sites and the effector binding site within the GPCR, resulting in downstream activation of signaling pathways (this is known as orthosteric agonism). (modified from (Wootten et al., 2013)).

Another advantage is that allosteric modulators with limited positive or negative cooperativity would impose a 'ceiling' on the magnitude of their allosteric effect. This property allows for a high degree of titratability of the pharmacological effect, which means that large doses of allosteric modulators can be administered with a lower propensity towards target-based toxicity than orthosteric agonists or antagonists. Moreover, limited cooperativity modulators introduce a new level of pharmacological responsiveness, whereby they can allow for a subtle re-setting of endogenous ligand activity.

Furthermore, there is growing evidence showing that some allosteric modulators mediate receptor activation in their own right in addition to modulating orthosteric ligand pharmacology (Knudsen et al., 2007; Zhang et al., 2005). This is known as allosteric agonism. For instance, McN-A-343 and AC-42 inhibited the binding of N-methylscopolamine to rat M2 and human M1 mAChRs and played a partial agonist role for the receptors in the absence of orthosteric ligand (Langmead et al., 2006; Valant et al., 2008).

Cinacalcet (Sensipar™) was the first allosteric GPCR modulator to gain FDA marketing approval (Jensen and Brauner-Osborne, 2007). Cinacalcet is a PAM of the Ca^{++} sensing receptor (CaR), a member of the type C family of GPCRs. Ca^{++} binds to the amino-terminus of CaR and a critical role of this receptor is to regulate circulating Ca^{++} levels to maintain control of parathyroid hormone (PTH) release and levels. Cinacalcet is a small molecule that binds to the transmembrane region of the receptor to alter conformation to enhance affinity for Ca^{++} . The compound is therapeutically useful because in kidney disease, hypocalcemia results in excessive PTH which causes bone fractures, pain and cardiovascular disorders. Cinacalcet increases sensitivity of CaR to Ca^{++} to suppress excess PTH release despite hypocalcaemia. Thus, Cinacalcet is an allosteric modulator that binds to the transmembrane regions and the

hydrophobic pocket of the CaR distal to the orthosteric binding domains in the N-terminus (Jensen and Brauner-Osborne, 2007).

The approval of Cinacalcet was followed by marketing approval from the FDA for Maraviroc (Celzentry™) for treating HIV disease (reviewed in (Wood and Armour, 2005)). Maraviroc is an allosteric inhibitor or NAM of the CCR5 receptor. Maraviroc was shown to be an allosteric inhibitor by its ability to differentially affect the binding of different chemokines to the receptors. Its therapeutic mechanism of action is believed to involve preventing HIV entry into immune cells via the CCR5 receptor.

1.1.7 Functional selectivity of GPCRs

Recent pharmacological studies have shown that GPCRs adopt multiple conformations that can lead to different downstream signaling events. These different conformations are dependent on the type of ligand bound, causing ligand-biased signal transduction (Kenakin, 2007) and functional selectivity (Kenakin and Miller, 2010). This concept is based on the idea that GPCRs can couple to different G proteins, activate selective pathways (either G protein or β -arrestin) and ultimately activate multiple downstream signaling pathways (Figure 1.8), and selectively engage different kinases that can direct phosphorylation at different sites. Evidence has emerged that in addition to β -arrestin's role in cellular internalization, it can also act as a signaling protein, recruiting its own subset of signaling molecules (Violin and Lefkowitz, 2007). Thus GPCR function can be mediated via G protein-dependent mechanisms or through G protein-independent mechanisms such as those involving β -arrestin (Violin and Lefkowitz, 2007). Biased agonism thus provides pathway-selective drugs that can have the potential to separate therapeutic drug effects from their unwanted negative side effects.

One of the early examples of biased agonism was that of serotonin type 2A and 2C receptors (5-HT_{2A/2C}) (Berg and Clarke, 2009; Berg et al., 1998). They observed that different agonists could preferentially activate either phospholipase C-mediated inositol phosphate (IP) accumulation or phospholipase A2-mediated arachidonic acid (AA) release. This was determined by measuring the maximal response (E_{\max}) of each agonist and was found to be different for the two pathways measured, whereas the relative potency remained unchanged. Consequently, they found that the agonists lysergic acid diethylamide (LSD) and bufotenin favored AA release while 3-trifluoromethylphenylpiperazine (TFMPP) and quipazine preferentially activated the IP pathway (Berg and Clarke, 2009; Berg et al., 1998).

An increasing number of studies have also identified “ β -arrestin-biased” synthetic ligands for the Angiotensin 1A receptor (Wei et al., 2003) and the β 2AR (Drake et al., 2008; Wisler et al., 2007), and the parathyroid hormone (PTH) receptor (Gesty-Palmer et al., 2006). As an example, the PTH receptor plays an important role in regulating the continuous process of bone formation and resorption, with recombinant human PTH 1-34 (Forteo®) currently approved for the treatment of osteoporosis. Previous studies have shown that β -arrestin 2 plays a role in regulating PTH-receptor-mediated signaling (Castro et al., 2002; Ferrari et al., 1999; Ferrari and Bisello, 2001; Sneddon and Friedman, 2007; Vilaradaga et al., 2001; Vilaradaga et al., 2002), with effects on bone formation and resorption (Bouxsein et al., 2005; Ferrari et al., 2005), as well as related gene expression (Bianchi and Ferrari, 2009; Pierroz et al., 2009). Furthermore, studies have shown that the PTH receptor can stimulate both G protein- and β -arrestin-mediated signals, and that these signals can be selectively engaged by both G protein- and β -arrestin-biased ligands (Bisello et al., 2002; Gesty-Palmer et al., 2006). Most recently, it has been shown that the β -arrestin-biased ligand (D-Trp12,Tyr34)-PTH(7–34), which simultaneously stimulates receptor-

mediated β -arrestin signaling and blocks G protein signaling, promotes anabolic bone formation in the absence of bone resorption, and that this activity is abolished in β -arrestin 2 knockout mice (Gesty-Palmer et al., 2009). These studies have thus identified a novel β -arrestin-mediated pathway and a unique β -arrestin-biased ligand that positively affects anabolic bone formation. Targeting of this newly identified mechanism of action could represent a novel therapeutic strategy for the treatment of osteoporosis. Such potential has also been highlighted for other GPCRs, notably β_2 AR for the treatment of heart failure (Galandrin and Bouvier, 2006; Wisler et al., 2007) and for GPR109A (the target for niacin) for the treatment of dyslipidemia (Richman et al., 2007; Walters et al., 2009). Even more recently, evidence has emerged for the functional selectivity of certain allosteric modulators. For example, 1-(4-ethoxyphenyl)-5-methoxy-2-methylindole-3-carboxylic acid, an allosteric modulator of the chemoattractant receptor-homologous molecule on T helper 2 cells (CRTH2), is inactive against prostaglandin D₂-induced G-protein-linked pathways but is a potent antagonist of G-protein-independent, β -arrestin coupling to the same receptor (Mathiesen et al., 2005). In another recent study, performed on native metabotropic glutamate receptor 5 (mGluR5) expressed in cultured rat cortical astrocytes, the allosteric modulator, CPPHA, had different effects on calcium mobilization and ERK1/2 phosphorylation, even though both pathways were linked to the endogenous mGluR5 receptor (Zhang et al., 2005). The ability to modulate selected signaling pathways that are associated with a given GPCR introduces an additional opportunity for fine-tuning intracellular signaling with allosteric modulators. Many more examples of β -arrestin biased ligands that possess therapeutic potential at GPCRs have been described and reviewed (Whalen et al., 2011).

The idea that different kinases can be selectively engaged to direct phosphorylation at different sites based on the ligand bound or selectively activate certain signaling pathways is an avenue of

receptor regulation about which little is known. This notion has been supported by recent studies on the β 2AR and CXCR4R receptor where receptor phosphorylation by different members of the GRK family can mediate different signaling outcomes (Busillo et al., 2010; Violin et al., 2006). Furthermore, studies on the M3-muscarinic receptor have demonstrated that phosphorylation of this receptor subtype by protein kinase CK1 α regulates receptor signaling to the phospholipase C and ERK pathways (Budd et al., 2001; Luo et al., 2008), whereas phosphorylation mediated by protein kinase CK2 has no impact on ERK/phospholipase C but rather regulates the coupling of the receptor to the JNK pathway (Torrecilla et al., 2007). These studies suggest that one mechanism by which receptors might direct signaling to one pathway (i.e. JNK) in preference to another (i.e. ERK) might be via specific phosphorylation profiles of the receptor. By adopting a specific “phosphorylation signature,” a receptor could favor coupling to a particular pathway. In this way, the phosphorylation profile of a receptor could act as a “bar code” that encodes a particular signaling outcome (Kim et al., 2005; Ren et al., 2005; Tobin et al., 2008; Zidar et al., 2009). This suggests that one mechanism underlying functional selectivity may be centered on the capacity of ligands to promote receptor phosphorylation at specific sites (Butcher et al., 2011) and consequently recruit either G proteins or arrestin to mediate their signaling.

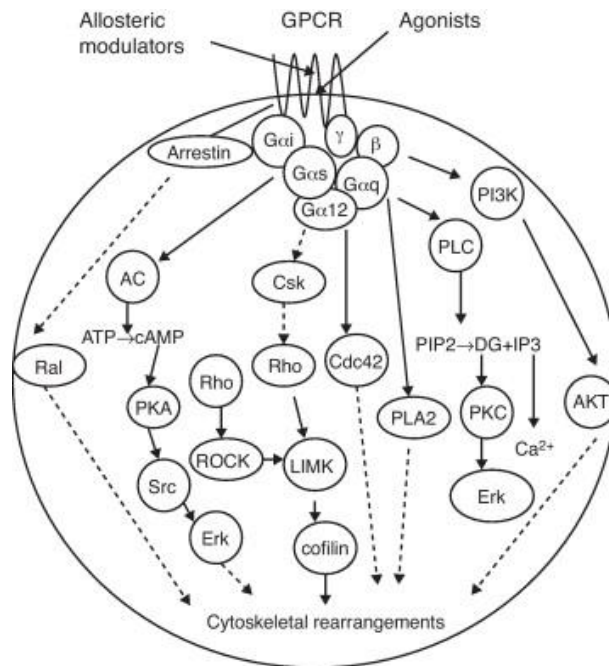


Figure 1.8 A generalized model of the signaling of a GPCR. GPCRs can activate different G protein families (i.e. Gi/o, Gs, and Gq). Activated G proteins result in the inhibition or activation of an array of effector pathways including adenylyl cyclase (AC), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways. Subsequent activation of the MAPK and PI3K pathways results in the phosphorylation of extracellular signal-regulated kinases (ERKs) and protein kinase B (PKB, also known as Akt), respectively. G protein independent signaling by arrestins is also possible. (modified from (Gao and Jacobson, 2013)).

1.1.8 GPCR biosynthesis and cell surface trafficking

The biosynthesis of GPCRs begins at the endoplasmic reticulum (ER), where they are synthesized, folded and assembled. Properly folded receptors are transported from the ER through the Golgi complex to the plasma membrane by passing a quality control process and undergoing post-translational modifications (e.g. glycosylation, methylation, and

palmitoylation). The first step in ER assembly is ER-membrane insertion. Some GPCRs possess cleavable signal peptides in their amino termini and these sequences help target the receptors for insertion into the ER membrane. However, the majority (about 90%) of GPCRs, especially those of the rhodopsin-like family of receptors, do not contain a signal peptide (Andersson et al., 2003; Wallin and von Heijne, 1995). For such receptors, the TM domains are believed to function as a reverse signal anchor sequence to initiate the protein's assembly process in the ER membrane (Friedlander and Blobel, 1985). Signal sequences have been found present in GPCR subfamilies that have large amino-termini, i.e. receptors with signal peptide sequences likely have an amino terminus that contributes to ligand binding, and which therefore, comprises a stable fold that would otherwise be an impediment to the translocation of the receptor (Schulein et al., 2012). Membrane translocation of the extracellular amino terminus across the ER membrane can take place either co- or post-translationally, as has been suggested for opsin (Kanner et al., 2002), possibly in a C to N-terminal direction (Nilsson et al., 2000). Several factors including the hydrophobicity and the length of the amino terminus play a role in its membrane translocation efficiency. Statistical analyses have shown that GPCRs without a signal peptide tend to have shorter amino termini (40 amino acids on average) than GPCRs with signal sequences (200 amino acids) (Wallin and von Heijne, 1995). There are, however, exceptions to this rule. For example, the human cannabinoid receptor 1 (CB1) has an amino terminus of 116 amino acids and lacks a cleavable signal peptide. This receptor shows very poor ER translocation and it was shown that introducing a signal sequence or shortening its amino terminus greatly improved its stability and cell surface expression (Andersson et al., 2003).

Evidence has emerged that several GPCRs can exist as dimers (hetero- or homo-dimers) or higher ordered oligomers (Breitwieser, 2004; Franco et al., 2003; Milligan et al., 2003) and that

this dimerization plays a role in the expression and function of the GPCRs. Interestingly, the dimerization of many GPCRs was found to happen as early as the ER and that this dimerization was key for cell surface expression (Herrick-Davis et al., 2006; Karpa et al., 2000; Kaykas et al., 2004; Margeta-Mitrovic et al., 2000). For example, the homodimerization of the β 2AR was found to be a prerequisite for the targeting of the receptor to the cell surface (Salahpour et al., 2004). A possible explanation for this prerequisite requirement with some GPCRs was that the formation of dimers masks an ER retention signal that exists within the carboxyl terminus of the receptor and this would then allow trafficking of the receptor to the cell surface (Margeta-Mitrovic et al., 2000).

The appropriate localization of a receptor to the cell surface is essential to the function of a GPCR because it makes the receptor accessible to requisite ligands and signal transduction machinery. This targeted cell-surface is coordinated by many regulatory factors (reviewed in (Wu, 2012)). First, GPCR export to the cell surface is regulated by multiple proteins, such as receptor activity modifying proteins (RAMPs), ER chaperones, and accessory proteins. These proteins may stabilize receptor conformation, facilitate receptor maturation, and promote receptor delivery to the plasma membrane. Second, recent studies have indicated that the exit of GPCRs from the ER may be directed by specific motifs embedded within the receptors. Third, post-translational modifications, such as N-linked glycosylation, have long been proven to be involved in the delivery of some GPCRs to the cell surface. Fourth, GPCR cell-surface targeting depends on the microtubule networks and GPCRs may directly interact with tubulin to control their cell-surface movement. The functional importance of GPCR localization is emphasized by diseases that ensue from receptor mislocalization such as retinitis pigmentosa (RP) (Humphries et al., 1992), where there is progressive photoreceptor degeneration and eventual retinal

dysfunction due to intracellular accumulation of mutant rhodopsin (reviewed in (Dejneka and Bennett, 2001)), and X-linked nephrogenic diabetes insipidus (NDI), which is caused by an improper response of the kidney to the antidiuretic hormone arginine vasopressin. This leads to a decrease in the ability of the kidney to concentrate urine by removing free water (Oksche and Rosenthal, 1998) and results from intracellular accumulation of the V2 vasopressin receptor.

1.1.9 GPCR lifecycle

As soon as the GPCR is at the cell surface, it can be activated by ligands that include light-sensitive compounds, odors, neurotransmitters, ions, chemokines, nucleotides, hormones, and growth factors. The classical view of the activation of GPCRs is that upon ligand binding, a conformational change is induced in the structure of the GPCR which leads to the activation of their respective heterotrimeric guanine-nucleotide binding protein (G protein) by enabling a GTP for GDP exchange on the α -subunit of the coupled G protein. These activated G proteins can, in turn, stimulate various downstream signaling pathways or effector molecules such as adenylate cyclase before the intrinsic GTPase activity of the α -subunit hydrolyzes the bound GTP to GDP and inactivates the G protein. Upon prolonged exposure to ligand, most GPCRs show a rapid loss of responsiveness (desensitization). Their signaling is terminated by phosphorylation which typically occurs on serine and threonine residues within intracellular domains, typically IC3 or the carboxyl terminus. This phosphorylation plays an important role in receptor regulation and function. As was first described for rhodopsin, phosphorylation is a crucial mechanism in turning off receptor signaling. The phosphorylation that leads to receptor uncoupling from G protein is usually catalyzed by members of the GPCR kinase (GRK) family. This phosphorylation modifies the intracellular face of the receptor and leads to arrestin recruitment and consequent receptor desensitization and internalization by sterically blocking the receptors from coupling to G

proteins (Lefkowitz, 1998). Phosphorylation of GPCRs with protein kinase C (PKC) and protein kinase A (PKA) has also been reported (Garcia et al., 1998; Lefkowitz et al., 2002; Smith, 1994; Smith and Goldin, 1992).

Fundamental to the process of receptor internalization are 2 families of proteins, the GRKs and the β -arrestins, both of which were initially identified in studies of GPCR desensitization and which are involved in removal of ligand-activated GPCRs from the plasma membrane. The GRK family of kinases is composed of seven members that share significant amino acid and structural homology (reviewed in (Ferguson, 2001; Penela et al., 2006)). GRK2 and GRK3 reside in the cytosol in the absence of agonist and translocate to the membrane following GPCR stimulation. GRK2/3 translocation and membrane localization are mediated in part by their binding to heterotrimeric G protein $\beta\gamma$ subunits (Pitcher et al., 1992). GRK5 and GRK6, on the other hand, are constitutively localized to the plasma membrane. Whereas GRK6 palmitoylation is essential for membrane association (Stoffel et al., 1994), localization of GRK5 to the plasma membrane is believed to be attributable to an electrostatic interaction between the highly basic carboxyl terminus of GRK5 and phospholipids at the plasma membrane (Kunapuli et al., 1994; Premont et al., 1994). The β -arrestin proteins exist in 2 isoforms in humans, namely β -arrestin 1 and β -arrestin 2, both of which show ubiquitous tissue distribution. In addition to their well-described role in limiting receptor-G protein interaction, the β -arrestin proteins also serve to both recruit and physically bridge the receptor to the endocytic machinery. In addition, the β -arrestin proteins themselves are able to interact directly with the essential components of the clathrin-coated vesicle (CCV) coat machinery, namely the heterotetrameric AP-2 complex (Laporte et al., 1999), as well as clathrin (Goodman et al., 1996), and these interactions are critical both for recruitment

of the receptor into clathrin-coated pits as well as for receptor internalization (Doherty and McMahon, 2009; Wolfe and Trejo, 2007).

Internalized receptors can then either traffic to lysosomes for degradation or follow a recycling route back up to the cell surface (resensitization; Figure 1.9) (Claing et al., 2002; Gainetdinov et al., 2004). Recent work has established that agonist stimulation of the β 2-AR promotes direct association of the extreme carboxyl terminus of the receptor with the first PDZ domain within Na^+/H^+ -exchanger regulatory factor-1 (NHERF1) (Hall et al., 1998b).

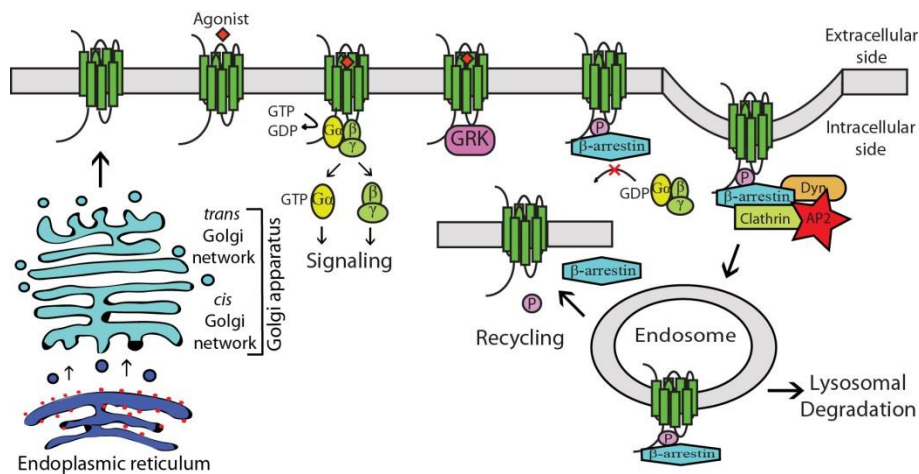


Figure 1.9. Model of the GPCR life cycle. GPCRs are synthesized, folded, and assembled at the endoplasmic reticulum (ER). Properly folded receptors are transported from the ER through the Golgi complex to the plasma membrane. Upon agonist stimulation, GPCRs bind and activate a G protein, which in turn dissociates to impact downstream signaling pathways. Prolonged exposure to agonist results in desensitization and internalization of the receptor by phosphorylation and arrestin recruitment. (modified from (Stadel et al., 2011)).

Similar associations have been shown to occur for other GPCRs containing sequences that conform to the consensus motif D-S/T-X-L, including the purinergic P2Y1 receptor and the CFTR (Hall et al., 1998a), whereas other GPCRs such as the parathyroid hormone receptor have been shown to interact with both PDZ domains of both NHERF1 and NHERF2. NHERF was found to be of critical importance for proper intracellular sorting of the β 2-AR (Figure 1.10b). In addition to its ability to bind the extreme carboxyl terminus of the β 2-AR via its PDZ domain, NHERF is able via its ezrin–radixin–moesin (ERM) domain to bind to the actin cytoskeleton through association with ERM proteins. Mutations that were generated to disrupt the interaction of NHERF with either the β 2-AR carboxyl terminus or with ERM proteins lead to significant agonist-induced lysosomal degradation of the β 2-AR following endocytosis, rather than recycling (Figure 1.10b; (Cao et al., 1999)). Another protein found to be critical for receptor endocytosis is the N-Ethylmaleimide-Sensitive Fusion Protein (NSF), an ATPase essential for many intracellular transport functions (McDonald et al., 1999). Overexpression of NSF in HEK293 cells led to enhanced agonist-induced β 2-AR internalization and could rescue the effects of a β -arrestin1 mutant (S412D) previously shown to function as a dominant negative for β 2-AR internalization. β 2-AR is able, via its extreme carboxyl terminus, to bind directly to NSF (Cong et al., 2001). The β 2-AR/NSF interaction is agonist dependent and requisite for efficient agonist-mediated β 2-AR internalization. Importantly, whereas wild-type β 2-ARs recycle to the cell surface following exposure to the antagonist propranolol, β 2-ARs containing mutations in their distal carboxyl termini remain sequestered intracellularly, demonstrating the importance of the β 2-AR/NSF interaction for proper β 2-AR recycling. Thus, although it is clear that proteins that bind to the extreme carboxyl termini of GPCRs, such as NHERF and NSF, serve to regulate

the intracellular sorting of the receptors such as the β 2-AR, the extent to which the trafficking of other GPCRs is modulated by these or similar unidentified proteins remains to be elucidated.

Following internalization, GPCRs may be either recycled to the plasma membrane or undergo sorting to the endosomal/lysosomal pathway for degradation. Sorting nexin 1 (SNX1) has been demonstrated to interact with protease activated receptor 1 (PAR1) in an agonist-dependent manner (Wang et al., 2002). Overexpression of a SNX1 carboxyl terminal-deletion mutant did not significantly impair agonist-induced PAR1 internalization and accumulation in early endosomes but was able to impede endosome to lysosome sorting of PAR1 and thus markedly inhibit PAR1 degradation (Wang et al., 2002). Additional studies, in which depletion of endogenous SNX1 in HeLa cells via small interfering RNA technology markedly impaired agonist-induced PAR1 degradation, further supporting the suggestion that SNX1 plays a role in the intracellular trafficking of PAR1 from endosomes to lysosomes (Gullapalli et al., 2006). Whether SNX1 or other SNX proteins can mediate the endosomal to lysosomal sorting of GPCRs other than PAR1 remains to be determined but is certainly possible given the general conservation of trafficking themes shared across members of this large protein family.

A second class of proteins shown to regulate the movement of GPCRs both during and subsequent to endocytosis are the Rab proteins, a family of Ras-like small GTP binding proteins. Importantly, different Rab GTPase family members selectively associate with specific intracellular structures including both recycling and sorting endosomes, where they mediate multiple steps of vesicular membrane trafficking including vesicle budding, docking, and fusion (reviewed in (Seachrist and Ferguson, 2003)). Rab5 has been found to play a role in GPCR trafficking events associated with GPCR internalization and/or early endosomal formation. Multiple additional Rab family members have been implicated in other GPCR-trafficking events,

including Rab4 and Rab11 in receptor recycling, and Rab7 in endosomal to lysosomal sorting (Dale et al., 2004).

Another key cellular mediator in the GPCR postendocytic sorting process that was found to bind to the carboxyl-terminus of the dopamine receptor D2 (Bartlett et al., 2005), the CB1 receptor (Martini et al., 2007) and the δ -opioid receptor (Figure 1.10a; reviewed in (Whistler et al., 2002)) is the G-protein-coupled receptor-associated sorting protein (GASP1). For CB1, HEK293 cells overexpressing GASP1 displayed reduced lysosomal colocalization compared with cells expressing the CB1 receptor alone (Martini et al., 2007). This reduced lysosomal targeting led to the recycling and enhanced surface recovery of the receptor following treatment with inverse agonist, suggesting that overexpression of GASP1 promoted the redirection of internalized receptors to the recycling pathway. The opposite holds true for the δ -opioid receptor (Figure 1.10a).

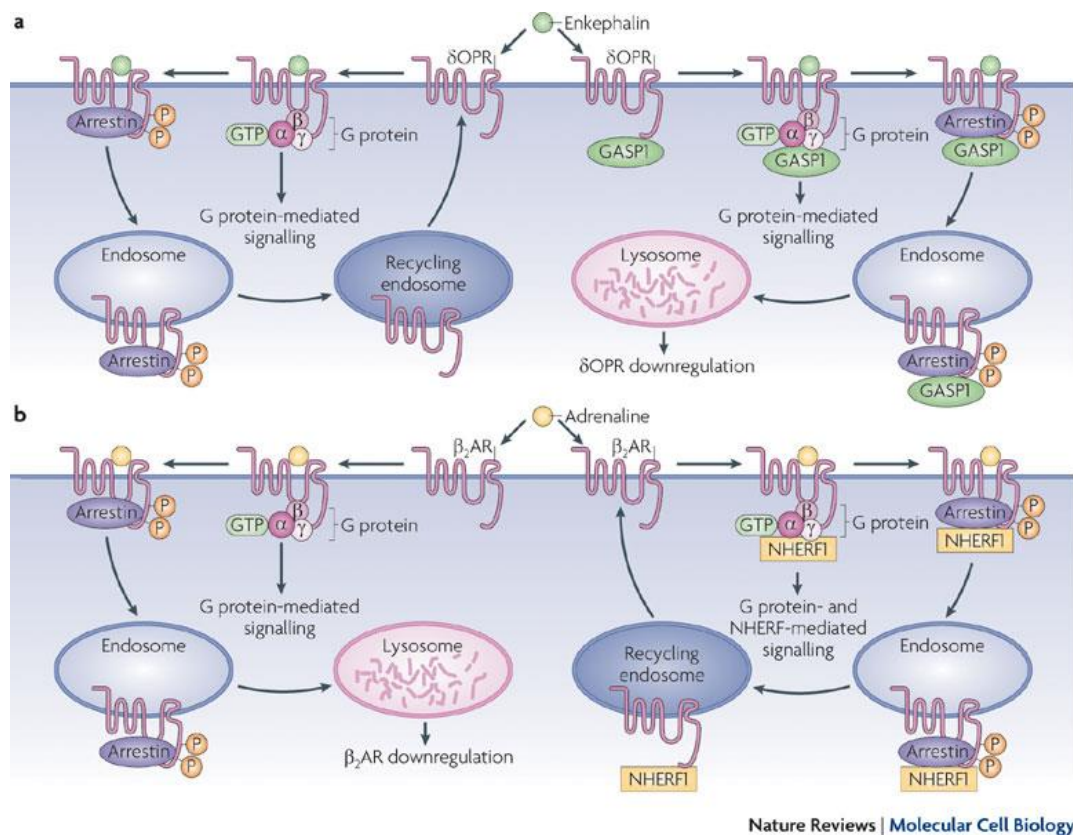


Figure 1.10 GPCR-interacting proteins can regulate the post-endocytic trafficking of GPCRs. Following agonist-induced receptor endocytosis, some GPCRs are targeted for proteolytic and/or lysosomal degradation, whereas other GPCRs rapidly recycle back to the plasma membrane. **(a)** The interaction between GPCR-associated sorting protein 1 (GASPI) and δ -type opioid receptor (δ OPR) promotes the endocytic targeting of agonist-internalized δ OPRs to lysosomes, where the receptors are degraded. However, in a distinct cellular compartment (or distinct cell type) that lacks GASPI, as shown on the left, δ OPRs are rapidly recycled back to the plasma membrane. **(b)** By contrast, the interaction between the GPCR-interacting protein Na⁺–H⁺ exchange regulatory factor 1 (NHERF1) and the β_2 -adrenergic receptor (β_2 AR) promotes the rapid recycling of receptors following agonist-promoted internalization. However, in a distinct cellular compartment (or distinct cell type) that lacks NHERF1, as shown on the left, β_2 ARs are preferentially targeted to lysosomes for degradation. (Ritter and Hall, 2009).

1.2 Cannabinoid receptors

The identification of the cannabinoid receptors grew out of a desire to understand the psychoactive effects of Δ^9 -tetrahydrocannabinol (THC), the main psychoactive component of *Cannabis sativa*. To date, two cannabinoid receptors, CB1 and CB2, have been described with regard to their primary structure (Figure 1.11), ligand binding properties and signal transduction systems (Howlett et al., 2002; Pertwee et al., 1995). The existence of these receptors was confirmed when it was shown that cannabinoids decreased cAMP in neuroblastoma cell cultures, suggesting the activation of a Gi/o-coupled receptor (Howlett, 1985; Howlett and Fleming, 1984; Howlett et al., 1986). Immunohistochemical and radioligand binding methods were also used to characterize a receptor that was present in high concentrations in certain brain regions (Devane et al., 1988). The cloning of the cannabinoid receptor 1 (CB1) provided the final evidence for its existence in 1990 when it was cloned from a cDNA library obtained by sequencing rat cerebral cortex (Matsuda et al., 1990). The cloning of the cannabinoid receptor 2 (CB2) followed closely thereafter in 1993 and was cloned from the HL60 promyelocytic human leukaemic cell line (Munro et al., 1993).

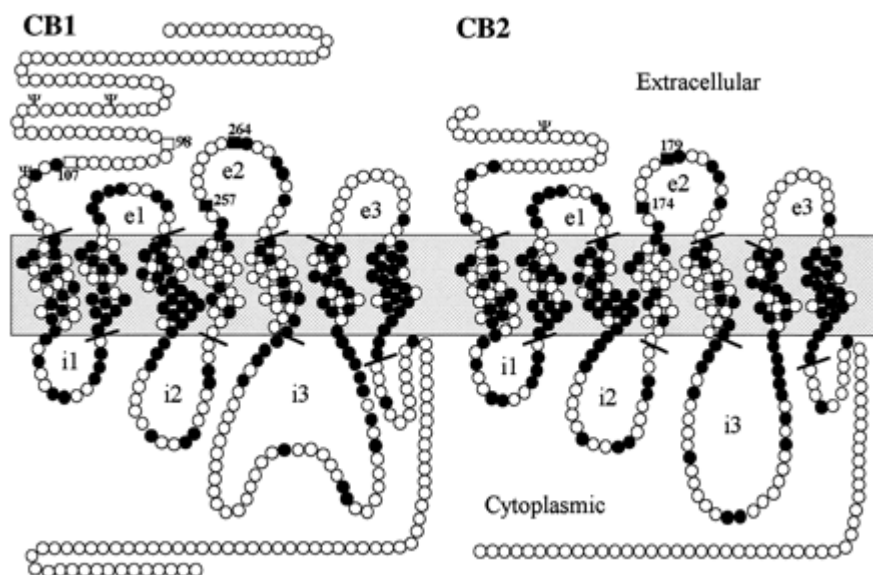


Figure 1.11 Schematic representations of the human CB1 and CB2 receptors. Black circles represent amino acids common to the two receptors, and white circles different amino acids. (Shire et al., 1996)

1.2.1 Tissue distribution of CB1 and CB2

The physiological distribution of CB1 receptors has been characterized in both rat and human brains (Glass et al., 1997; Herkenham et al., 1991a; Herkenham et al., 1991b; Herkenham et al., 1990b; Mailleux and Vanderhaeghen, 1992; Westlake et al., 1994). CB1 receptor expression was detected in regions influencing a number of key functions, including mood, motor coordination, autonomic function, memory, sensation and cognition. Electron microscopy studies demonstrated CB1 receptors predominantly on presynaptic terminals (Katona et al., 1999; Marsicano and Lutz, 1999; Tsou et al., 1999), but they were found also on postsynaptic structures and glia (Rodriguez et al., 2001). The presence of CB1 receptors in the hippocampus in high density is related to the disruptive effects of cannabinoids on memory and cognition

(Herkenham et al., 1991a; Herkenham et al., 1990b). Chronic exposure to THC or marijuana extracts were shown to permanently alter the structure and function of the hippocampus (Scallet, 1991) which is involved in learning and memory processes in both rats and humans. The occurrence of CB1 receptors in the basal ganglia and the effects of cannabinoids in these structures imply that the endogenous cannabinoids are involved in the fine-tuning of motor control. Evidence of decreased CB1 receptor expression and binding has been shown in neurodegenerative diseases, such as Parkinson's and Huntington's (Glass et al., 1993; Richfield and Herkenham, 1994; Sanudo-Pena et al., 1998).

There is also a high density of CB1 receptors in the rat cerebellum (Matsuda et al., 1993) and they may have a role in the ataxia, immobility, and catalepsy following acute THC administration (Navarro et al., 1998). In contrast, a relatively low density of CB1 receptors is found in the human cerebellum and is consistent with the more subtle effects observed in overall human motor functioning after marijuana use (Ameri, 1999; Herkenham et al., 1990a).

The CB1 receptors are perhaps most known for their important role in the central and peripheral regulation of food intake, fat accumulation and lipid and glucose metabolism (Svizenska et al., 2008). Alterations of these functions are associated with CB1 hyperactivity (Gelfand and Cannon, 2006) in both central nervous system (CNS) and peripheral tissues (adipocytes, skeletal muscle cells, liver, gastrointestinal tract). Stimulation of hypothalamic CB1 receptors interacts with neuropeptides regulating energetic homeostasis, food intake and lipogenesis in visceral tissues (Cota et al., 2003). Stimulation of CB1 receptors in the accumbens nucleus activates the dopaminergic reward pathway and thus the motivation to eat, as well as to smoke or intake drugs of abuse (Maldonado et al., 2006). CB1 receptors are also highly expressed in the areas that are involved in pain modulation, including the periaqueductal gray (Tsou et al., 1997) and the dorsal

horn of the spinal cord (Farquhar-Smith et al., 2000). More recent evidence has emerged that the CB1 receptor is also expressed in a number of peripheral tissues, such as the cardiovascular and reproductive systems as well as the gastrointestinal tract (Figure 1.12; (Batkai et al., 2001; Croci et al., 1998; Pertwee, 1997; Pertwee, 2001)).

Determination of the distribution of CB2 receptors has lagged considerably due to the lower abundance of CB2 receptors relative to CB1 as well as the difficulty in raising highly selective CB2 receptor antibodies (Mackie, 2008). Strong evidence exists of the distribution of CB2 receptors in peripheral tissues, and particularly in immune tissues such as the spleen, tonsils, thymus, mast cells and blood cells (Berdyshev, 2000; Munro et al., 1993; Sugiura and Waku, 2000; Wilson and Nicoll, 2001). The CB2 receptor was also recently found in the CNS, e.g., in rat microglial cells (Ashton et al., 2006) as well as neurons (Gong et al., 2006; Skaper et al., 1996) under certain conditions (Figure 1.12).

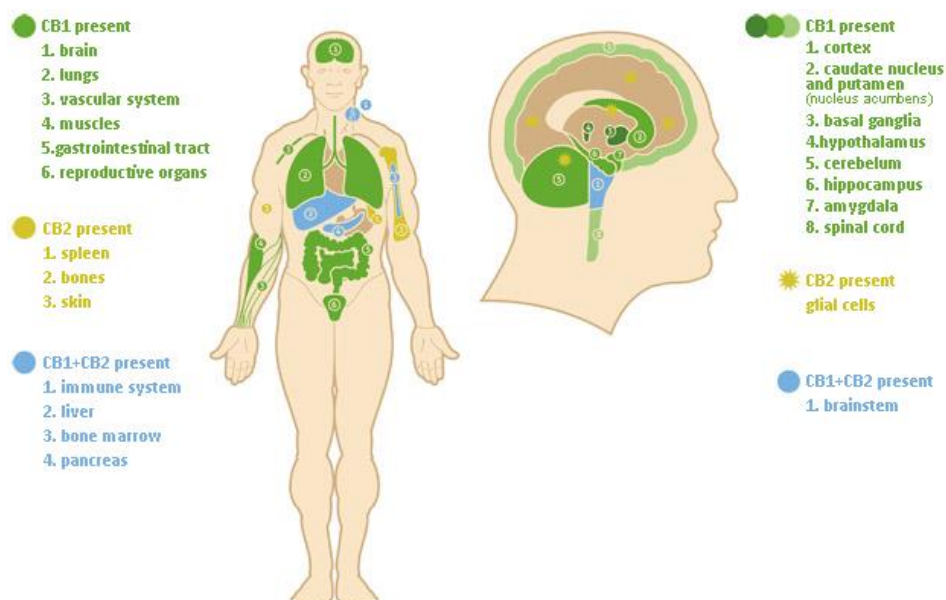


Figure 1.12 Tissue distribution of the human CB1 and CB2 receptors in the body. CB1 receptors are highly expressed in the hippocampus, basal ganglia, cortex and cerebellum. They

are less expressed in the amygdala, hypothalamus, nucleus accumbens, thalamus, periaqueductal grey matter and the spinal cord, as well as in other brain areas, mainly in the telencephalon and diencephalon. CB1 receptors are also expressed in several peripheral organs. Thus, they are present in adipocytes, liver, lungs, smooth muscle, gastrointestinal tract, pancreatic β -cells, vascular endothelium, reproductive organs, immune system, sensorial peripheral nerves and sympathetic nerves. The distribution of CB2 receptors is quite different and mainly restricted to the periphery in the immune system cells, such as macrophages, neutrophils, monocytes, B-lymphocytes, T-lymphocytes and microglial cells. Recently, CB2 receptor expression has also been shown in skin nerve fibers and keratinocytes, bone cells such as osteoblasts, osteocytes and osteoclasts, liver and somatostatin secreting cells in the pancreas. The presence of CB2 receptors has also been demonstrated at the CNS, in astrocytes, microglial cells and brainstem neurons. (online at <http://www.fundacion-canna.es/en/endocannabinoid-system>).

1.2.2 The endocannabinoid system

The endocannabinoid system is comprised of cannabinoid receptors, their endogenous ligands, i.e. endocannabinoids, and the enzymes required for their biosynthesis and degradation (Figure 1.13; (Freund et al., 2003; Howlett et al., 2002; Mackie, 2006)). Endocannabinoids are so named because they were first identified to activate the same receptors that were activated by the main psychoactive component of cannabis. Endocannabinoids produced by the brain include arachidonoyl ethanolamide (anandamide), 2-arachidonoyl glycerol (2-AG), noladin ether, virodhamine, and N-arachidonoyl dopamine (NADA). Unlike many neuromodulators,

endocannabinoids are not synthesized in advance and stored in vesicles. Rather, their precursors exist in cell membranes and are synthesized and cleaved “on demand” from cell membrane arachidonic acid derivatives by specific enzymes such as diacylglycerol lipase (DAGL). Depolarization of a postsynaptic neuronal membrane induces membrane-derived endocannabinoid production. Endocannabinoids are then capable of activating cannabinoid receptors on the membranes of presynaptic neurons resulting in a G protein-dependent reduction in neurotransmitter release into the presynaptic cleft. Intracellular endocannabinoid sequestration inactivates the cannabinoid receptors. Whether cannabinoid uptake is predominantly an active or passive process remains controversial. Hydrophobic endocannabinoids may passively internalize through the plasma membrane by means of simple diffusion. However, recent data suggests that both the 2-AG and anandamide internalization process is saturable and temperature dependent indicating the uptake may be protein-facilitated (reviewed in (Yates and Barker, 2009)). Endocannabinoids have a local effect and short half-life before being degraded by enzymes such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Alexander and Kendall, 2007; Basavarajappa, 2007; Okamoto et al., 2007; Simon and Cravatt, 2006). The concentration of 2-AG in the brain greatly exceeds anandamide levels by upwards of 150 times (Dinh et al., 2002). These two endocannabinoids are discussed in more detail in section 1.2.4.

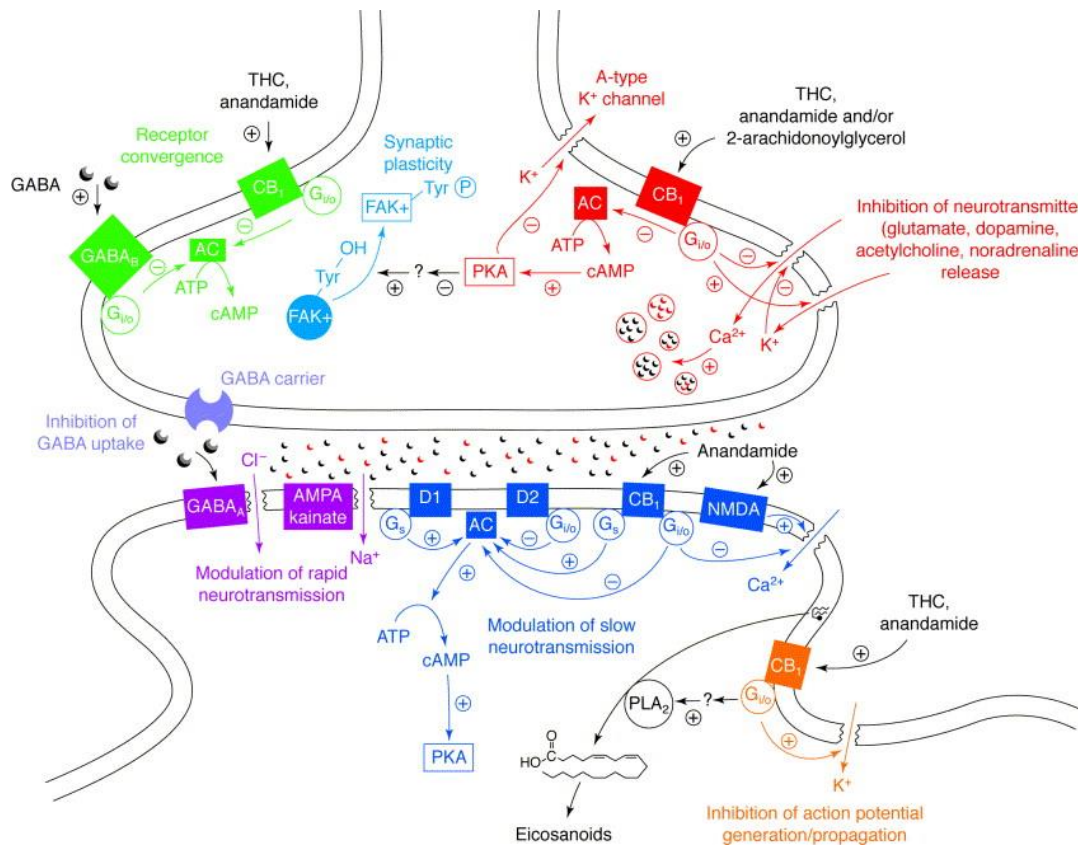


Figure 1.13 Molecular basis of endocannabinoid neuromodulatory action. At the presynaptic nerve terminal, anandamide and 2-arachidonoylglycerol (2-AG) activate G-protein-coupled CB1 receptors, modulating neuronal membrane permeability to Ca^{2+} and K^{+} ions and the activity of adenylyl cyclase (AC), thereby affecting neurotransmitter release or action. Once released by the depolarization of neurons, the two compounds, due to their lipophilicity, might behave, like other arachidonic acid (AA) derivatives, as autocrine or paracrine signals by acting on the same or neighboring neurons or on astrocytes. In the hippocampus, inhibition of AC and subsequently cAMP-dependent protein kinase (PKA), can also lead to modulation of synaptic plasticity, for example, through increased tyrosine phosphorylation and subsequent activation of focal adhesion kinase+ (FAK+). G_s -mediated activation of AC was observed upon G_i/o inhibition with pertussis toxin, and concurrent stimulation of D2 and CB1 receptors in striatal slices leads to AC

stimulation instead of inhibition. In hippocampal slices, a direct potentiation of NMDA receptors by anandamide has also been observed. By acting on CB1 receptor-coupled K⁺ channels, anandamide might also directly hyperpolarize smooth muscle cells. Anandamide also affects intracellular AA and Ca²⁺ concentrations in astrocytes via pertussis toxin-sensitive mechanisms. Abbreviations: AA, arachidonic acid; FAK+, focal adhesion kinase+; PLA2, phospholipase A2; THC, Δ⁹(-)-tetrahydrocannabinol. (Di Marzo et al., 1998).

1.2.3 Structural features of the cannabinoid receptors

CB1 splice variants. Multiple splice variants of human CB1 are thought to exist in vivo. These splice variants vary in the length of their extracellular amino terminus. The first variant to be cloned was CB1a in 1995 and was found to lack the first 61 amino acids of the amino terminus of CB1 (Shire et al., 1995). The second splice variant to be identified was CB1b which was shown to have a deletion of 33 amino acids from the CB1 amino terminus (Ryberg et al., 2005). The splice variants CB1a and CB1b lacked an affinity or efficacy for the endogenous cannabinoids (Ryberg et al., 2005) and showed only minimal binding to anandamide and 2-arachidonoylglycerol (Ryberg et al., 2005). The Kendall lab, however, showed that up to 89 amino acids from the amino terminus of CB1 can be deleted without affecting receptor binding to the ligand CP55,940 (Andersson et al., 2003).

Glycosylation. Glycosylation involves the addition of carbohydrate chains to target proteins. These added polysaccharide chains serve various functions including providing structural components to the cell wall and extracellular matrix, increasing protein stability at high temperatures, directing trafficking of the protein and proper folding and mediating cell signaling

(Lodish H, 2000). Within the amino terminus of CB1, there are 3 potential N-linked glycosylation sites (N-X-S/T, where X can be any amino acid). In rat brain, about 60-85% of cannabinoid receptors were found to be glycosylated. Rat CB1 specific bands were detected at 52 kDa, 59 kDa and 64 kDa, suggesting that glycosylation occurs at 2 sites based on the size differences between molecular weights (Song and Howlett, 1995). In 2003, truncation mutants of human CB1 were generated and it was determined by immunoblot analysis that N77 and N83 are likely the glycosylation sites (Andersson et al., 2003). The third potential glycosylation site on CB1, N113, was ruled out as a glycosylation site when the truncation the first 89 residues of the amino terminus and immunoblot analysis revealed a 53 kDa band corresponding to a non-glycosylated CB1 receptor (Andersson et al., 2003).

Loop regions. Even though sequence and structure diversity is more prominent in loop regions than in transmembrane domains, these loop regions nonetheless play important roles in receptor activation, signaling and cellular localization. The NMR solution structures of bovine rhodopsin (Yeagle et al., 1997a; Yeagle et al., 2000) intracellular IC1 and IC2 showed that these regions adopted a β -turn (Yeagle et al., 1997b) in the middle of each loop and that extracellular EC1, EC2 and EC3 regions adopted a helix-turn-helix motif. These regions are important for compact packing with TMs and receptor stability. IC3 is the longest loop among all of the loop regions of GPCRs. It has been shown to be important in signal transduction including G protein selectivity (Eason and Liggett, 1995; Eason and Liggett, 1996; Hallbrink et al., 2001; Jewell-Motz et al., 1997; Wade et al., 1999) and desensitization and internalization through arrestin binding (Bhaskaran et al., 2003; DeGraff et al., 2002; Lee et al., 2000; Peverelli et al., 2008; Schmidlin et al., 2003). An NMR study performed on a peptide corresponding to the CB1 IC3 has shown that IC3 contains three helical segments (Ulfers et al., 2002). Helical segments are extended from the

carboxyl terminal end of TM5 and from the amino terminal end of TM6, respectively (Ulfers et al., 2002). Interestingly, the squid rhodopsin crystal structure also revealed intracellular helical extensions from both TM5 and TM6, which may contribute to G protein selectivity (Murakami and Kouyama, 2008).

Helix 8 (H8). The cannabinoid receptors also possess an intracellular carboxyl-terminus domain (reviewed in (Stadel et al., 2011)). The CB1 carboxyl terminus has 73 residues (Bramblett et al., 1995) and the CB2 receptor carboxyl terminus has 59 residues (Munro et al., 1993). Although the human CB1 and CB2 receptors only differ in their carboxyl terminal length by 14 residues, there is no significant homology across these receptor subtypes. H8 (residues 401-412 in CB1) is found at the cytoplasmic end of TM7 (reviewed in (Stadel et al., 2011)). H8 has been shown in rhodopsin to undergo conformational changes upon receptor activation and this movement is believed to unmask critical G protein-binding domains (Abdulaev and Ridge, 1998). More direct evidence of the interaction of G-proteins and the carboxyl terminus of CB1 (residues 400-416) has been shown following co-immunoprecipitation and suggests that this part of the terminus which includes H8 is involved in G α i3/o recognition, binding and activation (Mukhopadhyay et al., 2000).

Palmitoylation. Residue Cys415 immediately following H8 in CB1 may serve as a potential palmitoylation site. Palmitoylation enhances the hydrophobicity of proteins and contributes to their membrane association and appears to play significant roles in subcellular trafficking of proteins between membrane compartments (Rocks et al., 2005) and protein-protein interactions (oyoti Basu, 2004). When mutating this residue to alanine on a cysteine-less CB1 receptor, wild-type-like ligand binding and G protein activation were observed (Fay et al., 2005) suggesting this residue may not be palmitoylated. In contrast, mutating Cys313 or Cys320 in the carboxyl

terminus of CB2 resulted in receptors that bound ligand similarly to the wild-type receptor but had significantly less ability to reduce cAMP production. This suggests that Cys313 and Cys320 may indeed be palmitoylation sites (Feng and Song, 2001). The distal region of the carboxyl terminus has also been found to impact receptor activation, signaling and subcellular localization.

Conserved motifs and the inactive CB1 model. Despite the lack of cannabinoid receptor crystal structures, the β 2AR has been used as a template in the creation of homology models of CB1 because it shares many structural features with CB1. Both CB1 and the β 2AR bind a small molecular-weight ligand, possess a disulfide bond within EC2 (Fay et al., 2005; Shire et al., 1996), lack secondary structure in the amino terminus, and lack the helix kink in TM1.

Many of the functional motifs that are conserved among most family A GPCRS exist in CB1 and CB2. These include the S(N)LAxAD in TM2, E/DRY in TM3, WX₍₉₎P in TM4, CWXP in TM6, and NPXXY in TM7. These motifs within the transmembrane helical domain contribute to helical stabilization through hydrogen bonding, water-mediated hydrogen bonding, aromatic stacking and lipid interactions.

Several modeling studies of CB1, using both the rhodopsin and β -adrenergic receptors as templates, have suggested the presence of the TM1-TM2-TM7 hydrogen bonding network that was mentioned earlier as being conserved in rhodopsin (Figure 1.2b). This network involves residues Asn134, Asp163 and Asn393 of the CB1 receptor, which are conserved in rhodopsin as Asn55, Asp83, and Asn302. Another hydrogen bonding cluster exists in CB1 between TM3 and TM5 that includes the interaction between Thr201 and Thr283, and between Thr197 and Tyr275. For the helical stability of the inactive CB1 receptor conformation, modeling studies have also

revealed two important salt bridges or ionic locks (Salo et al., 2004; Shim, 2009). The first of these is the highly conserved TM3-TM6 ionic lock which involves Arg214 of the E/DRY motif on TM3 and Glu338 on TM6 of the CB1 receptor. The second ionic lock involves interactions between Asp176 on TM2 and Lys192 on TM3. Site-specific mutational studies and molecular modeling analyses have indicated that Lys192 on TM3 plays a critical role in ligand binding (Chin et al., 1998; Salo et al., 2004; Shim and Howlett, 2006; Song et al., 1999). Thus, Lys192 likely mediates receptor activity through direct interaction and hydrogen bonding with ligands. Aromatic stacking within CB1 has also been shown to exist. Some of the aromatic stacking interactions that have been proposed include Tyr215, Phe208, Phe289, Tyr292, Tyr2296, Trp356, Phe170 and Phe200 (McAllister et al., 2003; Shim, 2009; Shim and Howlett, 2006). The interaction between Trp356 of the CWXXP motif and Phe200 in the inactive state of the receptor is highly conserved throughout family A GPCRs and is believed to also exist within CB1 (McAllister et al., 2004; Singh et al., 2002). Breaking this interaction upon receptor activation was found to change the angle of the proline kink in TM6, which is the basis of the rotamer toggle switch and accompanies rigid body movements of TM3 and TM6 (McAllister et al., 2004; Nygaard et al., 2009).

1.2.4 Cannabinoid ligands and receptor activation states

Interestingly, the cannabinoid receptors exhibit some basal activity (like many non-sensory class A GPCRs) indicative of G protein coupling in the absence of agonists (Landsman et al., 1997). In transfected HEK293 cells, up to 85% of CB1 receptors were found to be localized to endocytic intracellular vesicles with only a small population expressed on the cell surface (Leterrier et al., 2004). This cellular distribution of CB1 is consistent with patterns observed in a variety of cell types including CHO cells and hippocampal neuronal cells (Martini et al., 2007;

Rozenfeld and Devi, 2008). Cannabinoid receptors can be activated by four major chemical groups of agonists: classical cannabinoids, nonclassical cannabinoids, aminoalkylindoles and eicosanoids ((Pertwee, 2006); Figure 1.14).

Classical and nonclassical cannabinoids. The classical cannabinoids consist of dibenzopyran derivatives and are either plant-derived cannabinoids or synthetic analogues of these. The most notable examples are Δ^9 -tetrahydrocannabinol (THC; derived from *Cannabis sativa*), Δ^8 -tetrahydrocannabinol, or HU210. These ligands have three rings in their structure, one of which is a pyran ring along with an alkyl chain on C-3 of the A ring. Nonclassical cannabinoids are quite similar in structure to classical cannabinoids but lack a pyran ring and have either two or three rings along with the C-3 alkyl chain. Several of the compounds of this group were developed by a Pfizer research team. The most widely used of these is CP55940 which has CB1 and CB2 affinities in the low nanomolar range (K_i = 0.5 nM and 0.69 nM, respectively) and exhibits relatively high efficacy at both of these receptor types (reviewed in (Pertwee et al., 2010)). One of the most investigated pharmacophores is the lipophilic chain or alkyl chain projecting from the C-3 of the phenol group common to both classical and nonclassical agonists. An extensive study was performed on the length and nature of the side chain of Δ^8 -tetrahydrocannabinol that suggested that an alkyl chain methylated at the second carbon retained CB1 agonism if the chain was between 3 to 10 carbon atoms in length. Attempts have been made to modify the CB1-CB2 selectivity of classical agonists which intrinsically bind both cannabinoid receptors by modifying the length of this C-3 side chain. Some evidence suggests shortening the side chain can enhance CB2 selectivity (reviewed in (Poso and Huffman, 2008)). Removal of the hydroxyl group at the C-1 position could also be used to augment CB2 selectivity (Huffman et al., 2006) presumably because Lys 192 of CB1 is thought to hydrogen

bond to this phenolic hydroxyl group. However, mutation of the corresponding residue in CB2 (Lys 109) to a residue which precludes hydrogen bonding to the C-1 hydroxyl group did not significantly affect CP55940 binding (Tao et al., 1999).

Aminoalkylindoles. The aminoalkylindole agonist was developed by Sterling Research after observations were made that certain aminoalkylindoles were able to inhibit adenylyl cyclase by activating G proteins. The structure of WIN 55212-2 is completely different than any of the other compound groups. Like CP55940, it exhibits relatively high efficacy at CB1 and CB2 receptors (K_i = 1.89 nM and 0.28 nM, respectively) but has a slightly greater affinity for CB2 than CB1 receptors (Chin et al., 1999; Song et al., 1999). This selectivity was studied by creating a chimeric cannabinoid receptor and swapping TM3 of CB1 with the corresponding domain of CB2. This resulted in enhanced WIN55212-2 binding affinity to CB2. A mutagenesis study performed by Song and colleagues (Song et al., 1999) suggested that a Phe present in TM5 of CB2 corresponding to a Val residue in CB1 confers the selectivity of the compound. Molecular modeling of CB2 points to Phe197 as being able to form an aromatic stacking interaction with the indole ring of WIN55212-2, an interaction lacking with CB1's corresponding Val residue.

Endocannabinoids. The two most common and investigated members of the endogenous, lipid-derived agonists have been anandamide and 2-AG (Figure 1.14). Anandamide binds somewhat more selectively to CB1 than to CB2 receptors (61 nM and 279 nM, respectively). 2-AG has been found to possess affinity for CB1 and CB2 receptors (58.3 nM and 145 nM, respectively) similar to those of anandamide but to exhibit higher CB1 and CB2 efficacy than anandamide (Pertwee et al., 2010). Other endocannabinoids also include 2-arachidonoyl glyceryl ether (noladin ether) (Fezza et al., 2002), N-arachidonyldopamine (NADA) as well as O-arachidonoyl ethanolamine (virodhamine) (Porter et al., 2002). Because these ligands are substrates for

enzymes such as fatty acid amide hydrolase (FAAH), an enzyme typically present in membrane preparations, a C-2 methylated anandamide, known as methanandamide, was created to circumvent FAAH. Methanandamide binds CB1 with an affinity comparable to that of anandamide but the added methylate precludes its hydrolysis by FAAH (Pertwee et al., 2010).

Inverse Agonists. Inverse agonists of the CB1 receptor have attracted considerable attention in drug discovery because of their ability to regulate appetite and manage substance addiction (Janero, 2012; Janero and Makriyannis, 2009; Silvestri and Di Marzo, 2012a). The first specific cannabinoid inverse agonist to be developed was the diarylpyrazole, SR141716A (rimonabant) (Rinaldi-Carmona et al., 1994) and was the only CB1 inverse agonist to be approved and briefly clinically marketed in Europe for the control of obesity (Moreira and Crippa, 2009). It was removed from use shortly after due to its severe neuropsychiatric side effects including mood-depressant actions (Despres et al., 2005; Traynor, 2007). SR141716A is a highly potent and selective CB1 ligand that blocks and reverses CB1-mediated effects both *in vitro* and *in vivo* (Howlett et al., 2002; Pertwee, 1999; Pertwee, 2005b) and binds to CB1 with $K_i = 1.8$ nM. Three analogs of SR141716A that have also been used to block CB1-mediated effects are AM251 ($K_i = 7.49$ nM), AM281 ($K_i = 12$ nM) and LY320135 ($K_i = 141$ nM), each with less affinity for CB1 (Pertwee et al., 2010). The structures of these compounds are shown in Figure 1.14. The most notable CB2-selective inverse agonists are the Sanofi-Aventis diarylpyrazole, SR144528 ($K_i = 0.28$ nM) (Rinaldi-Carmona et al., 1998a) and 6-iodopravadoline (AM630; $K_i = 31.2$ nM) (Ross et al., 1999). Both compounds bind with much higher affinity to CB2 than to CB1 receptors. These inverse agonists produce inverse cannabimimetic effects by reducing the constitutive activity of CB2 receptors.

Antagonists. VCHSR (Figure 1.14), an antagonist analog of SR141716A lacking hydrogen bonding potential at the C-3 substituent displayed no inverse agonism in cells expressing the wild-type CB1 receptor HURST 2006 (Hurst et al., 2006). Interestingly, VCHSR bound the wild-type cannabinoid receptor and the K192A receptor with affinities similar to that of SR141716A. Further synthesis of SR141716A analogs was studied to determine if the carboxamide group or the piperidine nitrogen of the C-3 substituent of SR141716A were necessary for its high affinity binding to the cannabinoid receptor. These analogs lacked hydrogen bonding potential at either of the two positions. The results demonstrated that the carboxamide group rather than the amide nitrogen of the C-3 substituent of SR141716A likely hydrogen bonds with Lys192. Molecular modeling studies suggest that the carboxamide group of SR141716A forms a hydrogen bond with Lys 192 in an inactive receptor, explaining the loss of inverse agonism when a hydrogen bond between Lys 192 and SR141716A is precluded.

As previously mentioned, the alkyl chain of cannabinoid receptor agonists has been shown to play a role in the efficacy of the ligand. Shortening the alkyl chain of Δ^8 -tetrahydrocannabinol to two carbons, with a dimethyl group on the first carbon, resulted in the compound JWH179 that bound CB1, but failed to exhibit a functional response (Huffman et al., 2003). Besides shortening the chain, the addition of a triple bond, as in O-2050, renders it less flexible and has been shown to abolish agonism (Gardner and Mallet, 2006). Taken together these results suggest a flexible alkyl chain on classical and nonclassical cannabinoid receptor agonists longer than two carbons is a prerequisite for receptor activation.

Cannabinoid Receptor Ligands

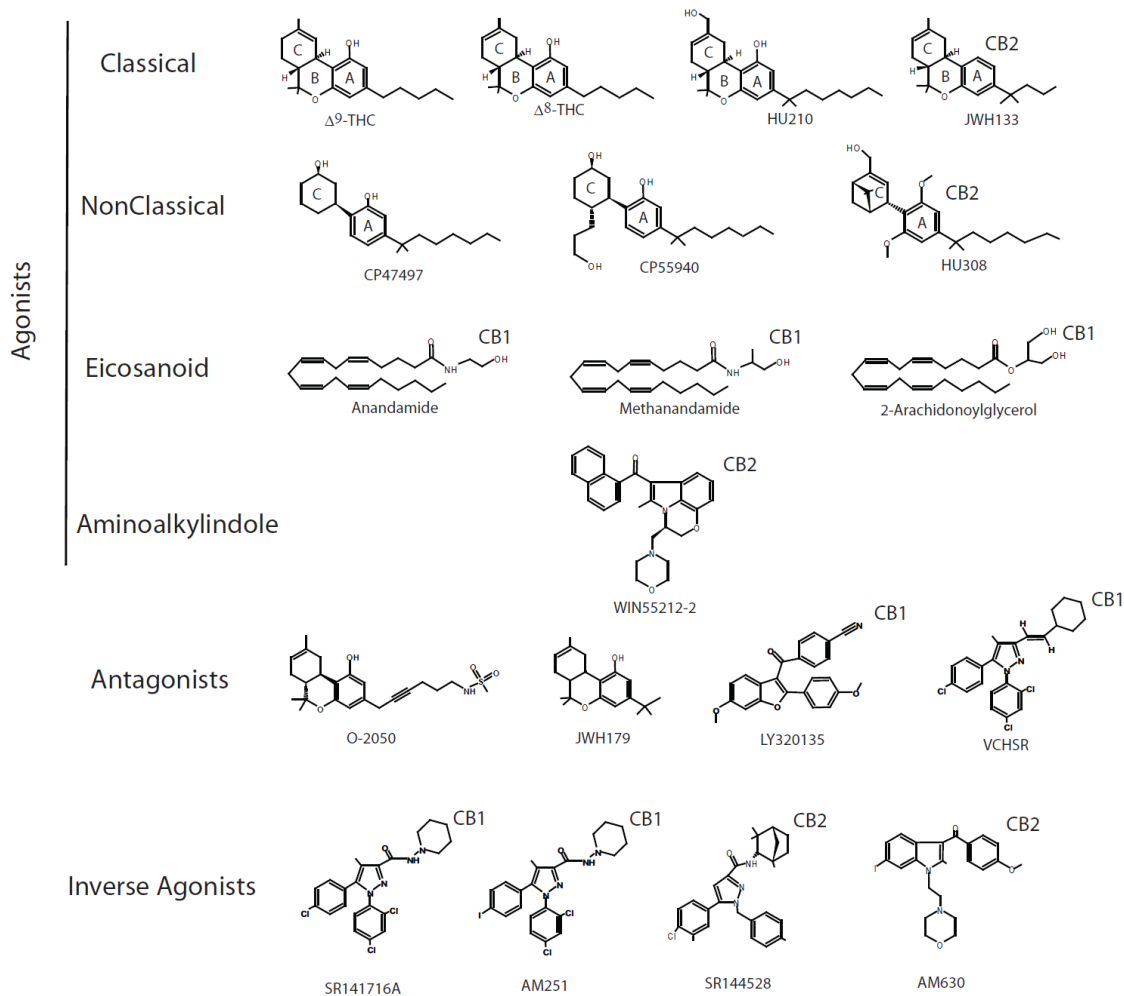


Figure 1.14 Cannabinoid receptor ligands. Structures of common CB1 and CB2 agonists, antagonists, and inverse agonists. Ligands which are thought to selectively bind CB1 or CB2 have either a “CB1” or “CB2” adjacent to their structures, respectively.

CB1 active state model. The inactive states of GPCRs have been shown to possess many structural similarities. However, when the receptor is activated by structurally distinct ligands with various potencies, the receptor may adopt multiple activation conformations with diversity in ligand binding site interactions. Many studies on Class A GPCRs indicate that the ligand binding pocket is primarily within the core of the helical bundle of the receptors. This is unlike class B or C GPCRs that bind glycoprotein hormones or neurotransmitters through their extracellular region. Based on molecular modeling studies of CB1, it is believed that there is a primary hydrophobic binding pocket, mainly formed by TM3, TM5, TM6 and TM7 (Figure 1.15). The key residues of this pocket are Phe189, Phe200, Trp279, Leu276, Tyr275, Lys192, Pro358, Ile353, Trp356, Leu359, Ser383, Cys386, Leu387 and Leu360 (Gonzalez et al., 2008; Montero et al., 2005; Padgett et al., 2008; Salo et al., 2004; Shim and Howlett, 2006). Residues Ile175 and Asp176 at the end of TM2 of CB1 have been determined to play a role in transmitting a signal across the receptor following ligand binding (Kapur et al., 2008). The single-site mutations of both of these residues resulted in a significant decrease in inverse agonist SR141716A binding affinity (Kapur et al., 2008).

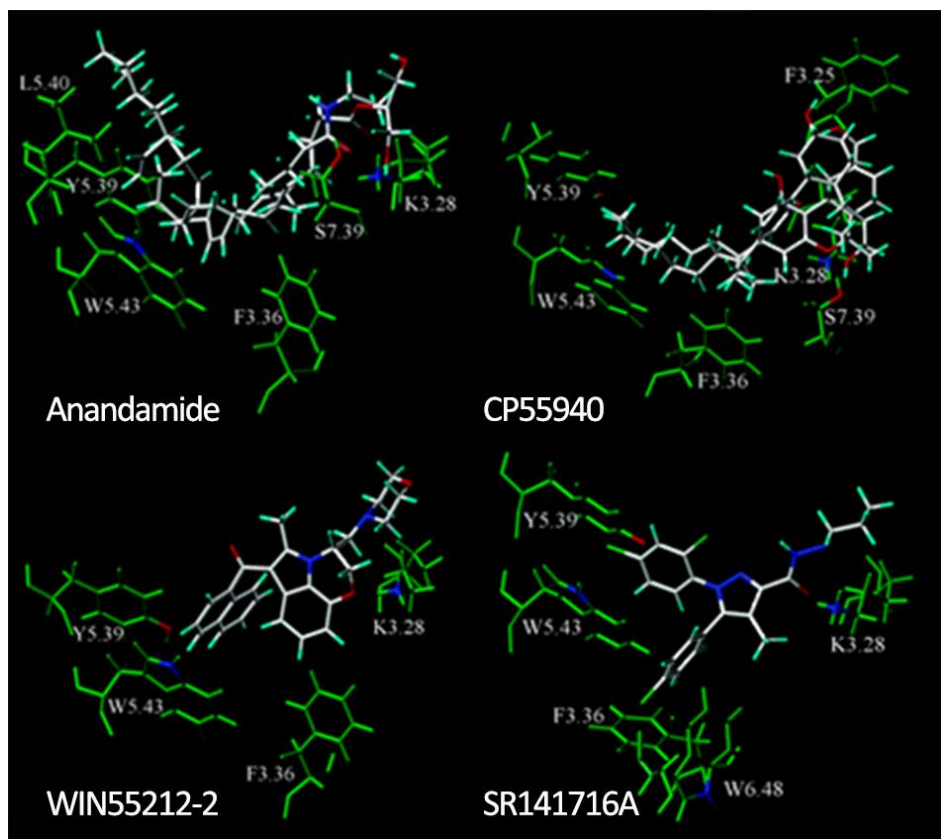


Figure 1.15 Docking conformations of the cannabinoid ligand. The key residues forming the primary binding pocket for anandamide, CP55940, WIN55212-2, and SR141716A are illustrated in green. The ligand is colored as follows: gray is carbon, red is oxygen, blue is nitrogen and green is chlorine. (modified from (Salo et al., 2004)).

Residues L207 and T210 in TM3 have also been implicated in cannabinoid receptor activation (D'Antona et al., 2006a; D'Antona et al., 2006b). A L207A mutant displayed increased basal levels of cAMP production and increased constitutive activity. Upon treatment of L207A with SR141716A, the basal levels of cAMP were decreased, ruling out the possibility that this mutant coupled to Gi and instead showing that this mutant constitutively couples to Gs. Interestingly,

upon treatment of L207A with the agonist CP55940, cAMP levels were also reduced. This finding suggests that CP55940 alters the structure of the L207A receptor shifting it from a Gs to a Gi favoring conformation. The study also showed that the noncovalent interactions of L207 and T210 also keep the receptor in the constitutively active R' state.

Mutations of residue T210 in TM3, to produce T210I and T210A, created the CB1 receptor in active and inactive states, respectively (D'Antona et al., 2006a). CB1 with the T210I mutation had an increased affinity for agonist while at the same time reducing affinity for inverse agonist. Pretreatment of T210I with guanosine-5'-O-(3-thiotriphosphate) (GTP γ S) significantly attenuated CP55940 binding. In addition, T210I displayed enhanced thermal lability over wild-type which provided further evidence that the T210I mutation increased CB1 constitutive activity. Molecular modeling performed with both the L207A and T210I mutant receptors suggest the mutation disrupts the highly conserved TM3-TM6 ionic lock between Arg214 of the E/DRY motif in TM3 and Asp338 in TM6. In contrast, CB1 with the T210A mutation decreased the constitutive activity of the CB1 receptor (D'Antona et al., 2006a). The mutant receptor exhibited enhanced affinity for inverse agonists and decreased agonist affinity. The mutation significantly reduced CP55940 binding sensitivity to GTP γ S suggesting an attenuation in G protein coupling and increased the thermal stability of T210A over wild-type CB1. These results suggest that residue T210 of TM3 is critical for regulating the equilibrium between the inactive state and the active state of the receptor.

1.2.5 Signaling of the cannabinoid receptors

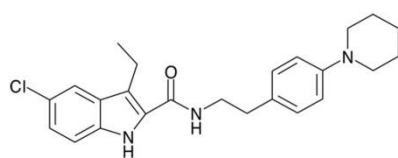
Both CB1 and CB2 receptors couple primarily to and signal through the pertussis toxin (PTX)-sensitive inhibitory Gi/o proteins (Howlett et al., 2002). The activation of these inhibitory G proteins by CB1 has been functionally linked to the inhibition of adenylate cyclase (Slipetz et al., 1995) and the cAMP pathway. Due to the decrease of cAMP accumulation, cAMP-dependent protein kinase (PKA) is inhibited by CB1 activation. In the absence of cannabinoids, PKA phosphorylates and inhibits the A-type and inwardly rectifying K⁺ channels (Howlett, 2005), thereby reducing outward potassium current. However, when cannabinoids are present, PKA phosphorylation of the channel is reduced and leads to an enhanced outward potassium current. CB1 activation has also been shown to be associated with the inhibition of N- and P/Q-type voltage-dependent Ca²⁺ channels (Howlett, 2005). Based on these findings, it has been suggested that cannabinoids suppress neuronal excitability and play a role in regulating neurotransmitter release. The CB1 receptor has also been linked to the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase-1 and -2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK) (Ahn et al., 2012; Bouaboula et al., 1996; Turu and Hunyady, 2010). The CB1 receptor can also interact with other partners such as β -arrestins, adaptor protein AP-3, GPCR-associated sorting protein 1 (GASP1) and the adaptor protein FAN to control receptor signaling or trafficking (reviewed in (Howlett et al., 2010; Smith et al., 2010)). Other events following CB1 receptor activation include activation of focal adhesion kinase and the expression of immediate early genes (Bouaboula et al., 1995; Howlett and Fleming, 1984; Mackie and Hille, 1992; Mackie et al., 1995).

Stimulation of CB2 has similar consequences in that CB2 is also coupled to Gi proteins and therefore inhibits adenylyl cyclase and the cAMP pathway in various types of cells (Howlett,

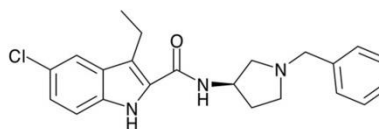
2005). This decreased cAMP production results in less activation of cAMP-dependent protein kinase (PKA), culminating in inhibition of A-type potassium channels and inhibition of some gene expression. CB2 stimulation can also result in the activation of AKT/protein kinase B which are involved in stimulating cell survival, migration and growth. It also stimulates MAPK cascades (i.e. Raf, MEK1/2 and ERK1/2) and can involve inwardly rectifying potassium channels as a signaling mechanism (Ho et al., 1999; McAllister et al., 1999). Since CB2 receptors are mainly located in immune cells, one of their functions in the immune system is the modulation of cytokine release. The activation of B- and T-cell CB2 receptors by cannabinoids leads to the inhibition of adenylyl cyclase in these cells and to a reduced response to immune challenge (Condie et al., 1996).

1.2.6 Allosteric modulators of CB1

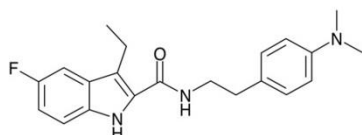
The discovery of a potential allosteric site on CB1 has generated significant interest, due to the anticipated therapeutic potential of allosteric-based drugs. A few compounds have been identified as allosteric modulators of the CB1 receptor including ORG27569, ORG29647, ORG27759, and PSNCBAM-1 (Horswill et al., 2007; Price et al., 2005) (Figure 1.16). As positive allosteric modulators, these compounds were found to increase the binding of the CB1 agonist [³H]CP55940 in membranes from cells expressing the CB1 receptor. Intriguingly, these studies suggested that these compounds also concurrently antagonized CP55940-induced signaling by inhibiting [³⁵S]GTPγS binding and reducing the E_{max} value in the mouse vas deferens assays (Price et al., 2005). The prototypical CB1 allosteric modulator is ORG27569 (Figure 1.16).



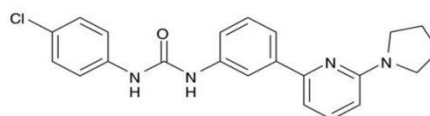
ORG27569



ORG29647



ORG27759



PSNCBAM-1

Figure 1.16 Structures of CB1 allosteric modulators. CB1 allosteric modulators enhance the affinity of certain cannabinoid receptor agonists while decreasing their ability to transduce a signal.

Not only has this modulator exhibited paradoxical effects of increasing the equilibrium binding of CP55940 while at the same time antagonizing its G protein-mediated efficacy, but it was also shown to decrease the binding of the inverse agonist [^3H]SR141716A to CB1 in both mouse brain membranes (Price et al., 2005) and in HEK293 membranes (Ahn et al., 2012). Despite this antagonizing effect on CP55940-induced G protein activation, the Kendall lab showed (Ahn et al., 2012) that upon co-treatment of HEK cells with CP55940 and ORG27569, a robust acceleration in the internalization process of the CB1 T210A (inactive) receptor occurred. High levels of receptor internalization began at 10 min and plateaued by 20 min, compared to the internalization upon treatment with CP55940 alone which plateaued at 40 min. Additionally, ORG27569 was found to play a role in cellular signaling. The CB1 wild-type receptor displayed CP55940-stimulated JNK phosphorylation with a peak at 5 min. This effect was blocked by 10

μ M SR141716A, indicating that the JNK phosphorylation is CB1-mediated. However, in agreement with the [35 S]GTP γ S binding data, ORG27569 abrogated the JNK phosphorylation induced by CP55940. These data (Ahn et al., 2012) suggest that JNK phosphorylation is only G protein-dependent via CB1 consistent with a previous report by Rueda and colleagues (Rueda et al., 2000). Moreover, the signaling pathway involving ERK1/2 which has been demonstrated to occur both G protein-dependently and G protein-independently by the μ -opioid receptor (Zheng et al., 2008), angiotensin AT1A receptor (Kendall et al., 2011), and parathyroid hormone receptor (Gesty-Palmer et al., 2006) was investigated. CP55940 induced a time-dependent stimulation of ERK1/2 with a maximum phosphorylation at 5 min. Interestingly, ORG27569 (10 μ M) alone caused a significantly higher increase in transient ERK1/2 phosphorylation at 5 min compared with the stimulation by CP55940 alone in cells expressing the CB1 wild-type receptor. When the cells were co-treated with ORG27569 and CP55940, a peak at 5 min was also observed that was somewhat diminished relative to ORG27569 alone, perhaps reflecting some competition between G protein-mediated and G protein-independent pathways (Ahn et al., 2012; Baillie et al., 2013). In addition, ORG27569 treatment alone also caused MEK1/2 and Src phosphorylation, components of the upstream MAPK pathway, to peak at 5 min and then diminish after 10 min (Ahn et al., 2013). CP55940 treatment alone resulted in MEK1/2 phosphorylation but interestingly failed to induce Src phosphorylation. Src phosphorylation is a necessary step in the activation of various mitogenic signaling pathways activated by GPCRs. It has been shown that non-receptor tyrosine kinases from the Src family are recruited to GPCRs via β -arrestin interactions (Barlic et al., 2000; Imamura et al., 2001).

Using a combination of computational methods together with mutagenesis, synthesis and pharmacological studies, one hypothesis regarding the ORG27569 binding site is that it is

located in the TM3-6-7 region of the CB1 receptor, partially overlapping the SR141716A binding site. This is consistent with equilibrium binding assays that suggest ORG27569 displaces SR141716A (Shore et al., 2014). The indole ring of ORG27569 was found to form several aromatic stacks with Phe379, placing a significant amount of ORG27569's steric bulk against the EC of TM6. This tight packing against TM6 is hypothesized to prevent TM6 from undergoing the necessary conformational changes required upon activation by an agonist, i.e. the "tripping" of the toggle switch within the orthosteric binding pocket that allows TM6 to flex in the CWXP hinge region and break the "ionic lock" between Arg135 and Glu134 at the intracellular end of the receptor. The ORG27569 indole ring also forms an aromatic stacking interaction with Phe268 an EC2 loop residue, which is believed to move down towards the TM core during receptor activation; this movement of EC2 places Phe268 in close proximity to CP55940. The aromatic stacking interaction of ORG27569 with Phe268 may sterically block this movement. This correlation between a conformational change in the EC2 loop and receptor activation has been observed for other numerous GPCRs. For example, the results of NMR studies have suggested that upon activation, rhodopsin's EC2 loop undergoes a necessary conformational change that is coupled to the breaking of the intracellular "ionic lock" (Ahuja et al., 2009). Additionally, the ORG27569 piperidine nitrogen forms an important hydrogen bond with Lys192, which was previously shown to be important for SR141716A's inverse agonism (Hurst et al., 2002). It is believed that with these interactions, ORG27569 promotes an intermediate conformation of the CB1 receptor (i.e. a conformation that is in between inactive and active) that accounts for its enhanced binding to agonist but its inability to signal in G protein-mediated pathways.

The indole-2-carboxamide class of compounds, to which ORG27569 belongs, have been noted to have great potential as CB1 allosteric modulators (Ahn KH et al., 2013; Piscitelli et al., 2012). It was previously noted that indole-2-carboxamides with lower alkyl group substitutions at C3 of the indole ring impact orthosteric ligand binding (Piscitelli et al., 2012). Thus, two novel structural analogs of ORG27569, ICAM-a and ICAM-b, were synthesized and studied by the Kendall lab (Ahn KH et al., 2013). In ICAM-b, the C3 alkyl chain was elongated from an ethyl to a *n*-pentyl group in comparison with ORG27569. In ICAM-a, only the nitrogen of the indole ring was methylated in comparison with ORG27569 (Figure 1.17). Although both compounds enhanced the level of specific [³H]CP55,940 binding, ICAM-b showed a more drastic effect than ICAM-a. ICAM-b also exhibited a high cooperativity for CP55,940, evident from a moderate K_B value of 470 nM and a remarkably high α value of 17.6. Consistent with previous allosteric modulators, ICAM-b inhibited CP55,940-induced [³⁵S]GTP γ S binding in a concentration-dependent manner evident by a progressive decrease in E_{max} values with an increasing concentration of ICAM-b for the CB1 receptor. In the same study (Ahn KH et al., 2013), it was demonstrated that β -arrestin 1, but not β -arrestin 2, is key for ICAM-b-induced ERK1/2 phosphorylation.

Given the vital role of CB1 in the CNS and its potential therapeutic value, the development of its allosteric modulators with distinct therapeutic properties resulting from receptor-selectivity and signaling-pathway-selectivity provides a promising avenue by which the current obstacles in cannabinoid-based drug discovery such as on- and off-target side effects can be overcome.

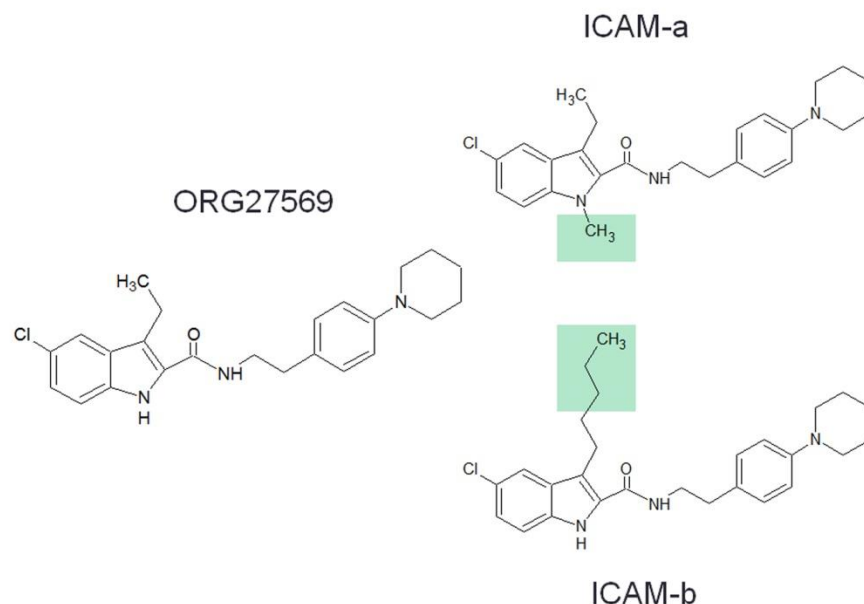


Figure 1.17 Novel indole-2-carboxamide analogs of ORG27569. In ICAM-b, the C3 alkyl chain was elongated from an ethyl to an *n*-pentyl group in comparison with ORG27569. In ICAM-a, only the nitrogen of the indole ring was methylated in comparison with ORG27569.

1.2.7 Functional selectivity of the cannabinoid receptors

Studies have reported agonist-specific differences in potency and/or efficacy towards activation of different G protein species upon CB1 receptor activation. For CB1 receptor-catalyzed activation of Gi, HU210, WIN55,212, and anandamide all elicited maximal activation, whereas Δ^9 -THC ($56 \pm 6\%$) caused only partial Gi activation. In contrast, only HU210 effected maximal CB1 stimulation of Go, with anandamide, WIN55,212-2, and Δ^9 -THC all stimulating between 60 and 75% compared with HU210. These data demonstrate that different agonists induce different conformations of the CB1 receptor, which in turn can distinguish between different G proteins (Glass and Northup, 1999). Other studies also demonstrated the concept of functional selectivity

at the CB1 cannabinoid receptor by co-immunoprecipitation studies that revealed that WIN 55,212-2 activates all subtypes of Gi proteins (Gi₁, Gi₂, Gi₃) while the unrelated cannabinoid ligand, desacetyllevonantradol, activates Gi₁ and Gi₂ while acting as an inverse agonist with respect to Gi₃ coupling (Mukhopadhyay and Howlett, 2005). Extending this concept to other types of G protein, agonist-selective regulation of Gs (Bonhaus et al., 1998) and Gq by WIN55,212 (Lauckner et al., 2005) coupling to the CB1 cannabinoid receptor have been reported. A mutagenesis study further supporting this model was performed. The intracellular carboxyl-terminal H8 of the CB1 cannabinoid receptor deviates from the highly conserved NPXXY(X)5,6F GPCR motif, possessing a Leu instead of a Phe. The study compared the signal transduction capabilities of CB1 with those of an L7.60F mutation and an L7.60I mutation that mimics the CB2 sequence. The two mutant receptors differed from wild type in their ability to regulate G-proteins in the [³⁵S]GTPγS binding assay. Hence it demonstrated that the Leu in the NPXXY(X)5,6L in H8 contributes to maximal activity of the CB1 receptor and thus provides a molecular basis for the differential coupling observed with chemically different agonists (Anavi-Goffer et al., 2007).

Further investigations via biochemical studies directly compared agonist efficacies and potencies at different levels of downstream signaling cascades. By comparing the transcriptional regulation achieved by HU210 and CP55,940, Bosier et al. reported these ligands to have different effects on the expression of tyrosine hydroxylase in a model of neuroblastoma cells (Bosier et al., 2007): while both ligands inhibited CRE-dependent gene transcription, CP55,940 acted as an antagonist of the AP-1-dependent gene transcription, whereas HU210 behaved as a full agonist (Bosier et al., 2008a). In addition, it was demonstrated that among the different MAPK family members,

HU210 was more efficacious in increasing ERK1/2 phosphorylation, while CP55,940 displayed a higher efficacy to activate JNK (Bosier et al., 2008b).

While we know that β -arrestins are involved in the desensitization and internalization of the CB1 receptor as scaffolding proteins (Breivogel and Childers, 2000; Daigle et al., 2008; Jin et al., 1999; Kouznetsova et al., 2002), there has been evidence that β -arrestins can also act as signaling molecules independent of G proteins. The signaling discussed earlier by ORG27569 on the CB1 receptor was reported by the Kendall lab (Ahn et al., 2013) to be mediated specifically by β -arrestin 1 in a G protein-independent manner. siRNA knockdowns of β -arrestin 1 resulted in abolishing ERK1/2, MEK1/2 and Src phosphorylation when treated with ORG27569 but had no significant effect on CP55940-induced phosphorylation. Knockdowns of β -arrestin 2, on the other hand, had no effect on the signaling of either compound, but did dramatically attenuate the internalization of CB1 following CP55940 treatment, co-treatment with ORG27569 and CP55940 and treatment with ORG27569 alone (Ahn et al., 2013). These observations provided the first evidence of signaling-pathway-selective allosteric modulation via CB1.

Interestingly, CB2 ligands show significant functional selectivity. For example, it was found that endocannabinoids activated distinct signaling pathways with varied rank order potencies in Chinese hamster ovary (CHO) cells transfected with CB2 receptors (Shoemaker et al., 2005). The endocannabinoid, 2-AG, was most potent in activating the ERK1/2-MAPK pathway but higher concentrations were needed to inhibit adenylyl cyclase and induce calcium transients. On other hand, noladin ether displayed higher potency in inhibiting adenylyl cyclase as compared to activating ERK1/2 and calcium transients. An even more extreme example of functional selectivity occurs for several commonly used CB2 agonists. Certain CB2 agonists (e.g., the aminoalkylindoles) inhibit adenylyl cyclase and activate ERK1/2, but failed to induce CB2

internalization or inhibition of voltage sensitive calcium channels in AtT20 cells (Atwood et al., 2012). This striking functional selectivity of CB2 receptor agonists enriches the possibilities for developing drugs targeting CB2 receptors (Atwood et al., 2012; Han et al., 2013). Arrestin-biased signaling has been found to produce useful therapeutic effects for other GPCR's (Wisler et al., 2007). To date no CB2 arrestin-biased agonists have been described, so it will be interesting to screen the rich repertoire of CB2 ligands synthesized to determine if arrestin-biased signaling exists for CB2 ligands.

CHAPTER 2

Allosteric modulators of the cannabinoid receptor 1 (CB1)

Parts of this chapter have been incorporated from published material (Mahmoud, M.M., Ali, H.I., Ahn, K.H., Damaraju, A., Samala, S., Pulipati, V.K., Kolluru, S., Kendall, D.A., Lu, D. "Structure–Activity Relationship Study of Indole-2-carboxamides Identifies a Potent Allosteric Modulator for the Cannabinoid Receptor 1 (CB1)." *Journal of Medicinal Chemistry* 56(20) (2013): 7965-7975). Compound synthesis was done by our collaborators from Dr. Dai Lu's lab.

This chapter deals with the identification of novel allosteric modulators of the CB1 receptor and the optimization of both their potency and efficacy, which are two independent parameters contributing to the pharmacological profile of allosteric modulators. The ubiquitous distribution of GPCRs in general and their involvement in virtually all physiological processes makes them extremely attractive targets for drug development. Modulation of interactions between activated GPCRs and their intracellular signal transducers, such as heterotrimeric G-proteins or kinases, is an attractive, yet largely unexplored, paradigm for the treatment of certain diseases. The importance of the regulation of cellular functions has been a topic of increased interest in the hopes of regulating downstream signaling for the treatment of a plethora of associated diseases such as congenital diseases due to constitutively active GPCRs, as well as tumors where GPCRs are often overexpressed. The multiple potential advantages that allosteric modulators present compared to orthosteric drugs have made them the focus of drug development efforts. In particular, the greater ease of achieving higher selectivity for the target because of the higher heterogeneity of the allosteric binding sites. A few compounds have been identified as allosteric modulators of the CB1 receptor and we demonstrate the ability to use these compounds to fine-

tune and specifically target downstream signaling pathways. Therefore, developing new modulators and understanding their regulation of receptor activity will contribute to our understanding of the ligand-specific modulation of CB1 receptor activity.

2.1 Abstract

The cannabinoid CB1 receptor is involved in complex physiological functions. The discovery of CB1 allosteric modulators generates new opportunities for drug discovery targeting the pharmacologically important CB1 receptor. 5-chloro-3-ethyl-*N*-(4-(piperidin-1-yl)phenethyl)-1*H*-indole-2-carboxamide (ORG27569; **1**) represents a new class of indole-2-carboxamides that exhibit allostery of CB1. To better understand the structure-activity relationships (SAR), a group of indole-2-carboxamide analogs were synthesized and assessed for allostery of the CB1 receptor. We found that within the structure of indole-2-carboxamides, the presence of the indole ring is preferred for maintaining the modulator's high binding affinity for the allosteric site, but not for generating allostery on the orthosteric site. However, the C3 substituents of the indole-2-carboxamides significantly impact the allostery of the ligand. A robust CB1 allosteric modulator 5-chloro-*N*-(4-(dimethylamino)phenethyl)-3-pentyl-1*H*-indole-2-carboxamide (**11j**; **LDK1240**) was identified. It showed an equilibrium dissociation constant (K_B) of 167.3 nM with a markedly high binding cooperativity factor ($\alpha=16.55$) and potent antagonism of agonist-induced GTP γ S binding.

2.2 Introduction

The endocannabinoid system is composed of at least two G-protein coupled receptors (GPCRs), CB1 and CB2, a group of endogenous lipid ligands and several catabolic and anabolic enzymes that are involved in the biosynthesis and degradation of the endogenous ligands (Battista et al., 2012). The CB1 receptors are mainly found on neurons of the central and peripheral nervous system (Herkenham et al., 1990b) where they attenuate neurotransmitter release. CB1 receptors are also present at much lower concentrations in some peripheral tissues,

including spleen, tonsil, reproductive organs, endocrine glands, arteries, bone marrow, lungs and heart (Galiegue et al., 1995a; Gerard et al., 1991a; Straiker et al., 1999). Consistent with its widespread distribution, the CB1 receptor regulates a wide variety of physiological functions including neuronal development, neuromodulatory processes, energy metabolism, cardiovascular, respiratory as well as reproductive functions (Howlett et al., 2004; Mackie, 2006; Pertwee, 2006). In addition, it also modulates cell proliferation, motility, adhesion and apoptosis (Guzman et al., 2002; Rajesh et al., 2008).

Like many GPCRs, the CB1 receptor displays a multiplicity of intracellular signal transduction mechanisms. It preferentially couples to $G_{i/o}$ type G proteins while its interaction with G_s (Glass and Felder, 1997) or G_q (Lauckner et al., 2005) is also possible. Activation of the CB1 receptor primarily leads to the inhibition of adenylyl-cyclase thereby reducing cAMP levels in cells. The CB1 receptor also modulates the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase-1 and -2 (ERK1/2), p38 MAPK, p42/p44 MAPK and c-Jun *N*-terminal kinase (JNK) (Turu and Hunyady, 2010). Additionally, CB1 can negatively couple to N- and P/Q-type voltage-gated Ca^{2+} channels and positively couple to A-type and inwardly rectifying K^+ channels (Howlett, 2005). CB1 also interacts with non-G protein partners such as β -arrestins, adaptor protein AP-3, GPCR-associated sorting protein 1 (GASP1) and the adaptor protein FAN to control receptor signaling or trafficking (Howlett et al., 2010; Smith et al., 2010).

The activation of the CB1 receptor by endogenous and exogenous agonists such as anandamide and (-)- Δ^9 -tetrahydrocannabinol (Δ^9 -THC) has been linked to a wide range of pharmacological effects (Pertwee, 1999). Growing evidence suggests that the CB1 receptor exists in multiple active conformations, each of which may display distinct abilities to regulate

individual signaling pathways (Hudson et al., 2010). Thus, the assumption that there is only one active conformation of the CB1 receptor is overly simplistic and is inadequate to explain all cannabinoid-mediated responses. Recently, several small molecules (Figure 2.1) have been identified as allosteric modulators of the CB1 receptor. The representative members include ORG27569 (**1**) (Price et al., 2005), (1-(4-chlorophenyl)-3-(3-(6-(pyrrolidin-1-yl)pyridin-2-yl)phenyl)urea (**2**, PSNCBAM-1) (Horswill et al., 2007), 3-(4-chlorophenyl)-5-(8-methyl-3-p-tolyl-8-azabicyclo[3.2.1]octan-2-yl)isoxazole (**3**, RTI-371) (Navarro et al., 2009) and the endogenous ligand (5*S*,6*R*,7*E*,9*E*,11*Z*,13*E*,15*S*)-5,6,15-trihydroxyicosa-7,9,11,13-tetraenoic acid (**4**, lipoxin A4) (Pamplona et al., 2012). The identification of these ligands indicates the existence of allosteric site(s) on the CB1 receptor and suggests new opportunities for fine-tuning the signaling pathways of the CB1 receptor.

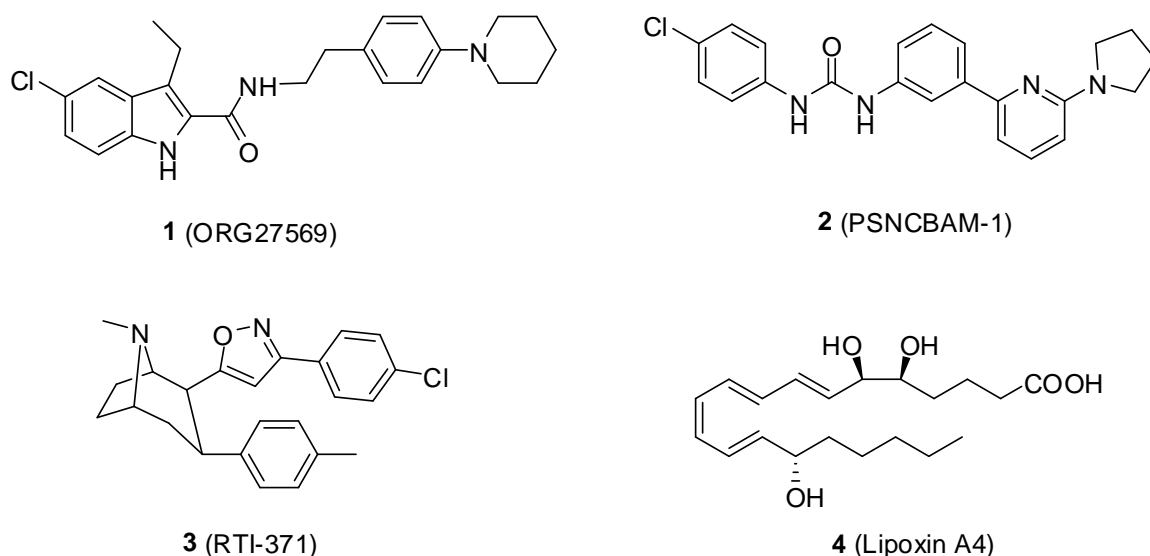


Figure 2.1 Structures of representative allosteric modulators of the CB1 receptor

Indole-2-carboxamide **1** (ORG27569) was the first allosteric modulator identified for the CB1 receptor. It increases specific binding of the tritium labeled CB1 agonist 2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol ([³H]CP55,940) but decreases the binding of the tritium labeled CB1 inverse agonist 5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide ([³H]SR141716A) in membranes from cells expressing the CB1 receptor. Despite acting as an enhancer of agonist binding, it acts as an inhibitor of agonist-induced G_oi protein coupling (Ahn et al., 2012; Price et al., 2005). It was further demonstrated that the ORG27569-induced conformational change of the CB1 receptor leads to cellular internalization of the receptor and downstream activation of ERK signaling (Ahn et al., 2012), both of which have been conventionally recognized as the consequences of CB1 receptor activation. More recent studies showed that the ORG27569-induced activation of ERK1/2 signaling is G_i-independent (Ahn KH et al., 2013; Baillie et al., 2013) and mediated by β -arrestins (Ahn et al., 2012). Hence, ORG27569 not only acts as an allosteric agonist which is able to induce receptor internalization and ERK1/2 phosphorylation on its own, but also functions as an antagonist of CB1 agonist-induced [³⁵S]GTP γ S binding. This supports the notion that allosteric modulators can selectively regulate the functions of the CB1 receptor. Consequently, more functionally-defined pharmacological effects can be achieved for the physiologically and pharmacologically important CB1 receptors. Along with ORG27569, a few other indole-2-carboxamides (Ahn KH et al., 2013; Piscitelli et al., 2012; Price et al., 2005) have also shown allosteric modulation of the CB1 receptor. To study the structure-activity relationships (SAR) of this class of compounds, we synthesized a series of analogs of ORG27569 with the general structure shown in Figure 2.2, and individual structures shown in Figures 2.3 and 2.4.

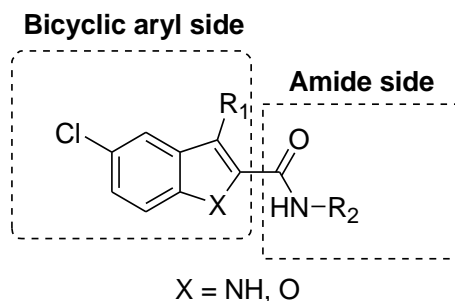


Figure 2.2 General structures of indole- and benzofuran-2-carboxamide analogs.

Previous results from us (Ahn KH et al., 2013) and others (Piscitelli et al., 2012) indicated that the alkyl substituents at the C3 position have a profound influence on the ligand's allostery. For instance, replacing compound ORG27569's C3 ethyl group with a *n*-pentyl group led to the enhancement of the allosteric effects, which is reflected by an improvement in the cooperativity factor α from 6.95 (**1**; ORG27569) to 17.6 (**11a**; ICAM-b) (Ahn KH et al., 2013). The α cooperativity factor is a value that quantifies the magnitude by which the affinity of one ligand is changed by the other ligand when both are bound to the receptor simultaneously to form the ternary complex. When α is 1.0, the test modulator does not affect the binding of the orthosteric ligand. If α is less than 1.0, the test modulator reduces orthosteric ligand binding (negative allosteric modulation on orthosteric ligand binding). If α is greater than 1.0, the modulator increases orthosteric ligand binding (positive allosteric modulation) (Christopoulos and Kenakin, 2002). This prompted us to continue the investigation of the C3 substituents. Therefore, alkyl groups of different lengths, a cyclic ring and an aromatic ring were introduced at the C3 position to elucidate the SAR. Since we previously found that **11a** (ICAM-b) displayed a remarkably enhanced binding cooperativity factor α (Ahn KH et al., 2013), we chose to use it as

a template to vary the amines of the amide side, with the aim of improving the affinity of the allosteric modulator to its allosteric site, which is denoted by its equilibrium dissociation constant (K_B) (Christopoulos and Kenakin, 2002). The above efforts yielded compounds **11b-11k** (Figure 2.3, Table 2.1, Table 2.2 and Table 2.4). Next, to elucidate the significance of the indole ring in the indole-2-carboxamide structure, we replaced the indole ring of **11a** (ICAM-b) with other bicyclic aromatic rings such as the benzofuran ring. This effort led to the compounds represented by the structure **13a-13d** (Figure 2.4, Table 2.3 and Table 2.4).

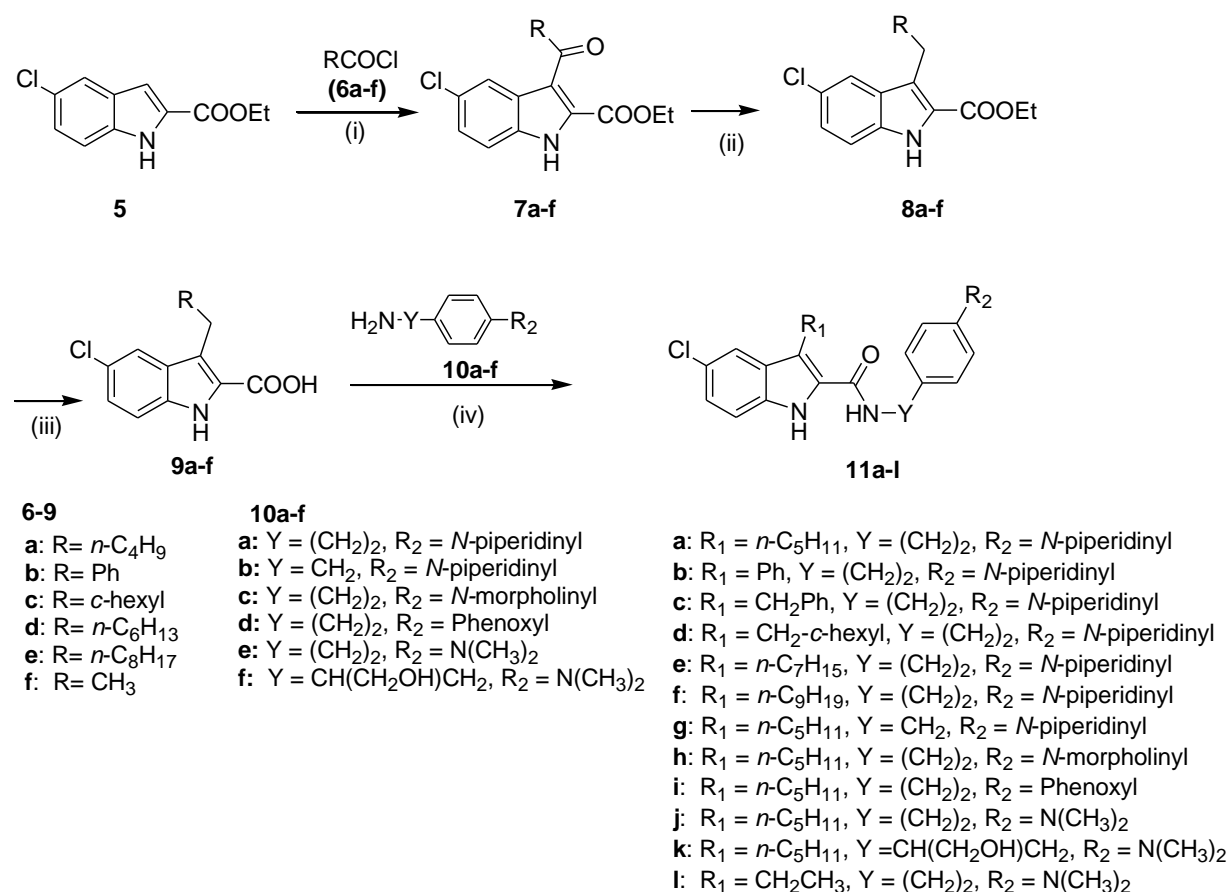


Figure 2.3 Syntheses of indole-2-carboxamide analogs (11a-l). *Reagents and Conditions:* (i)

AlCl₃, 1,2-dichloromethane, reflux, 2-3 h; (ii) (Et)₃SiH, CF₃COOH, 0 °C - rt, 4-12 h; (iii) NaOH,

EtOH, reflux, 2 h; (iv) BOP, DIPEA, DMF, rt, 4-12 h.

2.3 Materials and Methods

Chemistry. The syntheses of the indole-2-carboxamide analogs (**11**) were achieved through the methods illustrated in Figure 2.3.

The C3 substituents were introduced through acylation of the commercially available ethyl 5-chloroindole-2-carboxylate (**5**) by Friedel-Crafts reaction catalyzed by aluminum chloride. Acylation of **5** with the selected acyl chloride (**6**) provided the desired 3-acyl-5-chloroindole-2-carboxylates (**7**). Reduction of the carbonyl group of **7** by triethylsilane generated the C3 alkylated 5-chloroindole-2-carboxylates (**8**), which were then hydrolyzed in basic conditions to yield the key intermediate acids (**9**). The final compounds (**11a-11l**) were prepared by coupling commercially available amines (**10a-f**) with the indole-2-carboxylic acids (**9a-f**) and commercially available 5-chloro-3-phenyl-2-carboxylic acid individually in the presence of BOP and diisopropylethyl amine (DIPEA) in anhydrous DMF at room temperature. Similarly, benzofuran-2-carboxamides (**13a-d**) were prepared according to the methods depicted in Figure 2.4.

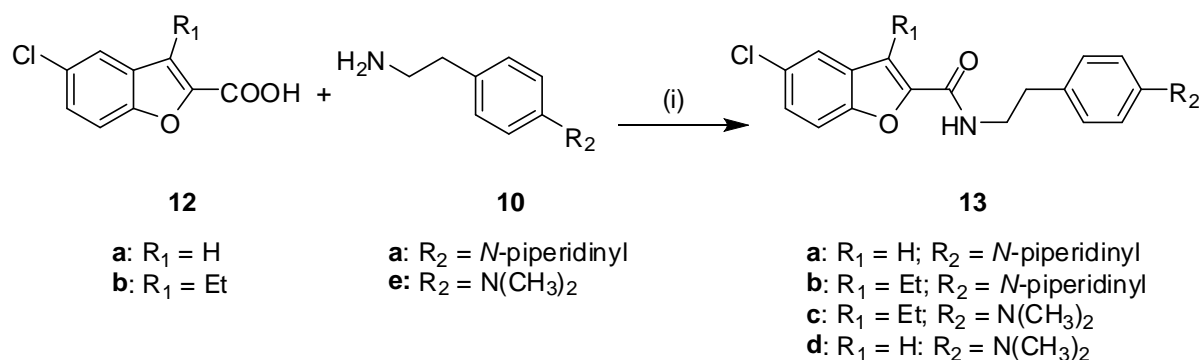


Figure 2.4 Syntheses of benzofuran-2-carboxamide analogs **13a-d**. *Reagents and Conditions:*

(i) BOP, DIPEA, DMF, rt, 4-12 h.

The commercially available 5-chlorobenzofuran-2-carboxylic acid **12a** (AlfaAesar, Ward Hill, MA) or 5-chloro-3-ethyl-benzofuran-2-carboxylic acid **12b** (Princeton BioMolecular Research, Monmouth Junction, NJ) was coupled with selected amines (**10a** or **10e**) in the presence of BOP and DIPEA in anhydrous DMF at room temperature to provide the desired benzofuran-2-carboxamides (**13a-13d**). Most of the final compounds of the **11** and **13** series had acceptable solubility in organic solvents and were therefore purified through chromatography. However, compounds **11b**, **11c** and **11d** had very poor solubility in most of the common organic solvents, such as acetone, ethyl acetate, tetrahydrofuran, dichloromethane, diethyl ether, ethanol and methanol. They were therefore purified using a combination of trituration, recrystallization and chromatography as detailed in the synthesis section.

Compounds. Tested compounds (**11a-11l** and **13a-13d**) were synthesized for this study except **1**, which was purchased from Tocris Bioscience (Minneapolis, MN). Compounds **11a** (Price et al., 2005) and **11l** (Piscitelli et al., 2012) have previously been reported and were used in this study for comparison.

CB1 Expression and Membrane Preparation. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 3.5 mg/ml glucose at 37 °C in 5% CO₂. For transient receptor expression, HEK293 cells were plated at 800,000 cells/100-mm dish on the day prior to transfection. The cells were transfected using the calcium phosphate precipitation method (Chen and Okayama, 1987). 24 h post-transfection, membranes of transfected cells were prepared as described previously (Ahn et al., 2009a).

Radioligand Binding Assay. Membranes from HEK293 cells expressing the CB1 wild-type receptor were prepared as previously described (Ahn et al., 2009a). In assays used to determine the cooperativity between allosteric and orthosteric ligands, approximately 4 µg of membrane

was incubated at 30 °C for 60 min with a fixed tracer ($[^3\text{H}]\text{CP55,940}$; 147.9 Ci/mmol, PerkinElmer Life Sciences (Boston, MA)) concentration typically at its K_d (2.5 nM) which was determined from a saturation binding isotherm. Binding assays were performed with at least nine concentrations of unlabeled allosteric compound (ranging between 100 pM and 100 μM) as described previously (Ahn et al., 2012). Nonspecific binding was determined in the presence of 1 μM unlabeled CP55,940. Reactions were terminated by adding 250 μL of TME buffer containing 5% BSA followed by filtration with a Brandel cell harvester through Whatman GF/C filter paper. Radioactivity was measured using liquid scintillation counting. The total assay volume and the amount of membrane samples were adjusted to avoid ligand depletion by keeping the bound ligand less than 10% of the total.

GTP γ S Binding Assay. GTP γ S binding assays were performed as described previously (Ahn et al., 2012). Briefly, 7.5 μg of membranes were incubated for 60 min at 30°C in a total volume of 200 μL GTP γ S binding assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl_2 , 0.2 mM EGTA, and 100 mM NaCl) with unlabeled CP55,940 (at least nine different concentrations were used ranging between 100 pM and 100 μM), 0.1 nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ (1250 Ci/mmol; PerkinElmer Life Sciences, Boston, MA), 10 μM GDP (Sigma, St. Louis, MO), and 0.1% (w/v) BSA in the absence and presence of varying concentrations of the allosteric compounds as indicated. The basal GTP γ S binding was measured in the absence of orthosteric ligand and with 5 μM GDP to increase the window of basal activity. Nonspecific binding was determined with 10 μM unlabeled GTP γ S (Sigma, St. Louis, MO). The reaction was terminated by rapid filtration through Whatman GF/C filters. The radioactivity trapped in the filters was determined by liquid scintillation counting.

Ligand and GTP γ S Binding Data Analysis. All ligand binding assays and GTP γ S binding assays were carried out in duplicate. Data are presented as the mean \pm S.E. value or the mean with the corresponding 95% confidence limits from at least three independent experiments. The interactions between the orthosteric radiolabeled agonist [3 H]CP55,940 and the test modulators were analyzed using Prism 6.0 (GraphPad Software Inc., San Diego, CA) according to the following allosteric ternary complex model of interaction (Price et al., 2005).

$$Y = \frac{[A]}{[A] + \frac{K_A(1 + [B]/K_B)}{(1 + \alpha[B]/K_B)}}$$

Equation 1.

Where Y denotes the specific bound orthosteric ligand divided by the total concentration of orthosteric ligand $[A]$. $[B]$ denotes the concentration of the allosteric ligands. K_A and K_B are equilibrium dissociation constants for their respective ligands, and α is a cooperativity factor that quantifies the allosteric interaction in terms of magnitude and direction between the two ligands when they simultaneously occupy the receptor. When α is 1.0, the tested modulator does not change orthosteric ligand binding. If α is less than 1.0, the tested modulator reduces orthosteric ligand binding (negative allosteric modulation on orthosteric ligand binding). If α is greater than 1.0, the modulator increases orthosteric ligand binding (positive allosteric modulation). For this analysis, the value of $[A]$ was kept constant and was the average of radioactive orthosteric ligand concentration employed in the binding assays. K_A was also fixed as a constant and was determined from previous CP55,940 saturation binding assays, whereas values of α and K_B were determined by nonlinear regression.

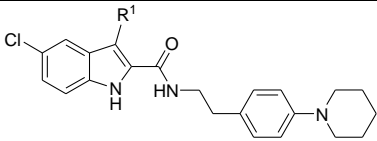
Synthesis. All chemical reagents and solvents were purchased from Sigma-Aldrich Chemical Company unless specified otherwise and used without further purification. All anhydrous reactions were performed under a static argon atmosphere in dried glassware using anhydrous solvents. Organic phases in work up were dried over anhydrous Na₂SO₄, and removed by evaporation under reduced pressure. The crude compounds were purified by a Combiflash *Rf* chromatography system (Teledyne Technologies, Inc, Thousand Oaks, CA) unless specified otherwise. Purities of the intermediates were established by Thin-layer Chromatography (TLC), melting point, ¹HNMR, and mass spectrometry. Analytical Thin-layer Chromatography (TLC) was run on pre-coated silica gel TLC aluminum plates (Whatman[®], UV₂₅₄, layer thickness 250 μm), and the chromatograms were visualized under ultraviolet (UV) light. Melting points were determined on a capillary Electrothermal[®] melting point apparatus and are uncorrected. ¹HNMR spectra of intermediates were recorded on a Bruker Avance DPX-300 spectrometer operating at 300 MHz. ¹HNMR spectra of final compounds were recorded on a Bruker AV-500 spectrometer operating at 500 MHz. All NMR spectra were recorded using CDCl₃ or DMSO-d₆ as solvent unless otherwise stated and chemical shifts are reported in ppm (parts per million) relative to tetramethylsilane (TMS) as an internal standard. Multiplicities are indicated as br (broadened), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broadened singlet) and coupling constants (*J*) are reported in hertz (Hz). Low resolution mass spectra were performed at the School of Chemical Sciences, University of Illinois at Urbana-Champaign. The purity of each tested compound was analyzed by combustion elemental analysis and was confirmed to be greater than 99%. Corresponding combustion elemental analysis was conducted in Roberson Microlit laboratories (Madison, NJ). The specific details for the synthesis of each compound can be found in the original publication.

2.4 Results and Discussion

To investigate the allostery of the newly synthesized compounds for the CB1 receptor, we evaluated each compound's binding cooperativity factor α to quantify the magnitude of the allosteric effect on orthosteric ligand binding. Some of the compounds that showed α values comparable to that of ORG27569 were selected for further testing their capability to modulate the agonist CP55,940-induced G protein coupling. Additionally, to evaluate the effectiveness of the modulator binding to the allosteric site, their equilibrium dissociation constants K_B were also determined. The α and K_B values were analyzed according to the allosteric ternary complex model ((Price et al., 2005); See equation (1) in experimental section).

The results for compounds with structural variation at the C3 position are shown in Table 2.1. We found that replacing the C3 alkyl group of ORG27569 with a phenyl ring completely abolished the allosteric effect of the ligand (**11b** (LDK1225) compared to previously tested ORG27569 (Ahn et al., 2012) and **11a** (ICAM-b) (Ahn KH et al., 2013)), whereas a benzyl group at C3 was somewhat tolerated but with a significantly increased K_B value indicative of lower affinity (**11c**; LDK1230). Replacing the phenyl ring of the benzyl group in ligand **11c** (LDK1230) with a cyclohexyl group (**11d**; LDK1232) further increased K_B although allosteric modulatory effects were retained. These data suggest that aliphatic or aromatic rings on the C3 alkyl group significantly decrease the affinity of the allosteric ligand. Therefore, linear alkyl chains as C3 substituents were employed for further exploration. The C3-*n*-heptyl (**11e**; LDK1218) and C3-*n*-nonyl (**11f**; LDK1219) analogs of ORG27569 were synthesized. Although **11e** and **11f** displayed appreciable K_B values, the α values substantially decreased relative to **11a** (ICAM-b), suggesting that a C3-*n*-pentyl is optimal for maximum allosteric modulatory effects.

Table 2.1 Allosterism of indole-2-carboxamide analogs **11a-f**

				
Cmpd	R ¹	K _B (nM) ^a	α ^b	[³⁵ S]GTPγS binding ^c
1 ^d	Et	217.3 (170.3-277.2)	6.95	Antagonizing
11a ^e	<i>n</i> -C ₅ H ₁₁	469.9 (126.2-1750)	17.60	Antagonizing
11b	Ph	ND	1.0	-
11c	CH ₂ Ph	1067 (347.3-3344)	11.37	-
11d	CH ₂ - <i>c</i> -hexyl	2408 (570.2-8151)	9.84	-
11e	<i>n</i> -C ₇ H ₁₅	651.2 (81.51-5203)	7.38	-
11f	<i>n</i> -C ₉ H ₁₉	259.7 (87.56-770)	6.80	Antagonizing

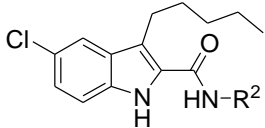
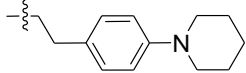
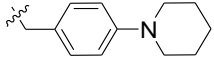
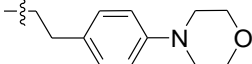
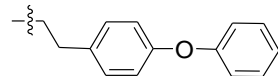
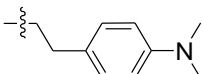
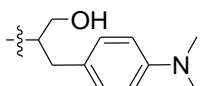
^a K_B: equilibrium dissociation constant.^b α: binding cooperativity factor between CP55,940 and the tested allosteric modulators.^c Effects on CP55,940-induced [³⁵S]GTPγS binding in the presence of allosteric modulator.^d Data cited for **1** (ORG27569) is from our earlier report and given for comparison (Ahn et al., 2012).^e Data cited for **11a** (ICAM-b) is from our earlier report and given for comparison (Ahn KH et al., 2013).ND: no detectable modulation of [³H]CP55,940 binding using up to 32 μM of test compound.

- : not performed.

Overall, this series of analogs indicates that the type and size of the substituents at the C3 position of indole-2-carboxamides have a critical role in the allosteric effects on the CB1 receptor. Our results support the hypothesis of the presence of a hydrophobic sub-pocket in close

proximity of the C3-position of ORG27569 when it is bound to the allosteric site. This sub-site could only be occupied by linear alkyl groups but not cyclic structures.

Table 2.2. Allostery of indole-2-carboxamide analogs **11g-k**

				
Cmpd	R ²	K _B (nM) ^a	α ^b	[³⁵ S]GTPγS binding ^c
11a^d		469.9 (126.2-1750)	17.06	Antagonizing
11g		ND	1.0	-
11h		508.4 (96.34-2683)	10.0	Antagonizing
11i		2335 (695.3-7542)	13.15	-
11j		167.3 (23.39-1197)	16.55	Antagonizing
11k		1018 (81.51-5203)	13.27	-

^a K_B: equilibrium disassociation constant.

^b α: binding cooperativity factor between CP55,940 and the tested allosteric modulators.

^c Effects on CP55,940-induced GTPγS binding in the presence of allosteric modulator.

^d Data cited for **11a** (ICAM-b) is from our earlier report and given for comparison (Ahn KH et al., 2013).

ND: no detectable modulation of [³H]CP55,940 binding using up to 32 μM of test compound.

- : not performed.

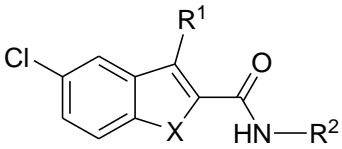
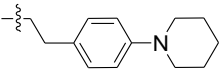
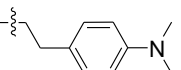
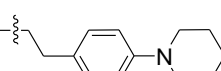
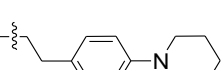
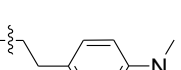

Among the C3-alkyl analogs of ORG27569, the previously tested compound **11a** (ICAM-b) (Ahn KH et al., 2013) exhibited the most robust allostery ($\alpha=17.6$) of the CB1 receptor. In an earlier communication, we reported that **11a** (ICAM-b) promoted the phosphorylation of CB1 downstream effectors ERK1/2 in a G_i -independent but β -arrestin-1 mediated manner (Ahn KH et al., 2013). Although a markedly high α factor was achieved, **11a** (ICAM-b) exhibited moderately weak affinity for CB1, as reflected by its relatively high K_B value (469.9 nM). Hence, this compound was selected for further modification on the amide side with the aim of exploring SAR and improving the affinity to the allosteric site on CB1. This effort led to the synthesis of the compounds presented in Table 2.2.

When a methylene group of the ethylene linker between the piperidinyphenyl group and the amide bond was removed, it resulted in complete elimination of the allostery of the ligand (**11g** vs **11a**). This indicates that the substituted phenyl ring on the amide side must be separated from the amide bond by at least a 2-carbon linkage. This ethylene moiety was therefore retained for the rest of this series of compounds (**11h-11k**). When the piperidiny group was replaced by a structurally related but more hydrophilic morpholinyl group (**11h**), the K_B value increased and the cooperativity factor α decreased. This suggests that the hydrophilic substitution on the amino group decreased the affinity of the ligand for the allosteric site and reduced the allosteric modulation of the orthosteric site. Replacing the piperidiny group with a phenoxy group (**11i**) resulted in a significant increase in the K_B value, but the high binding cooperativity (α) was retained. Next, swapping the cyclic piperidiny group with an acyclic dimethyl amino group on the phenyl ring led to **11j** (LDK1240) which exhibited the most robust allostery ($K_B = 167.3$ nM; $\alpha= 16.55$) reported so far for the CB1 receptor. A low K_B value and a high α factor, as achieved in **11j**, are significant and indicate the low concentration of compound **11j** that is needed to

achieve the increase in affinity this allosteric modulator has on CP55,940 binding; i.e. a smaller concentration of **11j** (compared to all CB1 allosteric modulators) is needed to achieve its robust allosteric effect on agonist binding. This is a key factor that is taken into consideration in therapeutic drug treatments. Moving forward to introduce a hydroxymethyl group at the α position of the ethylene linker of **11j** (LDK1240) reduced its affinity, but a relatively good binding cooperativity (α value) was maintained (**11k**; LDK1243). Overall, in this series of compounds, we found that structural modifications on the amide side generally do not significantly affect the cooperativity factor α , whereas the influence on the K_B of the allosteric modulators is much more profound.

To date, the majority of structural modifications on ORG27569 have been focused on the substitution of the indole ring (Ahn KH et al., 2013; Price et al., 2005) and the variation of the amide side (Piscitelli et al., 2012). To unveil the importance of the indole ring on the K_B and α values, we synthesized several benzofuran-2-carboxamides and compared them with their indole-2-carboxamide counterparts. The results are shown in Table 2.3.

Table 2.3. Allostery of indole-/benzofuran-2-carboxamide analogs (**11l** and **13a-d**)

						
Cmpd	X	R ¹	R ²	K _B (nM) ^a	α ^b	[³⁵ S]GTPγS binding ^c
1^d	NH	Et		217.3 (170.3-277.2)	6.95	Antagonizing
11l^e	NH	Et		207.4 (155.9-2759)	19.68	-
13a	O	H		ND	1.0	-
13b	O	Et		2594 (1005-6696)	18.04	Antagonizing
13c	O	Et		627.1 (151.5-2596)	11.59	-
13d	O	H		ND	1.0	-

^a K_B: equilibrium disassociation constant.^b α: binding cooperativity factor between CP55,940 and the tested allosteric modulators.^c Effects on CP55,940-induced GTPγS binding in the presence of allosteric modulator.^d Data cited for **1** is from our earlier report and given for comparison (Ahn et al., 2012).^e Compound **11l** was reported earlier (Piscitelli et al., 2012) but was synthesized here and pharmacologically characterized for comparison with other analogs under the same conditions.ND: no detectable modulation of [³H]CP55,940 binding using up to 32 μM of test compound.

- : not performed.

We learned that replacing the indole ring of ORG27569 with a benzofuran ring (**13b**; LDK1210) resulted in a significant loss of the affinity of the ligand (K_B = 2594 nM), however, the binding cooperativity with the orthosteric ligand was markedly enhanced (**13b** vs ORG27569). Similarly, replacing the indole ring of a potent CB1 allosteric modulator reported

earlier (**11l**) (Piscitelli et al., 2012) with a benzofuran ring (**13c**; LDK1238) led to a 3-fold increase in K_B while the high binding cooperativity ($\alpha=11.59$) was well-preserved. These results suggest that the bicyclic aryl moiety (i.e. the indole ring, Figure 2.2) significantly impacts the ligand's affinity compared to its impact on the binding cooperativity. The indole ring, therefore, accounts more for the ligand's capability to bind to the allosteric site, but is less influential on the ligand's capability to modulate the orthosteric site. In the benzofuran-2-carboxamides, removal of the C3 alkyl group (i.e. **13c** vs **13d**) eliminated the allosteric effects completely.

To further characterize the nature of the compounds that show positive allosteric modulation on the binding of the CB1 orthosteric agonist [^3H]CP55,940, compounds **11f** (LDK1219), **11h** (LDK1231), **11j** (LDK1240) and **13b** (LDK1210) were chosen for their strong allosteric properties (i.e. low K_B value or high α value) as representatives from each series of analogs and further tested for their effects on CP55,940-induced and basal G protein coupling using a [^{35}S]GTP γ S binding assay. Figure 2.5 shows that **11f**, **11h**, **11j** and **13b** decrease agonist-induced G protein coupling to the CB1 receptor in a concentration-dependent manner similar to the effects exhibited by the previously reported ORG27569, **2** (PSNCBAM-1), **11a** (ICAM-b) and **4** (Lipoxin A4) (Ahn KH et al., 2013; Ahn et al., 2012; Horswill et al., 2007; Pamplona et al., 2012; Price et al., 2005). Remarkably, compound **11j** exhibited a robust inhibitory effect in CP55,940-induced GTP γ S binding (Figure 2.5C) evident by a progressive decrease in the E_{max} values with an increasing concentration of **11j** (LDK1240). For example, at 0.1 μM of **11j**, the CP55,940-induced [^{35}S]GTP γ S binding was substantially reduced ($E_{max} = 53.4 \pm 3.1$ fmol/mg) compared with that in the absence of **11j** ($E_{max} = 113.0 \pm 2.6$ fmol/mg) while compounds **11f**, **11h** and **13b** exhibited comparable levels of reduction at 3.2 μM and higher ($E_{max} = 61.4 \pm 2.0$, 58.2 ± 1.07 and 59.5 ± 1.4 fmol/mg, respectively; Figure 2.5A, B and D). Treatment with 1 μM

of **11j** showed complete inhibition of CP55,940-induced [35 S]GTP γ S binding ($E_{max} = 36.04 \pm 2.0$ fmol/mg) while at least 10 μ M of **11f**, **11h** and **13b** was needed to achieve a similar effect. Because of the robust allosterity that compound **11j** displayed, the basal levels of G protein coupling in the absence of CP55,940 (Figure 2.5E) were also examined. Compound **11j** also antagonized the basal level of [35 S]GTP γ S in a concentration-dependent manner. Treatment with 10 μ M of **11j** resulted in complete inhibition of this basal activity. While this pattern of G protein coupling is consistent with an inverse agonist's profile in its ability to inhibit basal and CP55,940-induced GTP γ S binding, when collectively taken with its enhancement of the orthosteric agonist CP55,940 binding, it indicates that these compounds are allosteric modulators that are likely able to selectively regulate the functions of the CB1 receptor. To date, compound **11j** (LDK1240) is the most effective allosteric antagonist of CB1 G protein coupling and surpasses both ORG27569 and **11a** (ICAM-b) which we also found inhibited G protein coupling at 3.2 μ M (Ahn KH et al., 2013; Ahn et al., 2012). Moreover, this pattern is consistent with the robust allosterity of compound **11j** as reflected by its binding parameters (Table 2.2).

A recent study of the CB1 receptor explored the mechanism of action of ORG27569 using a site-directed fluorescence labeling approach (Fay and Farrens, 2012). The results showed that the agonist CP55,940 induces a movement in the cytoplasmic end of TM6, which accompanies G protein coupling. Upon co-treatment with ORG27569, this agonist-induced movement is blocked. This state of CB1 may explain how ORG27569 and its analogs can elicit differential effects on CB1 agonist affinity and efficacy (i.e. a positive allosteric modulator of binding but an antagonist of G protein coupling activity).

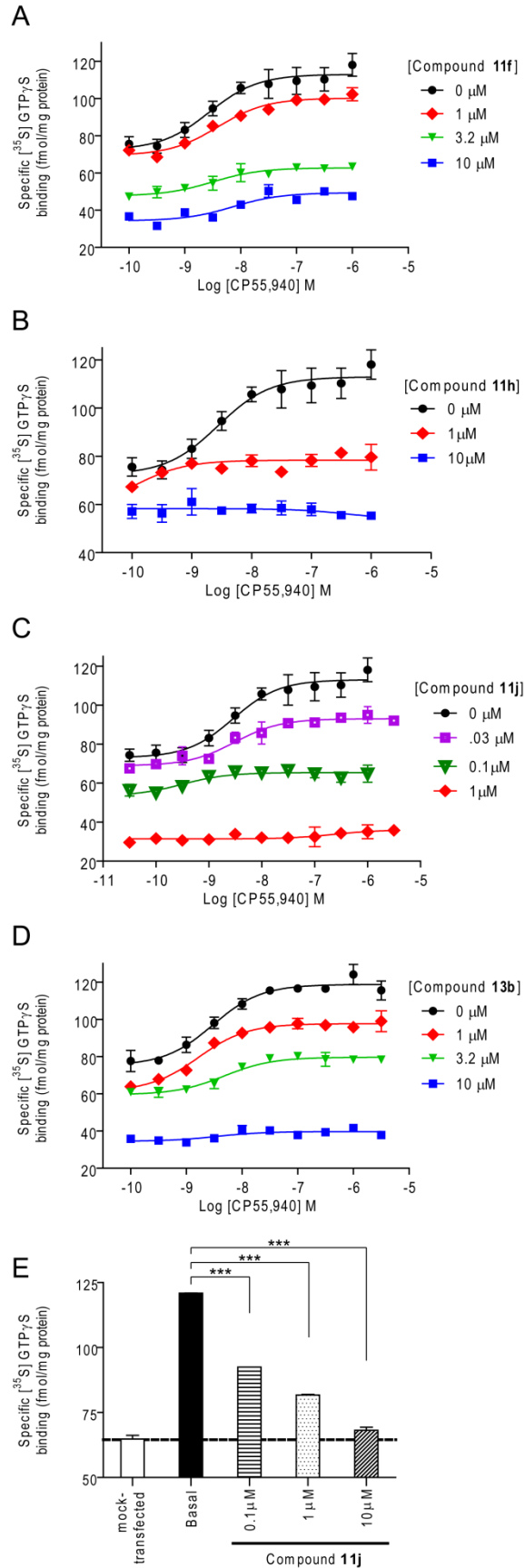


Figure 2.5 Dose-response curves for CP55,940-induced [³⁵S]GTPγS binding to human embryonic kidney (HEK293) cell membranes expressing the CB1 wild-type receptor in the absence and presence of compounds **11f** (A), **11h** (B), **11j** (C) and **13b** (D) at the indicated concentrations. The basal level of [³⁵S]GTPγS binding was also measured in the absence of any orthosteric ligand and the inhibition of it was tested at the indicated concentration of compound **11j** (E). Data are presented as specific binding of GTPγS to the membranes. Non-specific binding was determined in the presence of 10 μM unlabeled GTPγS. Each data point represents the mean ± S.E. (*error bars*) of at least three independent experiments performed in duplicate. Statistical significance of the differences in each concentration of **11j** compared to basal (i.e. no ligand) in (E) was assessed using one-way analysis of variance and Bonferroni's post-hoc test; ***, *p* < 0.005. The *dotted line* in (E) indicates the level of non CB1-mediated GTPγS binding obtained from [³⁵S]GTPγS binding to the mock-transfected membrane sample.

2.5 Summary

We synthesized a group of analogs of the CB1 allosteric modulator ORG27569 to understand the structure activity relationship of indole-2-carboxamides in allosteric modulation of the CB1 receptor. Within the structure of indole-2-carboxamides, the presence of the indole ring seems to be more influential on the ligand's binding affinity (K_B) to the allosteric site than on the binding cooperativity (α) between the allosteric site and the orthosteric site. The C3 alkyl groups on the indole-2-carboxamides profoundly impacted the allostery of the ligand. Through structural modification, we identified a robust CB1 allosteric modulator **11j** (LDK1240) which showed an equilibrium dissociation constant K_B of 167.3 nM with a markedly high binding

cooperativity factor ($\alpha=16.55$) and potent inhibition of GTP γ S binding. Although a small binding constant K_B for the allosteric modulator is not necessary for maximum binding cooperativity with the CB1 orthosteric ligand, CB1 modulators with high binding affinities to the allosteric site and high cooperativity towards the orthosteric site are desirable for therapeutic applications.

2.6 Supporting Information

Details of synthesis and compound characterization of intermediates **7a-f** to **9a-f** can be found in the supporting information for the associated paper. This material is available free of charge via the internet at <http://pubs.acs.org>.

Table 2.4. A guide to compound names and numbers

	Compd.	Chemical Name
ORG27569	1	5-chloro-3-ethyl-N-(4-(piperidin-1-yl)phenethyl)-1H-indole-2-carboxamide
ICAM-b	11a	5-chloro-3-pentyl-N-(4-(piperidin-1-yl)phenethyl)-1H-indole-2-carboxamide
LDK		
1225	11b	5-chloro-3-phenyl-N-(4-(piperidin-1-yl)phenethyl)-1H-indole-2-carboxamide
1230	11c	3-benzyl-5-chloro-N-(4-(piperidin-1-yl)phenethyl)-1H-indole-2-carboxamide
1232	11d	5-chloro-3-(cyclohexylmethyl)-N-(4-(piperidin-1-yl)phenethyl)-1H-indole-2-carboxamide
1218	11e	5-chloro-3-heptyl-N-(4-pipridin-1-yl)phenethyl)-1H-indole-2-carboxamide
1219	11f	5-chloro-3-nonyl-N-(4-pipridin-1-yl)phenethyl)-1H-indole-2-carboxamide
1221	11g	5-chloro-3-pentyl-N-(4-(piperidin-1-yl)benzyl)-1H-indole-2-carboxamide
1231	11h	5-chloro-N-(4-morpholinophenethyl)-3-pentyl-1H-indole-2-carboxamide
1224	11i	5-chloro-3-pentyl-1H-indole-2-carboxylic acid [2-(4-phenoxy-phenyl)-ethyl]-amide
1240	11j	5-chloro-N-(4-(dimethylamino)phenethyl)-3-pentyl-1H-indole-2-carboxamide
1243	11k	5-chloro-N-(1-(4-(dimethylamino)phenyl)-3-hydroxypropan-2-yl)-3-pentyl-1H-indole-2-carboxamide
1202	13a	5-chloro-N-(4-(piperidin-1-yl)phenethyl)benzofuran-2-carboxamide
1210	13b	5-chloro-3-ethyl-N-(4-(piperidin-1-yl)phenethyl)benzofuran-2-carboxamide
1238	13c	5-chloro-N-(4-(dimethylamino)phenethyl)-3-ethylbenzofuran-2-carboxamide
1239	13d	5-chloro-N-(4-(dimethylamino)phenethyl)benzofuran-2-carboxamide

2.7 Conclusions and Future Directions

The results from this study along with another indole-2-carboxamide study published from our lab (Khurana et al., 2014) elucidated key structural requirements of indole-2-carboxamides for allosteric modulation of the CB1 receptor. The critical structural factors include (see Figure 2.2): (1) the chain length of the C3-alkyl group is critical with n-propyl being preferred for allosteric modulation of orthosteric ligand binding and n-hexyl being preferred for enhancing affinity of the allosteric modulator to the CB1 receptor, (2) an electron withdrawing group needs to reside at the C5 position of the indole ring (Figure 2.2), (3) the linker between the amide bond and the phenyl ring B (Figure 2.2) must be an ethylene group whereas shortening or elongating the linker abolishes allosteric effects, and (4) the substituent on the phenyl ring B explicitly influences both the binding to the allosteric site and the binding cooperativity with the orthosteric ligand, with the N,N-dimethyl amino group being preferred over the piperidinyl functionality of the prototypical CB1 allosteric modulator ORG27569. These SAR studies will guide the future design and synthesis of more potent CB1 allosteric modulators based on the indole-2-carboxamide scaffold.

In recent years, drug discovery targeting allosteric sites of GPCRs has gained momentum (Muller et al., 2012). This approach offers a number of potential advantages over drugs targeting the orthosteric site, such as improved selectivity across receptor subtypes and the capability to maintain the spatial and temporal signaling profile of the endogenous ligand. The nature of biased signaling of some CB1 allosteric modulators such as **1** (ORG27569) (Ahn et al., 2012; Baillie et al., 2013), **11a** (ICAM-b) (Ahn KH et al., 2013), and **4** (Lipoxin A4) (Figure 2.1) (Pamplona et al., 2012) suggested a possibility to selectively regulate specific functions of the CB1 receptor and this may be therapeutically beneficial. The activation and inactivation of the

CB1 receptor have been linked to therapeutic implications for many diseases such as drug addiction, anxiety, depression, obesity, neurodegeneration, cancer and inflammation. ORG27569 has been the prototype to date for allosteric modulators of the CB1 receptor. Growing evidence indicates that ORG27569 (Ahn et al., 2012; Baillie et al., 2013) and its analogs (Ahn KH et al., 2013) are functionally positive allosteric modulators of the CB1 receptor although they antagonize G_i-protein coupling activity to CB1. This class of compounds may selectively transduce the orthosteric agonist signals through the ERK1/2 phosphorylation pathway. Very recently, Pamplona et al demonstrated using *in vivo* experiments that the CB1 positive allosteric modulator Lipoxin A4 is protective against β -amyloid-induced neurotoxicity (Pamplona et al., 2012). The therapeutic potential of indole-2-carboximides represented by ORG27569 as neuroprotective agents remains to be explored.

Future optimization of the allosteric modulators presented in this chapter, to increase their binding affinity to CB1, reflected by a decreased K_B, while also maintaining a high binding cooperativity factor, is desirable for therapeutic applications. In addition, expanding the SAR studies for other scaffolds that have shown promise as allosteric modulators, including the diarylurea backbone of **2** (PSNCBAM-1) or the 3-phenyltropane backbone of **3** (RTI-371) (see Figure 2.1 for structures) will broaden possibilities and will aid in the future design and synthesis of more robust allosteric modulators and increase our understanding of ligand-specific modulation of the pharmacologically and physiologically vital CB1 receptor.

It would also be of great interest to perform *in vivo* studies of the indole-2-carboxamide compounds, namely compound **11j** (LDK1240), that have a higher binding affinity to CB1 than ORG27569 and also a higher cooperativity factor. The question of whether the actions of these allosteric modulators, that have been characterized *in vitro*, translate to the whole animal is

crucial to their benefit in therapeutics. An in vivo study (Horswill et al., 2007) of **2** (PSNCBAM-1; Figure 2.1) showed that in an acute rat feeding model, PSNCBAM-1 decreased food intake and body weight. A more recent study (Gamage et al., 2014) investigated the effects of compound **1** (ORG27569) in a well-established mouse behavioral assay. The study showed that similar to the orthosteric CB1 inverse agonist rimonabant (SR141716A), ORG27569 reduced food intake. The study also questioned whether the anorectic effect presented by ORG27569 occurred independently of the CB1 receptor (Gamage et al., 2014) and was non CB1-mediated since their results showed that ORG27569 did not elicit CB1-mediated effects alone and lacked efficacy in altering antinociceptive, cataleptic, and hypothermic actions of the orthosteric agonists anandamide, CP55,940, and Δ^9 -tetrahydrocannabinol. These findings question the utility of ORG27569 as a ‘gold standard’ CB1 allosteric modulator and underscore the need for the development of other CB1 allosteric modulators, such as those presented in this chapter, with pharmacology that translates from the molecular level to the whole animal.

CHAPTER 3

Novel Cannabinoid CB1 Receptor Inverse Agonists from the class of Benzyhydryl Piperazine Analogs

Parts of this chapter have been incorporated from published material (Mahmoud, M.M, Olszewska, T., Liu, H., Shore, D.M., Hurst, D.P., Reggio, P.H., Lu, D., and Kendall, D.A. (4-(Bis(4-fluorophenyl)methyl)piperazin-1-yl)(cyclohexyl)methanone hydrochloride (LDK1229): A New Cannabinoid CB1 Receptor Inverse Agonist from the class of benzhydryl piperazine analogs. *Molecular Pharmacology*, 87(2) (2015): 197-206). Compound synthesis and receptor modeling were done by our collaborators from the Lu lab and the Reggio lab, respectively.

This chapter involves the characterization of 30 new compounds (for structures and chemical names see Tables 3.5-3.7) from the class of benzhydryl piperazine analogs as inverse agonists of the CB1 receptor. SR141716A, the first potent inverse agonist of CB1 was used for the treatment of smoking cessation, obesity and associated metabolic disorders. However, limited use in Europe revealed that this drug induced significant psychiatric side-effects, namely anxiety and depression and it was, therefore, withdrawn from the market. The design for an inverse agonist that can avoid such adverse side effects has been an ongoing effort in the field. The research presented here aims at furthering our understanding of the ligand-specific modulation of CB1 receptor activity. Developing drugs that act in slightly different ways than classical drugs at the CB1 receptor would be a potential option to overcome the problems that have beset current endocannabinoid drugs. For instance, the non-psychomimetic major cannabis constituent, cannabidiol (CBD), has been suggested to be capable of antagonizing CB1 and CB2 receptor

agonists at reasonably low concentrations (Zuardi, 2008). In spite of its low affinity for CB1 ($K_i > 4000 \text{ nM}$) and CB2 receptors ($K_i > 2000 \text{ nM}$), CBD has shown several potential therapeutic properties, including antidepressive, anxiolytic (Crippa et al., 2004), antipsychotic (Zuardi et al., 2006), anti-diabetes (Weiss et al., 2006) and motor diseases effects (Zuardi et al., 2009). Thus, the objective of this chapter is to optimize CB1 inverse agonists that reduce the potential psychiatric side-effects of the classical CB1 receptor antagonists, such as rimonabant. This is done by optimizing the binding of the inverse agonists, i.e. high affinity, and to also develop peripherally restricted CB1 inverse agonists that do not produce undesired central nervous system side effects. These novel drugs can then be used to further our understanding of the ligand-specific modulation of CB1 receptor activity.

3.1 Abstract

Some inverse agonists of the cannabinoid CB1 receptor have been demonstrated to be anorectic antiobesity drug candidates. However, the first generation of CB1 inverse agonists, represented by rimonabant (SR141716A), otenabant and taranabant, are centrally active with a high level of psychiatric side effects. Hence, the discovery of CB1 inverse agonists with a chemical scaffold distinct from these holds promise for developing peripherally active CB1 inverse agonists with fewer side effects. We present here the binding data for 30 new compounds from the class of benzhydryl piperazine analogs. From these, we characterized the CB1 inverse agonist (4-(Bis(4-fluorophenyl)methyl)piperazin-1-yl)(cyclohexyl)methanone hydrochloride (LDK1229). This compound binds to CB1, more selectively than CB2, with a K_i value of 220 nM. Comparable CB1 binding was also observed by analogs 1-(Bis(4-fluorophenyl)methyl)-4-cinnamylpiperazine dihydrochloride (LDK1203) and 1-(Bis(4-fluorophenyl)methyl)-4-tosylpiperazine hydrochloride (LDK1222) which differed by the substitution on the piperazine ring where the piperazine of LDK1203 and LDK1222 are substituted by an alkyl group and by a tosyl group, respectively. LDK1229 exhibits efficacy comparable to SR141716A in antagonizing the basal G protein coupling activity of CB1 as indicated by a reduction in $GTP\gamma S$ binding. Consistent with inverse agonist behavior, increased cell surface localization of CB1 upon treatment with LDK1229 was also observed. Although docking studies showed that LDK1229 forms similar interactions with the receptor as SR141716A, the benzhydryl piperazine scaffold is structurally distinct from the first generation CB1 inverse agonists; it offers new opportunities for developing novel CB1 inverse agonists through optimization of molecular properties such as the polar surface area and hydrophilicity, to reduce the central activity observed with SR141716A.

3.2 Introduction

The cannabinoid receptors are members of the class A superfamily of G protein coupled receptors (GPCRs). The cannabinoid receptor 1 (CB₁) is present in high abundance throughout the central nervous system (Howlett, 1995) but is also expressed in a number of peripheral tissues, such as the cardiovascular and reproductive systems as well as the gastrointestinal tract (Batkai et al., 2001; Croci et al., 1998; Engeli et al., 2005), and is involved in substance addiction, chronic pain, memory and metabolic and inflammatory disorders (Howlett et al., 2004; Mackie, 2006; Pertwee, 2006). A second subtype of the cannabinoid receptors, the cannabinoid receptor 2 (CB₂), is predominantly found in immune cells and non-neuronal tissues (Galiege et al., 1995b) and is implicated in a variety of modulatory functions, including immune suppression, induction of apoptosis, and induction of cell migration (Basu and Dittel, 2011).

The CB₁ receptor preferentially couples to the G_{i/o} type of G proteins (Howlett and Fleming, 1984) and has been functionally linked to the inhibition of adenylate cyclase (Slipetz et al., 1995) and the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase-1 and -2, p38 MAPK, and c-Jun *N*-terminal kinase (Ahn et al., 2012; Bouaboula et al., 1996; Turu and Hunyady, 2010). In addition, it is associated with the inhibition of N- and P/Q-type voltage-dependent Ca²⁺ channels and the stimulation of A-type and inwardly rectifying K⁺ channels (Howlett, 2005).

As is common for many GPCRs (De Lean et al., 1980), the CB₁ receptor may exist in multiple activation states that are promoted by its binding to different ligands. Upon binding to receptor agonists, such as the endogenous arachidonylethanolamide and 2-arachidonyl glycerol, or the synthetic agonists CP55,940 and WIN55,212-2, the active form of the receptor

predominates. Interestingly, the CB₁ receptor possesses agonist-independent constitutive or basal activity which can be inhibited by inverse agonists (Pertwee, 2005a). This ligand-independent activity led to a receptor model that accounts for multiple activation states (Gether and Kobilka, 1998; Ghanouni et al., 2001) with distinct biochemical characteristics, including extent and selectivity of G protein or β -arrestin coupling (Kenakin, 1995).

Inverse agonists of the CB₁ receptor have attracted considerable attention in drug discovery because of their ability to regulate appetite and manage substance addiction (Janero, 2012; Janero and Makriyannis, 2009; Silvestri and Di Marzo, 2012b). Consequently, considerable effort has been invested in discovering compounds that can regulate the constitutive activity of the CB₁ receptor. However, SR141716A (rimonabant) (Rinaldi-Carmona et al., 1994), the only CB₁ inverse agonist to be briefly clinically marketed in Europe for the control of obesity (Moreira and Crippa, 2009), was removed from use due to its severe neuropsychiatric side effects including mood-depressant actions (Despres et al., 2005; Traynor, 2007). The first generation of CB₁ inverse agonists are commonly derived from diaryl analogs of pyrazole (e.g. SR141716A) or pyrazole bioisosteres such as imidazole, triazole, thiazole and pyrazoline (Lange and Kruse, 2005; Lange and Kruse, 2008; Muccioli and Lambert, 2005). However, most of these are brain penetrant due to their physicochemical nature (Chorvat, 2013) and will likely generate unwanted side effects in the central nervous system. In an effort to develop new CB₁ inverse agonist scaffolds, we analyzed the common pharmacophore of this class of compounds that was proposed by Lange et al (Lange and Kruse, 2005). This showed that the biaryl groups connecting to a central heteroaromatic ring are pivotal in forming aromatic stacking interactions with CB₁ receptors (McAllister et al., 2004; Shim et al., 2012). This brought our attention to the benzhydryl piperazine scaffold, which exhibits a similar structure to the common pharmacophore

of CB₁ inverse agonists in lieu of the biaryl heteroaromatic ring moiety. Hence, we synthesized a group of benzhydryl piperazine analogs, and demonstrated that some of the synthesized analogs, LDK1203, LDK1222 and LDK1229, exhibited inverse agonist binding profiles for the CB₁ receptor. In addition to its binding profile to the CB₁ receptor, LDK1229's inverse agonism is evident from its effect on basal as well as agonist-induced G protein coupling, and its impact on the internalization and cell surface expression of CB₁. Docking and mutational studies using a model of the inactive CB₁ receptor showed that LDK1229 forms interactions with the receptor consistent with inverse agonist SR141716A. Discovering new and improved means for inhibiting the activity of CB₁ is critical for understanding the constitutive activity of CB₁ and for developing new therapeutic agents for treating substance addiction and disorders associated with CB₁ activity.

3.3 Materials and Methods

Synthesis. The benzhydryl piperazine analogs LDK1203, LDK1222 and LDK1229 were synthesized by alkylation, tosylation and acylation of the 1-(4,4'-difluorobenzhydryl)piperazine (Figure 3.1A). The employed 1-(4,4'-difluorobenzhydryl)piperazine (III) was prepared by monoalkylation of piperazine with 4,4'-difluorobenzhydryl chloride (II) that was obtained by halogenation of 4,4'-difluorobenzhydryl methanol (I) with oxalyl chloride as previously reported (Weïwer et al., 2012). The obtained free bases of the benzhydryl piperazine analogs were then converted to their corresponding hydrochloric salts by reacting with an ethereal solution of HCl. The chemical identity of the newly synthesized compounds is as follows: LDK1203 is 1-(Bis(4-fluorophenyl)methyl)-4-cinnamylpiperazine dihydrochloride, LDK1222 is 1-(Bis(4-fluorophenyl)methyl)-4-tosylpiperazine hydrochloride and LDK1229 is (4-(Bis(4-

fluorophenyl)methyl)piperazin-1-yl)(cyclohexyl)methanone hydrochloride. The structure confirmation data for the free bases of LDK1203, LDK1222 and LDK1229 can be found in the original publication.

Plasmid Construction. All mutants were generated by site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA) using the human CB1 cDNA cloned into pcDNA3.1 as a template, according to the manufacturer's instructions. All mutations were confirmed by DNA sequencing.

CB₁ Expression and Membrane Preparation. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 3.5 mg/ml glucose at 37°C in 5% CO₂. For transient expression of the receptors, HEK293 cells were seeded at 800,000 cells/100-mm dish on the day prior to transfection and transfected with 5-10 µg of the wild type or mutant human CB1 receptor cloned into pcDNA3.1 using the calcium phosphate precipitation method (Chen and Okayama, 1987). At 24 h post-transfection, membranes of transfected cells expressing either the wild-type or mutant receptors were prepared as described previously (Ahn et al., 2009a).

Radioligand Binding Assay. In the homologous and heterologous competition binding experiments, approximately 7.5 µg of wild-type CB₁, CB₂ or mutant CB₁ membrane was incubated at 30°C for 60 min with a fixed tracer ([³H]CP55,940, 141.2 Ci/mmol, [³H]SR141716A, 56 Ci/mmol, or [³H]WIN55,212-2, 52.2 Ci/mmol, PerkinElmer Life Sciences (Boston, MA)) concentration typically at its K_d, which was determined from saturation binding isotherms (see results for details). Binding assays were performed with at least nine concentrations of unlabeled competitor ligand (ranging between 100 pM and 100 µM) as described previously (Ahn et al., 2012). Nonspecific binding was determined in the presence of 1 µM unlabeled CP55,940, SR141716A or WIN55,212-2. Reactions were terminated by adding

300 μ L of TME buffer containing 5% BSA followed by filtration with a Brandel cell harvester through Whatman GF/C filter paper. Radioactivity was measured by liquid scintillation counting. The total assay volume and the amount of membrane samples were adjusted to avoid ligand depletion by keeping the bound ligand less than 10% of the total.

GTP γ S Binding Assay. GTP γ S binding assays were performed as described previously (Ahn et al., 2012). Briefly, 7.5 μ g of membranes were incubated for 60 min at 30°C in a total volume of 200 μ L GTP γ S binding assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 0.2 mM EGTA, and 100 mM NaCl) with unlabeled test compounds as indicated, 0.1 nM [³⁵S]GTP γ S (1250 Ci/mmol; PerkinElmer Life Sciences, Boston, MA), 3 μ M GDP (Sigma, St. Louis, MO), and 0.1% (w/v) BSA. 3 μ M GDP was used to increase the window of basal activity. Nonspecific binding was determined with 10 μ M unlabeled GTP γ S (Sigma, St. Louis, MO). The reaction was terminated by rapid filtration through Whatman GF/C filters. The radioactivity trapped in the filters was determined by liquid scintillation counting.

Confocal Microscopy and Image Quantification. HEK293 cells were transfected with the CB₁ receptor carboxyl terminally fused to GFP using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The CB₁-GFP expressing cells were seeded onto 35-mm glass-bottomed dishes (Matek, MA) pre-coated with poly-D-lysine. Cells were treated with different compounds for various lengths of time and then washed twice with PBS, followed by fixation with 4% paraformaldehyde for 10 min at room temperature. Images were acquired using a Leica confocal laser scanning microscope and detection of GFP was carried out following excitation at 488 nm. Quantification of the fluorescence intensity was achieved by using the Quantitative Imaging of Membrane Proteins (QuimP) software (<http://go.warwick.ac.uk/bretschneider/quimp>) (Bosgraaf et al., 2009; Dormann et al., 2002), a set of plug-ins for the open source program

ImageJ (<http://rsb.info.nih.gov/ij/>). The Boa plug-in was used to detect the cell surface and checked against the cell edge in the transmitted image of each cell. The Ana plugin was then used to read the cell contours produced by the Boa plugin and compute the ratio of fluorescence intensity on the cell surface to the average intensity of the cell interior fluorescence. The results are representative of at least four independent transfections and 6 different images for each condition. Untransfected cells exhibited no apparent fluorescence under the experimental conditions that were used. The parameters for all of the acquired images and their consequent analysis were kept constant throughout.

Ligand and GTP γ S Binding Data Analysis. All ligand binding assays and GTP γ S binding assays were carried out in duplicate. Data are presented as the mean \pm S.E. value or the mean with the corresponding 95% confidence limits from at least three independent experiments. K_i values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) based on K_d values obtained from saturation binding analyses. The binding constants for the wild-type and mutant receptors were compared using analysis of variance (ANOVA) followed by Bonferroni's post hoc test for significance. p values of <0.05 were considered to be statistically significant.

Computational Methods

Conformational Analysis of LDK1229. To generate a library of low-energy conformers of LDK1229, the Spartan Conformation Distribution protocol was used (Wavefunction, Inc., Irvine, CA). In this protocol, the algorithm systematically searches through all rotatable bonds and ring conformations (*e.g.* alternate chair conformations for flexible rings). The energy of each conformer generated was calculated using the Merck Molecular Force Field (MMFF94S). This calculation yielded 68 unique conformations of LDK1229. The geometry and energy of these 68

conformations was refined by performing *ab initio* HF-6-31G* energy minimizations on each conformer. To calculate the difference in energy between the global minimum energy conformer and its final docked conformation, rotatable bonds in the global minimum energy conformer were driven to their corresponding value in the final docked conformation and the single-point energy of the resultant structure was calculated at the HF 6-31G* level.

Template Rationale. Our CB₁ inactive state model was initially constructed by using the 2.8 Å x-ray crystal structure of bovine rhodopsin (Rho) as a template (Palczewski et al., 2000). We chose rhodopsin for several reasons: **(1)** Rhodopsin has an intact “ionic lock” (R3.50²¹⁴-E/D6.30³³⁸), which is the hallmark of the Class A GPCR Inactive state. **(2)** The cannabinoid receptors and rhodopsin have very hydrophobic binding pockets. Crystal structures reveal that the N-terminus of rhodopsin/opsin is closed over the binding pocket, preventing access from the extracellular milieu (Palczewski et al., 2000; Park et al., 2008; Scheerer et al., 2008b). It is very likely that CB₁, with its 112 residue N-terminus, is also closed off to the extracellular milieu. Instead, rhodopsin/opsin have been reported to have lipid portals that are used for entry and exit via the lipid bilayer for 11-cis-retinal/trans-retinal as they are shuttled into and out of the receptor (Hildebrand et al., 2009). There is evidence from simulations (Hurst et al., 2010) and from experimental covalent labeling studies (Pei et al., 2008; Picone et al., 2005) that the cannabinoid receptors also possess a portal between TMH6 and TMH7 through which ligands enter. **(3)** In addition, the cannabinoid receptors and rhodopsin share an unusual, GWNC motif sequence motif at the extracellular (EC) end of TMH4. Here a Trp forms an aromatic stacking interaction with Y5.39²⁷⁵. This interaction influences the EC positions of TMH3-4-5.

CB₁ Inactive State Model. A sequence alignment between the sequence of the human CB₁ receptor (Gerard et al., 1991b) and the sequence of bovine rhodopsin was constructed, using

highly conserved residues as an alignment guide; in addition, the hydrophobicity profile of the sequence was also considered when constructing the sequence alignment. Residues in the bovine rhodopsin structure were then mutated to those of human CB₁. The Monte Carlo/simulated annealing technique Conformational Memories was used to sample the conformational space of TMH6 (Barnett-Norris et al., 2002); this is because TMH6 is known to undergo a functionally-necessary conformational change upon G protein-mediated signaling. For the inactive state model, the chosen TMH6 conformer was one that enabled the formation of a salt bridge between the highly conserved TMH6 residue D6.30³³⁸ and the highly conserved TMH3 residue R3.50²¹⁴. This salt bridge (also termed the “ionic lock”) has been shown to be important in maintaining the inactive state in the β_2 adrenergic (Ballesteros et al., 2001) and 5HT-2 α receptors (Visiers et al., 2002). Extracellular and intracellular loops were added to the model using Modeller (Marcu et al., 2013). SR141716A was docked (within the TMH3-4-5-6 region) in this CB₁ inactive state model and the energy of the complex was minimized, as previously described (Hurst et al., 2006).

Glide Docking of LDK1229 at the CB₁ Receptor (Inactive Conformation). The docking program, Glide (version 5.7, Schrödinger, LLC, New York, NY, 2011), was used to explore possible receptor binding modes of LDK1229. First, the SR141716A-CB₁ binding site was chosen as a starting point for Glide docking studies because of the similar pharmacology between SR141716A and LDK1229. Second, LDK1229 displaces SR141617A in competitive binding experiments, suggesting some commonality between their binding sites. Thus, Glide was used to generate a grid centered on the center-of-mass of our previously reported binding site for SR141716A at the CB₁ receptor (Hurst et al., 2002; McAllister et al., 2004; McAllister et al., 2003). The grid dimensions were 26 Å x 26 Å x 26 Å; this grid size allowed Glide to thoroughly

explore the receptor for possible binding site(s). In addition, the results of previously reported mutagenesis and synthetic studies suggest that SR141716A forms an important interaction with K3.28¹⁹² that is necessary for its ability to act as an inverse agonist of G protein-mediated signaling (Hurst et al., 2006; Hurst et al., 2002). Therefore, Glide was required to dock LDK1229 in such a way so that it formed a hydrogen bond with K3.28¹⁹². The only other constraint used was the requirement that LDK1229 must be docked within the exploration grid. Standard precision was selected for the docking setup. 68 conformations of LDK1229 were flexibly docked using Glide. *The best Glide dock was chosen for subsequent calculations.* The chosen Glide dock was minimized, using the minimization protocol, described below.

Receptor Model Energy Minimization Protocol. The energy of the LDK1229-CB₁ complex, including loop regions, was minimized using the OPLS 2005 force field in Macromodel 9.9 (Schrödinger, LLC, New York, NY, 2011). An 8.0-Å extended non-bonded cutoff (updated every 10 steps), a 20.0-Å electrostatic cutoff, and a 4.0-Å hydrogen bond cutoff were used in the calculation. The minimization was performed in two stages. In the first stage, the TMH backbone was frozen; this constraint was used to preserve secondary structure while allowing the ligand and TMH side chains to relax. In addition, loop residues were frozen, until they could be minimized using an appropriate dielectric (in the next stage of the minimization). No constraints were placed on the ligands during this stage. The minimization consisted of a conjugate gradient minimization using a distance-dependent dielectric of 2.0, performed in 1000-step increments until the bundle reached the 0.05 kJ/mol gradient. In the second stage of the calculation, the helices and ligand were frozen, but the loops were allowed to relax. The generalized Born/surface area continuum solvation model for water as implemented in Macromodel was

used. This stage of the calculation consisted of a Polak–Ribier conjugate gradient minimization in 1000-step increments until the bundle reached the 0.05 kJ/mol gradient.

3.4 Results

Chemistry. The free base forms of LDK1203, LDK1222 and LDK1229 were synthesized according to the scheme illustrated in Figure 3.1A, then purified by Combiflash chromatography followed by conversion to hydrochloric salts to increase shelf life and aqueous solubility. Confirmation data for their structure by mass spectroscopy and ^1H NMR are presented in Materials and Methods. Representative first generation inverse agonists, Rimonabant, Otenabant, Taranabant and Ibipinabant are shown for comparison (Figure 3.1B).

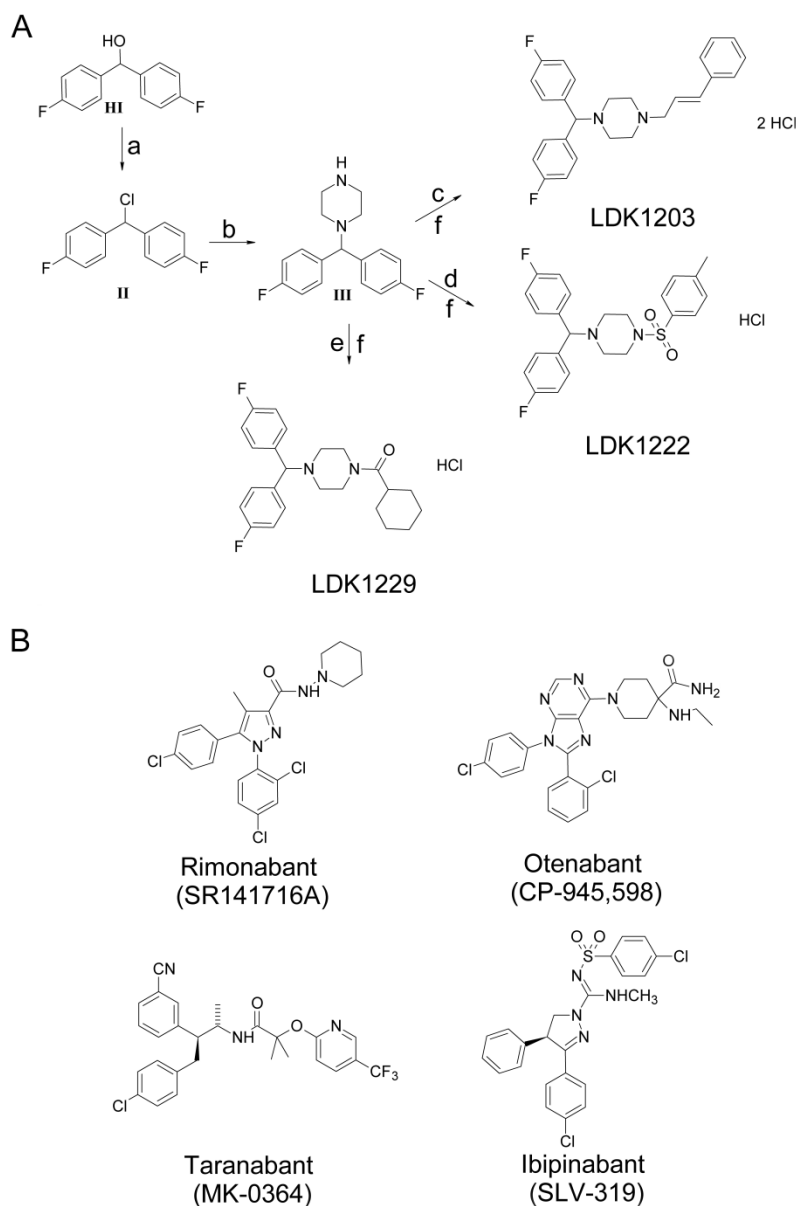


Figure 3.1 Compound structures. (A) Synthesis of benzhydryl piperazine analogs LDK1203, LDK1222 and LDK1229. a) Oxalyl chloride, DCM, cat. DMF; b) Piperazine, CH₃CN; c) 3-Bromo-1-phenyl-1-propene, K₂CO₃, DMF; d) TsCl, DIPEA, DCM; e) Cyclohexanecarbonyl chloride, DIPEA, DCM; f) 1.0 M HCl in ether. (B) Representative members of the first generation of CB1 inverse agonists are shown for comparison.

LDK1229 and its Analogs Exhibit Inverse Agonist Binding Profiles. To elucidate the nature of these new compounds, we performed ligand binding studies using membrane preparations from HEK293 cells transfected with CB₁. All three compounds competed with CP55,940 and SR141716A, an agonist and inverse agonist of CB₁, respectively. Using [³H]CP55,940 ($K_d = 2$ nM) as a tracer, LDK1203, LDK1222 and LDK1229 bound wild-type CB₁ with K_i values of 260 nM, 331 nM and 220 nM, respectively. Similarly while using [³H]SR141716A as a tracer ($K_d = 5$ nM), the compounds also bound with comparable affinities ($K_i = 297$ nM, 366 nM and 246 nM, respectively; Tables 3.1-3.3). The structures of LDK1203 and LDK1222 differed from LDK1229 by the substitution groups on the piperazine ring where the piperazine ring of LDK1203 was substituted by an alkyl group and the piperazine of LDK1222 was substituted by a tosyl group.

Since LDK1229 exhibited the strongest binding affinity among the compounds, it was further investigated for its capability to bind CB₁ receptors in the active and inactive states to confirm its nature as a CB₁ inverse agonist. The CB₁ receptor in the active and inactive states can be readily mimicked by our previously engineered mutant CB₁ receptor models (T210I and T210A) (D'Antona et al., 2006a). CB₁ with the T210I mutation was shown to adopt a fully active form in comparison with the CB₁ wild-type. In contrast, CB₁ with the T210A mutation adopts an inactive state. A GPCR in its active state displays enhanced affinity towards agonists but decreased affinity towards inverse agonists, whereas the inactive state of the receptor exhibits the opposite binding properties such that inverse agonists show higher binding affinity than agonists (Cotecchia et al., 1990; D'Antona et al., 2006a; McWhinney et al., 2000; Wade et al., 2001). Hence, our mutant CB₁ receptor models provide ideal tools to evaluate the inverse agonism of LDK1229.

Table 3.1 LDK1203 and LDK1222 binding to the wild-type CB1 receptor.

vs. [³ H] CP55,940				vs. [³ H] SR141716A		
K _i (nM) ^a				K _i (nM)		
CP55,940	LDK1203	LDK1222		SR141716A	LDK1203	LDK1222
CB1 wild-type	2.56 (1.45-4.51) ^b	260 (80.7-837)	331 (177-619)	5.23 (3.29-8.32) ^b	297 (192-458)	366 (144-928)

^aData are the median and corresponding 95% confidence limits of three independent experiments performed in duplicate. K_i values were determined from competition binding assays using [³H] CP55,940 or [³H] SR141716A as tracers at their respective K_d values as described in Results.

^bBinding values are from Ahn et.al., 2009 for comparison.

As expected for an inverse agonist, using [³H]SR141716A as the tracer, the binding affinity of LDK1229 to the inactive T210A receptor was enhanced ($K_i = 68$ nM) relative to its affinity to the wild-type CB₁ receptor ($K_i = 246$ nM; Table 3.2). A comparable trend is observed with the inverse agonist, SR141716A, that binds T210A with the highest affinity ($K_i = 1.47$ nM), and progressively weaker affinity for the wild-type CB₁ ($K_i = 5.23$ nM), and then the T210I receptor ($K_i = 14.7$ nM). The ratio of binding affinity for the wild-type receptor relative to the mutant receptor for LDK1229 and SR141716A also reflects the preference of these two compounds for the inactive T210A receptor (Table 3.2).

Table 3.2 LDK1229 binding to the T210A, wild-type and T210I CB₁ receptors using [³H]SR141716A as a tracer.

	K_i (nM) ^a			
	SR141716A	K_i ratio (wild-type : mutant)	LDK1229	K_i ratio (wild-type : mutant)
CB ₁ T210A	1.47 (1.14-1.90)	4:1	67.4 (34.7-131)	3:1
CB ₁ wild-type	5.23 (3.29-8.32) ^b	1:1	246 (164-367)	1:1
CB ₁ T210I	14.7 (9.61-23.1)	1:3	NB ^c	-

^aData are the median and corresponding 95% confidence limits of three independent experiments performed in duplicate. K_i values were determined from competition binding assays using [³H]SR141716A as tracers at its respective K_d values as described in Results.

^bBinding values are from Ahn et al. 2009 for comparison.

^cNB: no binding using up to 32 μ M of LDK compound.

Using [³H]CP55,940 as the tracer, and examining binding of the agonist CP55,940, the pattern is reversed (Table 3.3); CP55,940 binds T210A most weakly and then progressively exhibits enhanced affinity for the wild-type CB₁ and the T210I receptors. This is consistent with

its agonist properties. In contrast, LDK1229 binds CB₁ wild-type more tightly than the T210I receptor consistent with inverse agonist binding profiles (D'Antona et al., 2006a). For the constitutively active T210I receptor, a large decrease in the binding affinity of LDK1229 compared to the binding affinity of the agonist CP55,940 (5831 nM versus 0.835 nM) is observed. The ratio of binding affinity for the wild-type receptor relative to the mutant receptor for LDK1229 and CP55,940 is also given for comparison (Table 3.3). At the extremes, when using [³H]CP55,940 as a tracer, we cannot detect any specific binding with T210A, nor when using [³H]SR141716A as a tracer can we detect any specific binding with T210I, up to 32 μM of LDK1229. Taken together these data suggest that LDK1229's increased binding affinity to T210A over wild-type and its decreased binding affinity to T210I result from the compounds' inverse agonist mode of action.

Table 3.3 LDK1229 binding to the T210A, wild-type and T210I CB1 receptors using [³H]CP55,940 as a tracer.

	K _i (nM) ^a			
	CP55,940	K _i ratio (wild-type : mutant)	LDK1229	K _i ratio (wild-type : mutant)
CB1 T210A	7.00 (2.59-18.9)	1:3	NB ^c	-
CB1 wild-type	2.56 (1.45-4.51) ^b	1:1	220 (106-457)	1:1
CB1 T210I	0.835 (0.542-1.29)	3:1	5831 (907-7510)	1:27

^aData are the median and corresponding 95% confidence limits of three independent experiments performed in duplicate. K_i values were determined from competition binding assays using [³H]CP55,940 as tracer at its respective K_d values as described in Results.

^bBinding values are from Ahn et al. 2009 for comparison.

^cNB: no binding using up to 32 μM of LDK compound.

LDK1229 Inhibits Basal and Agonist-induced G Protein Coupling in a Concentration-dependent Manner. Given the inverse agonist properties observed in the binding profile of LDK1229 to the wild-type and mutant CB₁ receptors, [³⁵S]GTPγS binding assays were performed in its presence. This assay monitors the level of G protein activation by determining the extent of binding of the nonhydrolyzable GTP analog to Gα subunits. We investigated the effects of LDK1229 on the basal G protein coupling activity levels of the wild-type CB₁ receptor (Figure 3.2A). Interestingly, using 1 μM of LDK1229 in the absence of CP55,940 substantially reduced the basal level of [³⁵S]GTPγS binding from 110 fmol/mg to 70 fmol/mg. We also evaluated its impact on agonist CP55,940-induced [³⁵S]GTPγS binding in the presence of various concentrations of LDK1229. We observed a progressive decrease in the specific GTPγS binding with increasing concentration of LDK1229 up to 32 μM for the CB₁ wild-type receptor (Figure 3.2B).

Like LDK1229, LDK1203 produced an antagonizing effect on the basal and the agonist-induced levels of [³⁵S]GTPγS and is included for comparison (Figure 3.2). The antagonizing effect is consistent with the properties of an inverse agonist that promotes the inactive form of the receptor and suggests that this compound inhibits G protein coupling.

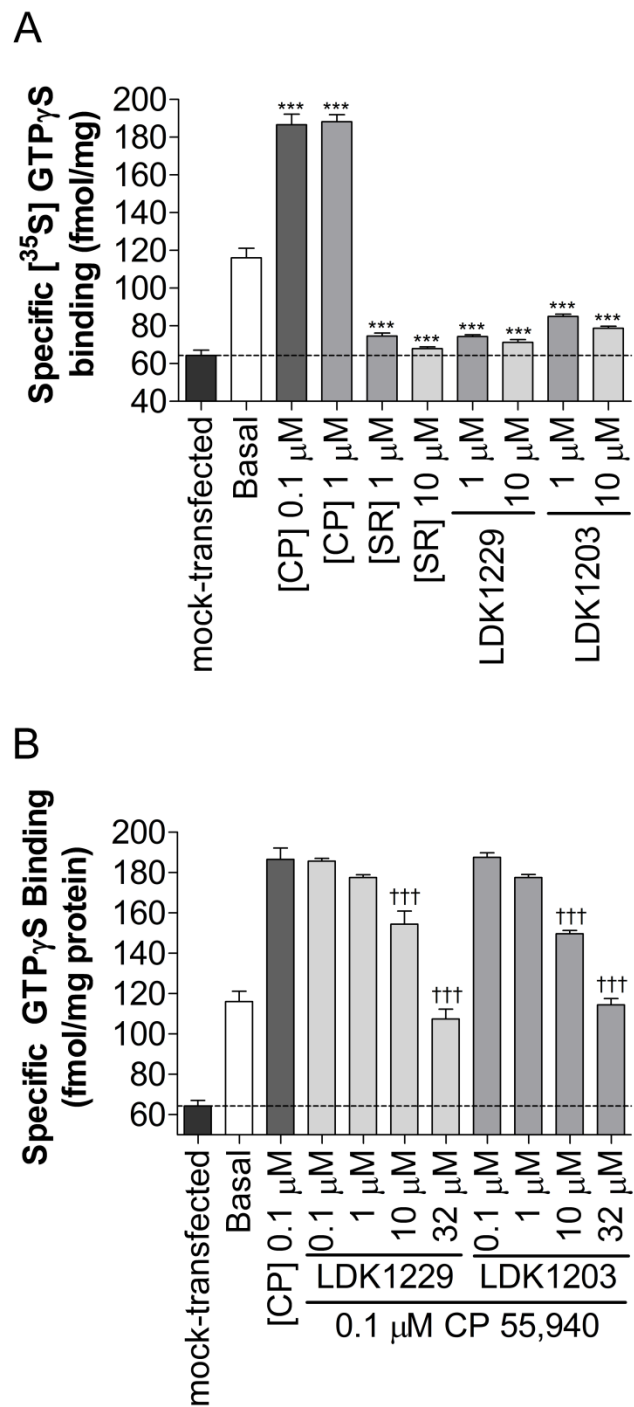


Figure 3.2 Effect of LDK1229 and LDK1203 on the stimulation of [³⁵S] GTP_γS binding to HEK293 cell membranes expressing the CB1 wild-type receptor. (A) The basal level of [³⁵S] GTP_γS binding was measured for the CB1 wild-type receptor. The stimulatory effect of

CP55,940 and the inhibitory effects of SR141716A, LDK1229 and LDK1203 on [35 S] GTP γ S binding were measured at the indicated concentrations. Statistical significance of the differences compared to basal was assessed using one-way analysis of variance and Bonferroni's post hoc test: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. **(B)** The inhibitory effects of both LDK1229 and LDK1203 on CP55,940-induced [35 S] GTP γ S binding in membrane preparations. Statistical significance of the differences compared to 0.1 μ M CP55,940 alone was assessed using one-way analysis of variance and Bonferroni's post hoc test: $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, and $\dagger\dagger\dagger p < 0.001$. Data are presented as specific binding of GTP γ S to the membranes. Nonspecific binding was determined in the presence of 10 μ M unlabeled GTP γ S. Each data point represents the mean \pm S.E. (*error bars*) of at least 3 independent experiments performed in duplicate. The *dotted line* indicates the level of non CB $_1$ -mediated GTP γ S binding obtained from GTP γ S binding to the mock-transfected membrane sample.

LDK1229 Promotes Cell Surface Expression of the CB $_1$ Receptor. Upon prolonged exposure to an agonist, GPCRs become desensitized and subsequently internalize in the cell. Inverse agonists, however, act in the opposite manner by promoting GPCR localization to the cell surface (Marion et al., 2004; Rinaldi-Carmona et al., 1998b). To assess the effect of LDK1229 on CB $_1$ cellular localization, we determined the cellular response of CB $_1$ upon LDK1229 treatment using confocal microscopy of cells expressing GFP-tagged CB $_1$ receptors. Previous observations (Leterrier et al., 2004; Martini et al., 2007; Rozenfeld and Devi, 2008) indicate that CB $_1$ is partially constitutive and that much of the wild-type CB $_1$ receptor (~85%) is localized on intracellular vesicles. Using the CB $_1$ -GFP chimera expressed in HEK293 cells and treated with vehicle alone (0.03% DMSO), we found that about 15% of CB $_1$ is present at the

plasma membrane but that the pattern of receptor fluorescence within the cells was intracellularly punctate, suggesting that a substantial proportion of receptors is internalized consistent with its basal activity (Figure 3.3). Treatment with 0.1 μ M of the agonist CP55,940 resulted in a further shift toward internalization and a punctate appearance of the CB₁-GFP receptor within the cells (data not shown). In contrast, treatment with 1 μ M SR141716A or 10 μ M LDK1229 resulted in approximately 20% cell surface localization evident within 3 h and an increase to about 65% of cell surface localization after 5 h (Figure 3.3B). This is strikingly different from the cell surface localization of 13% for basal cells at 5 h. Taken together, these data further support the inverse agonist activity of LDK1229.

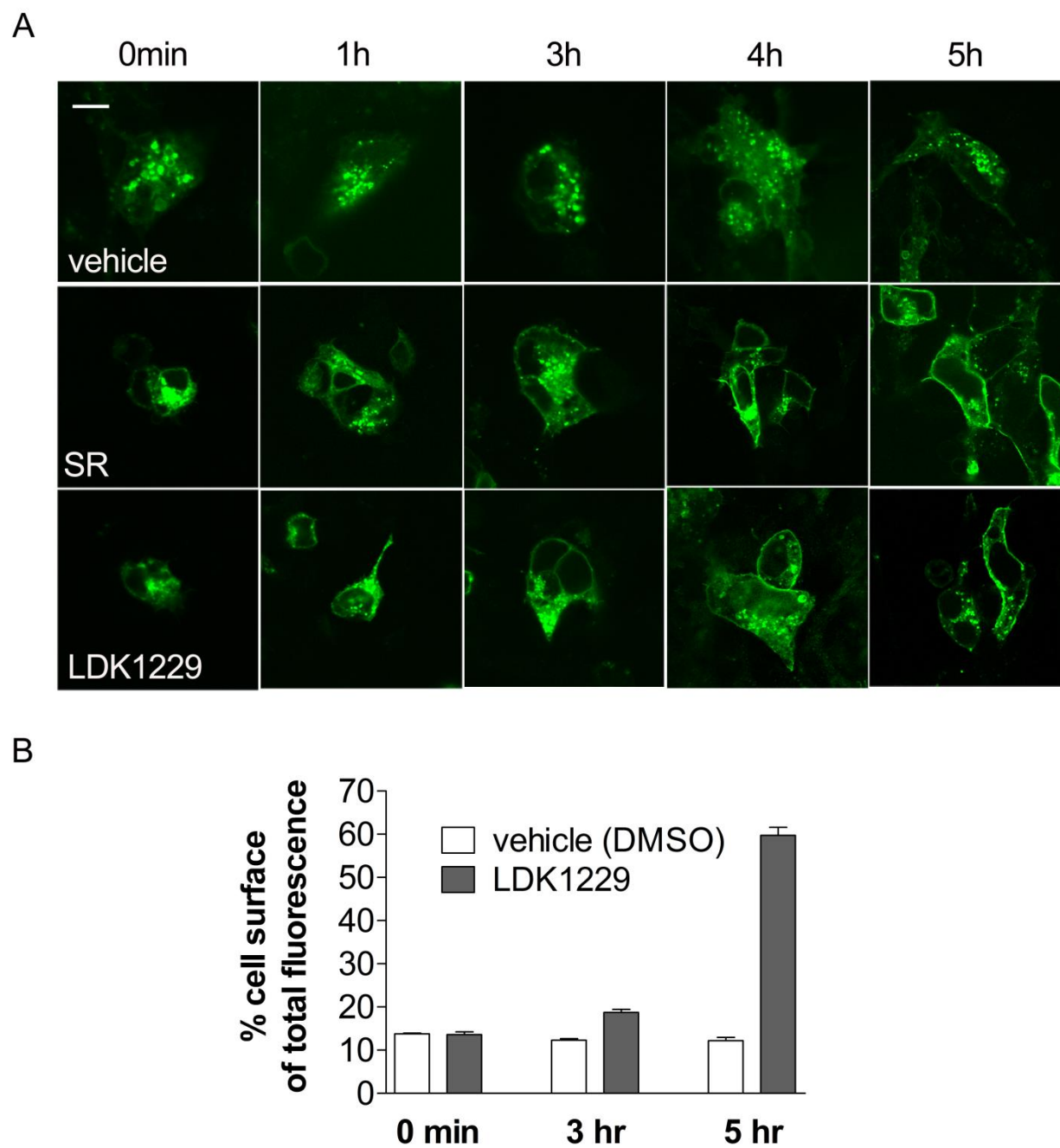


Figure 3.3 The effect of LDK1229 on the internalization of the CB1 receptor. (A) HEK293 cells expressing the CB1-GFP receptor were incubated with vehicle alone (0.03% DMSO), 0.5 μ M SR141716A or 10 μ M LDK1229. SR141716A is shown for comparison. (B) Quantification of CB1 receptors on the cell surface. The QuimP software with plug-ins was used as described in

Materials and Methods. Images are representative of at least four independent transfections. The scale bar is 5 μm .

LDK1229 Docked in the CB₁ receptor (Inactive) Model. Since LDK1229 exhibited the strongest binding affinity among the compounds, Glide docking studies were performed and suggest that LDK1229 binds in the TMH3-4-5-6 region of CB₁. This is the same region that forms the binding site for SR141716A at CB₁. Figure 3.4A and 3.4C illustrate SR141716A (shown in cyan) docked in the CB₁ inactive state model. The hydrogen bonding interaction is consistent with our prior CB₁ mutant cycle studies which indicated that the amide oxygen of SR141716A interacts directly with K3.28¹⁹². This interaction is critical for the inverse agonist properties of SR141716A (Hurst et al., 2006; Hurst et al., 2002).

At its binding site, SR141716A also forms several aromatic-stacking interactions. First, the SR141716A dichlorophenyl ring forms aromatic T-stacks with F3.36²⁰⁰ and W5.43²⁷⁹ (shown in orange, see Figure 3.4A). In addition, the SR141716A chlorophenyl ring forms off-set parallel aromatic stacks with Y5.39²⁷⁵ and W5.43²⁷⁹ (shown in orange); In addition, SR141716A forms hydrophobic interactions with W6.48³⁵⁶. These aromatic interactions are consistent with our prior mutagenesis studies (McAllister et al., 2004; McAllister et al., 2003) which indicated that SR141716A interacts within an aromatic microdomain in CB₁, which comprised of F3.36²⁰⁰, Y5.39²⁷⁵, W5.43²⁷⁹ and W6.48³⁵⁶. Finally, SR141716A forms hydrophobic interactions with L3.29¹⁹³, V3.32¹⁹⁶, and L6.51³⁵⁹ (shown in lime, see Figure 3.4C). In the ligand-free CB₁ receptor, the F3.36²⁰⁰/W6.48³⁵⁶ aromatic stacking interaction is instrumental to the maintenance of the CB₁-inactive state (McAllister et al., 2004). The binding of SR141716A stabilizes the

F3.36²⁰⁰/W6.48³⁵⁶ aromatic stacking interaction through the formation of an extensive network of aromatic stacks within the ligand-receptor complex (Shim et al., 2012).

Figures 3.4B and 3.4D illustrate the final docked conformation of LDK1229 (shown in lavender) in the CB₁ inactive state model (Data Supplement). Like SR141716A, the LDK1229 amide oxygen forms a hydrogen bond with K3.28¹⁹² (shown in yellow). The geometry of this hydrogen bond is not optimal, however, because the hydrogen bond distance is longer than found for SR141716A (2.8 Å vs. 2.6 Å) and because the hydrogen bond angle deviates more from linearity than that formed with SR141716A (157° vs. 171°). This suggests that the interaction between LDK1229 and K3.28¹⁹² is weaker than the interaction between SR141716A and K3.28¹⁹². The importance of this difference between SR141716A and LDK1229 is discussed in more detail below.

Like SR141716A, LDK1229 also docks in the CB₁ TMH3-4-5-6 aromatic microdomain, but the geometry of its interactions differ somewhat from SR141716A. The fluorophenyl ring of LDK1229 forms aromatic T-stacking interactions with W6.48³⁵⁶ and W5.43²⁷⁹ (shown in orange, see Figure 3.4 (B)). In addition, the other fluorophenyl ring of LDK1229 forms off-set parallel aromatic stacking interactions with Y5.39²⁷⁵ and W5.43²⁷⁹ (shown in orange). However, LDK1229 does not form an aromatic stacking interaction with F3.36²⁰⁰. This is due to a difference in how the two compounds are oriented within the receptor. Finally, LDK1229 also forms several hydrophobic interactions with residues F3.36²⁰⁰ (shown in orange), L3.29¹⁹³, V3.32¹⁹⁶, and L6.51³⁵⁹ (shown in lime).

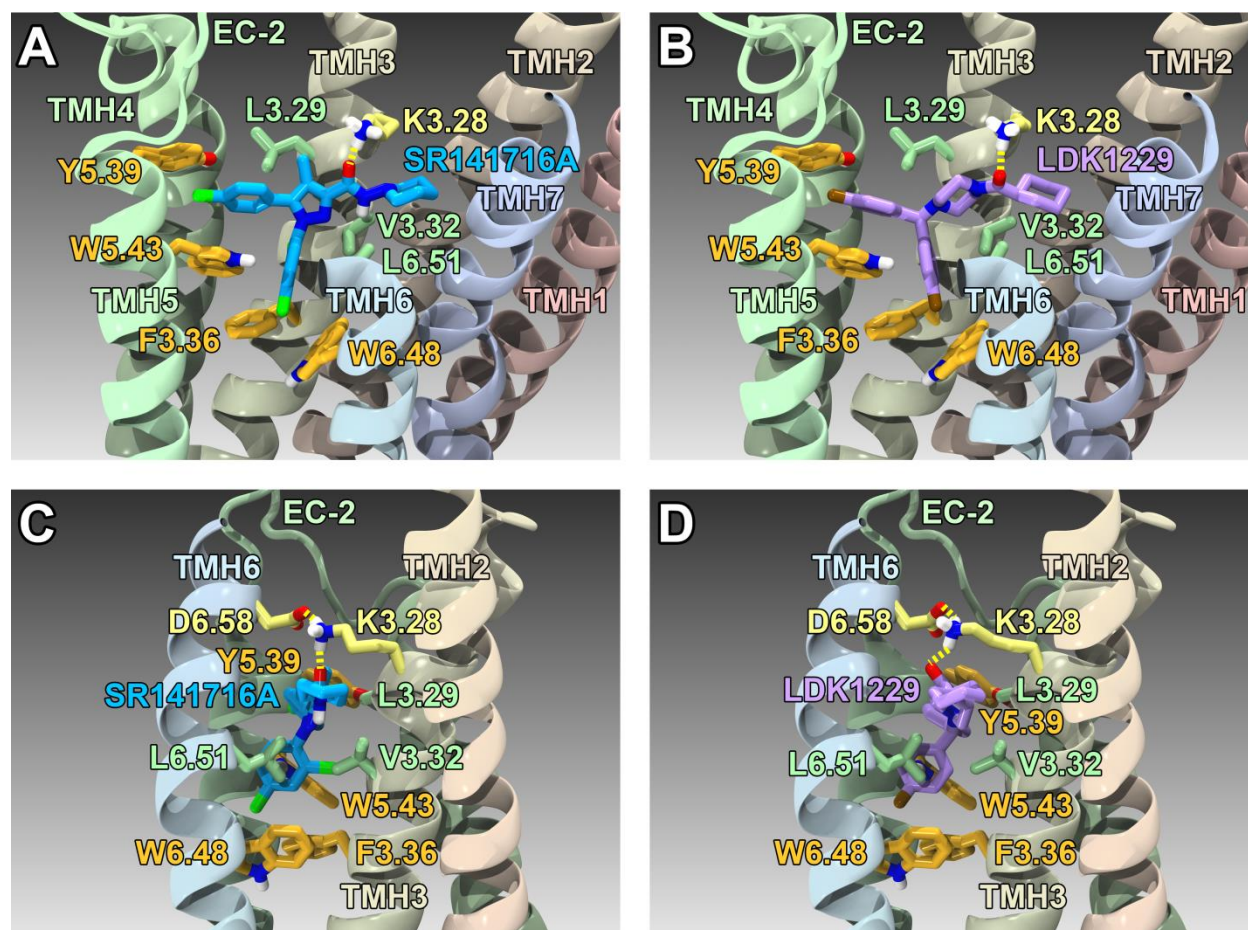


Figure 3.4 Model of LDK1229 docked in the inactive CB₁ receptor model. (A) and (C) illustrate SR141716A (shown in cyan) docked in the CB₁ inactive state model. (B) and (D) illustrate the final docked conformation of LDK1229 (shown in lavender) in the CB₁ inactive state model. Hydrogen bonds are shown in yellow, aromatic microdomain residues are shown in orange, and hydrophobic interactions are shown in lime.

Mutational Analysis of Computationally Predicted Residues in the LDK1229 binding pocket. To test the LDK1229 binding-site hypothesis generated by the receptor model, selective mutation of key residues in the CB₁ receptor were generated. The K3.28¹⁹²A, W5.43²⁷⁹A, W6.48³⁵⁶A, or C7.42³⁸⁶M mutant CB₁ receptors were generated. Our results showed

that the K3.28¹⁹²A mutant receptor resulted in total ablation of [³H]CP55,940 binding at wild-type CB₁ and that this residue is critical for CP55,940 binding consistent with previous data (Song and Bonner, 1996). Therefore, competition binding assays on the K3.28¹⁹²A mutant were performed using [³H]WIN55,212-2 as a tracer since [³H]WIN55,212-2 binding to the K3.28¹⁹²A mutant was not significantly different than its binding to the wild-type receptor ($K_d = 8.6$ nM versus $K_d = 4.5$ nM). Competition binding analysis revealed that the binding of LDK1229 ($K_i = 1316$ nM) was affected 6-fold by this mutation compared to wild-type ($K_i = 324$ nM; Table 4) suggesting that this residue may be important for a hydrogen bonding interaction with LDK1229. These results alone cannot tell us the specific region on LDK1229 with which K3.28¹⁹² interacts, but modeling studies reported here suggest that K3.28¹⁹² hydrogen bonds with the carboxyl oxygen of LDK1229. The K3.28¹⁹²A mutation results reported here are consistent with previous CB₁ K3.28¹⁹²A mutation studies which showed that K3.28¹⁹²A is an important interaction site (Hurst et al., 2002) for the carboxamide oxygen of SR141716A (Hurst et al., 2006).

Saturation (equilibrium) binding analysis for [³H]CP55,940 at the other three mutant receptors, W5.43²⁷⁹A, W6.48³⁵⁶A, and C7.42³⁸⁶M, showed that the affinity parameters obtained for the mutant receptors were not significantly different from the K_d at the wild-type CB₁ receptor (K_d for mutants = 6 nM, 4 nM, and 3 nM respectively) suggesting these receptors were properly folded. Therefore, competition binding assays on these mutants were performed using [³H]CP55,940 as a tracer. The W5.43²⁷⁹A mutation had the most profound effect on LDK1229 binding compared to any of the other mutations and showed only 39% displacement of [³H]CP55,940 when using 32 μ M of LDK1229. This result is consistent with the modeling studies that suggest that W5.43²⁷⁹A is central in the formation of the aromatic T-stacking interactions with the two fluorophenyl rings in LDK1229. The W6.48³⁵⁶A mutant affected

LDK1229 binding by 9-fold ($K_i = 1987$ nM) and the C7.42³⁸⁶M mutant by 5-fold ($K_i = 1010$ nM; Table 3.4). The result for the W6.48³⁵⁶A mutation further supports the modeling results which suggest that this mutation participates in direct aromatic stacking interactions with one of the fluorophenyl rings of LDK1229 consistent with prior studies (McAllister et al., 2003). As shown in Table 4, we found here that enlarging the residue at position 7.42 via the C7.42³⁸⁶M mutation results in a 5-fold loss in affinity for LDK1229 consistent with previous studies by Farrens and colleagues (Fay et al., 2005). Taken together, the results of all mutation studies are consistent with the modeling results reported here, specifically that LDK1229 occupies the same general binding region as SR1417167A.

Table 3.4. LDK1229 binding to the wild-type or mutant CB₁ receptors using [³H]CP55,940 or [³H]WIN55,212-2 as a tracer.

K_i ^a		
[³ H]CP55,940		
Receptor	nM	K_i ratio (wild-type : mutant)
CB1 wild-type	220 (106-457)	1:1
K3.28 ¹⁹² A	1316 (1115-1626) ^b	1:4 ^b
W5.43 ²⁷⁹ A	ND ^c	-
W6.48 ³⁵⁶ A	1987 (895-2736)	1:9
C7.42 ³⁸⁶ M	1010 (832-1154)	1:5

^aData are the median and corresponding 95% confidence limits of three independent experiments performed in duplicate. K_i values were determined from competition binding assays using [³H]CP55,940 as tracer at its respective K_d values as described in Results.

^bBinding was performed with [³H]WIN55,212-2 as a tracer. LDK1229 binding to CB1 wild-type using [³H]WIN55,212-2 as a tracer yielded a $K_i = 324$ nM (205-524).

^cND: not detectable; 39% displacement at 32 μ M.

LDK1229 has Diminished Activity for CB₂. Based on both binding and functional data, LDK1229 showed some selectivity for the CB₁ receptor over the CB₂ receptor (Figure 3.5). This is evident by LDK1229's 3-fold reduced binding affinity ($K_i = 633$ nM; Figure 3.5A) to the CB₂ receptor compared to the CB₁ receptor ($K_i = 220$ nM; Table 3.3) using [³H]CP55,940 as a tracer. This selectivity is also evident by the small magnitude of the reduction in basal and agonist-induced G protein coupling using CB₂. Using 1 μ M of LDK1229, in the absence of CP55,940, only reduced the basal level of [³⁵S]GTP γ S binding from 95 fmol/mg to 88 fmol/mg (Figure 3.5B) compared to the 2-fold decrease observed with CB₁ (Figure 3.2A). We also observed a small decrease in the CP55,940-induced [³⁵S]GTP γ S binding with an increasing concentration of LDK1229 (Figure 3.5B) with up to 32 μ M of LDK1229 (to 110 fmol/mg from the original 120 fmol/mg). The CB₂ selective inverse agonist SR144528 (Rinaldi-Carmona et al., 1998a) antagonized the basal level of [³⁵S]GTP γ S binding and is shown for comparison. We also found that LDK1229 can dock in the CB₂ inactive state model but in a higher energy conformation (data not shown). This is likely the origin of the 3-fold loss of affinity at CB₂.

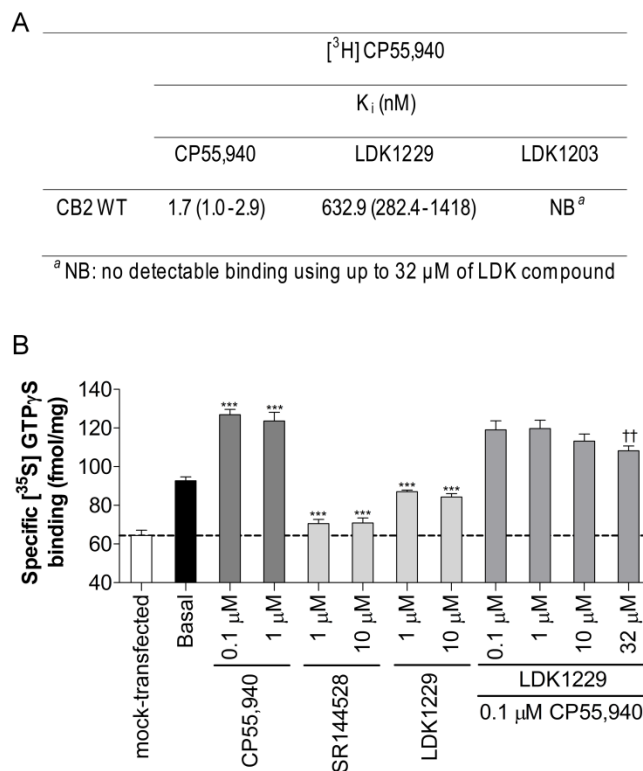


Figure 3.5 The binding parameters of LDK1229 and LDK1203 to CB2. (A) Binding of LDK1229 and LDK1203 to CB2 wild-type receptors and **(B)** their effect on the stimulation of $[^{35}\text{S}]$ GTP γ S binding. Statistical significance of the differences (*compared to basal, †compared to 0.1 μM CP55,940 alone) was assessed using one-way analysis of variance and Bonferroni's post hoc test: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; † $p < 0.05$, †† $p < 0.01$, and ††† $p < 0.001$.

3.5 Discussion and Future Directions

In an effort to develop new modulators of the CB₁ receptor, we synthesized a group of about 30 benzhydryl piperazine analogs (see Tables 3.5-3.7) including the compounds LDK1203, LDK1222 and LDK1229 and describe their inverse agonist properties in this study. In addition to their inverse agonist binding profiles to the CB₁ receptor and their preference to bind the inactive T210A CB₁ receptor over the constitutively active wild-type CB₁ or fully active T210I receptor (Tables 3.2-3.3), the inverse agonism exhibited by LDK1229 was also evident from its antagonistic effect on basal and agonist-induced G protein coupling (Figure 3.2), and its ability to increase the CB₁ localization to the cell surface (Figure 3.3). LDK1229 exhibited a lower affinity for the CB₂ receptor, with relative selectivity for the CB₁ receptor of 3-fold. Because the CB₁ receptor is constitutively active both *in-vitro* and *in-vivo* (Landsman et al., 1997; Meschler et al., 2000), discovering new and improved means for inhibiting the activity of the receptor is therapeutically useful and relevant for modulating activity of the CB₁ receptor system in the brain.

Our results show that the benzhydryl piperazine analogs represented by LDK1229 behave as inverse agonists of the CB₁ receptor. Structurally, the benzhydryl piperazine analogs are distinct from the first generation of CB₁ inverse agonists, which generally possess nitrogen-containing 5- or 6-member aromatic rings as their central connecting units (Chorvat, 2013; Lange and Kruse, 2005; Vemuri et al., 2008). In contrast, benzhydryl piperazine analogs have a central core of piperazine, which is non-aromatic and possesses basic amino group(s). A pharmacophore model of the first-generation of CB₁ inverse agonists was proposed, (Lange and Kruse, 2005) of which the biaryls (e.g. SR141716A) form favorable aromatic stacking interactions with two sub pockets surrounded by residues Y5.39²⁷⁵-W4.64²⁵⁵-F5.42²⁷⁸ and

residues W5.43²⁷⁹-F3.36²⁰⁰-W6.48³⁵⁶ of CB₁. The central core (e.g. pyrazole in SR141716A) then connects to a lipophilic moiety through a hydrogen bond acceptor (e.g. the carbonyl of SR141716A). The hydrogen bond acceptor stabilizes the D6.58³⁶⁶-K3.28¹⁹² salt bridge in the inactive state of the CB₁ receptor. The lipophilic moiety (e.g. the methylene groups of piperidine ring in SR141716A) fits in a pocket formed by V3.32¹⁹⁶-F2.57¹⁷⁰-L7.43³⁸⁷ and M7.44³⁸⁴ of the CB₁ receptor (Lange and Kruse, 2005). In spite of the structural difference between our benzhydryl piperazine analogs and the first generation of CB₁ inverse agonists, docking SR141716A and LDK1229 into the CB₁ inactive state model suggest that both compounds bind in similar receptor regions and form similar interactions with the receptor. This is also consistent with the results of competitive displacement assays that suggest LDK1229 displaces SR141716A, implying an overlapping binding site. However, the results of the docking studies suggest one major difference between SR141716A and LDK1229; the geometry of the SR141716A hydrogen bond with K3.28¹⁹² is much better than that of LDK1229. Conformational analysis of LDK1229 suggests that it does not have the conformational freedom to adopt a conformation that would allow it to improve its hydrogen bond geometry. In addition, the extra ring hydrogen (cyclohexyl ring vs. piperidine ring) introduced in LDK1229 forces the ligand to position itself differently. Together, these effects lead to a less favorable hydrogen bond with K3.28¹⁹². This is consistent with the results of the binding experiments that show that SR141716A has a higher affinity for CB₁ than does LDK1229. Notably, LDK1229 exhibits an efficacy comparable to SR141716A in antagonizing basal GTPγS binding to the CB₁ receptor although it shows a weaker binding affinity to the receptor (Table 2, Table 3 and Figure 2). One advantage of the benzhydryl piperazine analogs is that the piperazine ring provides two amino groups that can be readily used in further derivatization. This opens up rich opportunities for

structural modifications that may lead to the improvement of the pharmacokinetic properties of the drug molecules, such as their lipophilicity and polar surface area, which are critical factors influencing the brain barrier-penetration of these compounds.

Evidence has begun to emerge on the involvement of the endocannabinoid system in the regulation of metabolism in several peripheral organs crucial to energy storage and utilization (Di Marzo and Matias, 2005; Silvestri et al., 2011). Elevated peripheral endocannabinoid levels and increased CB₁ expression in these tissues have been observed in several studies of obese mice (Pagotto et al., 2006) as well as obese humans compared to leaner controls (Engeli et al., 2005). In light of the above, it has proven difficult to diminish the adverse central nervous system effects of brain-penetrant CB₁ inverse agonists (Jones, 2008). Not surprisingly, significant effort has been put forth to develop CB₁ neutral antagonists (Janero, 2012) or peripherally restricted CB₁ inverse agonists (Chorvat, 2013; Silvestri and Di Marzo, 2012b) that do not produce undesired central nervous system side effects. LDK1229 suggests a new lead for developing novel CB₁ inverse agonists and is attractive for the design and synthesis of peripherally active CB₁ inverse agonists, a class of potential therapeutic agents for the treatment of obesity and other related metabolic syndromes. In general, there are several strategies to reduce a ligand's permeability to the blood brain barrier (BBB) (Clark et al., 2005). These include increasing H-bonding capacity, molecular weight (MW) and polar surface area (PSA) as well as introducing acidic functional groups or increasing hydrophilicity. In comparison with the conventional scaffold of CB₁ inverse agonists (i.e. biaryl substituted heteroaromatic ring), the benzhydryl piperazine scaffold offers two basic nitrogen atoms, which provide extra opportunities to increase the overall H-bonding capability. Additionally, the two basic nitrogen functionalities, are considerably easy to modify to introduce various substitutions so that the MW

and PSA properties can be readily manipulated to gain suitable pharmacokinetic (PK) properties that reduce BBB penetration. Hence, this scaffold enriches the chances for PK-driven drug optimization to achieve the desired peripheral restriction of CB1 inverse agonists.

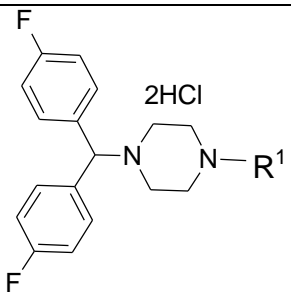
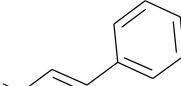
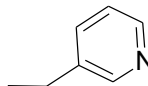
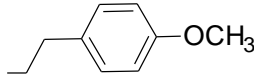
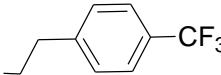
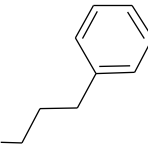
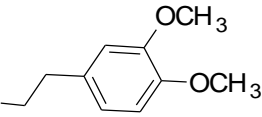
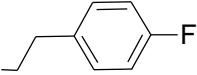
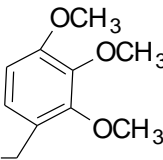
In a preliminary attempt, we designed and synthesized 3 compounds, LDK1270, LDK1273, and LDK1276, to be peripherally active (Tables 3.5-3.7). Although LDK1270 and LDK1273 showed no detectable binding with up to 32 μ M of LDK compound, LDK1276 showed promise in its ability to partially displace (41% displacement) the inverse agonist [3 H] SR141716A when using 32 μ M of LDK1276 in the presence of 5 nM of [3 H] SR141716A. The hydrophilic nature of these compounds (see structures in tables 3.5-3.7) may be the cause of their inability to penetrate the HEK293 membrane preparations and bind CB1 to fully displace the inverse agonist SR141716A.

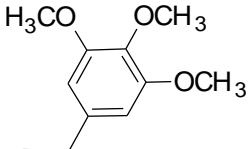
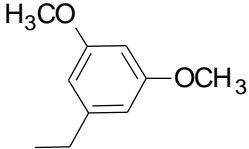
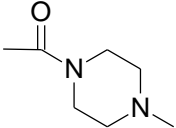
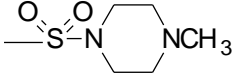
While the three peripherally active compounds identified thus far are previews of an early effort, they provide information on chemical templates and strategy—knowledge that can be of value for a new lead for optimizing and developing novel CB1 inverse agonists. The potential ability of these agents to treat obesity and other related metabolic syndromes in a unique and specific fashion versus available therapies makes the design of peripherally active compounds an enticing approach.

The modeling hypothesis presented in this manuscript proposes that the interaction of LDK1229 with the binding pocket of CB1 differs from the binding of SR141716A. This would suggest that the mode of action for the two inverse agonists is possibly different. Future studies, including the downstream signaling effects of LDK1229 compared to those of SR141716A, would provide more insight into whether LDK1229 will avoid the adverse side effects presented by SR141716A. In vivo animal studies of LDK1229 or its analogs will also give us more insight

into the physiological effects this inverse agonist presents in animal models. Another important avenue for future research will be to test LDK1229 against the endogenous endocannabinoids, and see whether they can compete with 2-AG and AEA and whether they are true inverse agonists in vivo for the confirmation of these results and their further characterization in the brain and clarification of their functional and therapeutic significance.

Table 3.5. Binding of benzhydryl piperazine analogs (entry numbers 1-12) to the wild-type CB₁ receptor.

				
Entry	LDK	R ¹	vs. [³ H] CP55940 nM	vs. [³ H] SR141716A nM
1	1203		259.9 (80.7-837.4)	296.8 (192.4-458.1)
2	1204		NB	NB
3	1205		1312 (700-2457)	-
4	1206		2035 (518.1-7992)	-
5	1207		746.2 (362.7-1535)	-
6	1208		1589 (1061-2378)	-
7	1209		1853 (1154-2976)	-
8	1211		686.5 (435.9-1081)	-

9	1233		1313 (855.4-1520)	-
10	1234		768 (227.2-2596)	-
11	1270*		NB	NB
12	1276*		-	partial [#]

NB: no detectable binding with up to 32 mM of LDK compound.

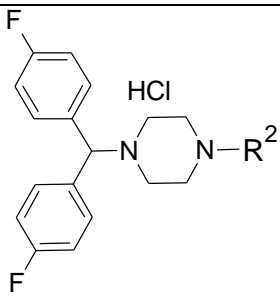
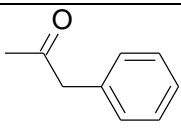
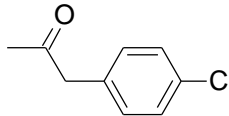
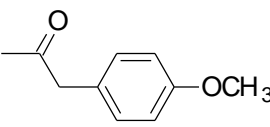
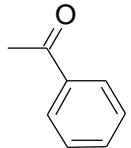
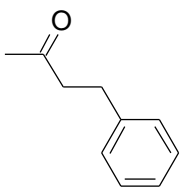
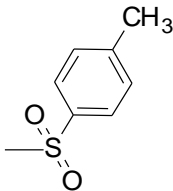
ND: not determined.

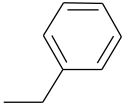
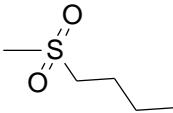
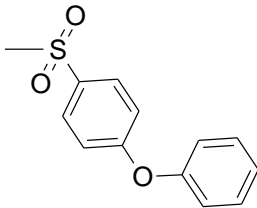
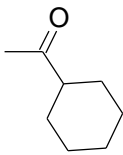
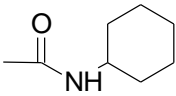
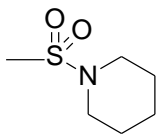
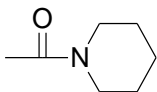
* designed to be peripherally active.

[#]41% displacement was observed at 32 mM of LDK1276 in presence of 2 nM of [³H] SR141716A.

- : not performed.

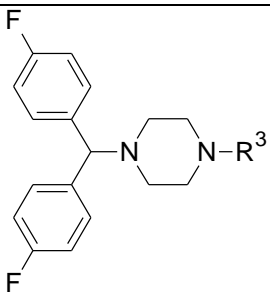
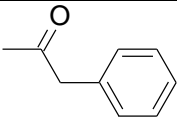
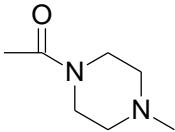
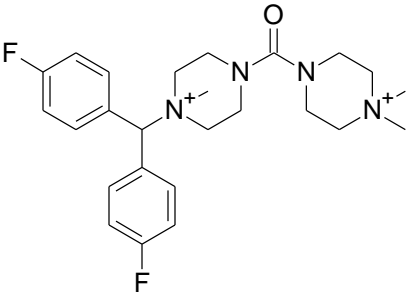
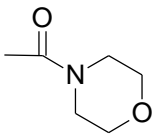
Table 3.6 Binding of benzhydryl piperazine analogs (entry numbers 13-25) to the wild-type CB₁ receptor.

				
Entry	LDK	R ²	vs. [³ H] CP55940 nM	vs. [³ H] SR141716A nM
13	1213		644.2 (365-1137)	-
14	1214		269.5 (176.8-410.8)	-
15	1215		1070 (692.5-1653)	-
16	1216		725.5 (213.3-2276)	-
17	1217		464.9 (156.7-1380)	-
18	1222		330.8 (176.6-619.1)	365.7 (144.1-928.2)

19	1223		530 (141.1-1991)	-
20	1227		276.8 (91.4-838.2)	-
21	1228		355.8 (156.9-806.6)	-
22	1229		219.7 (105.6-457)	245.5 (164.2-367.1)
23	1271		232.7 (148.7-364.1)	232.8 (127.6-425)
24	1275		252.1 (107-593.9)	292.8 (147.1-582.7)
25	1277		-	1077 (589.7-1968)

- : not performed.

Table 3.7 Binding of benzhydryl piperazine analogs (entry numbers 26-29) to the wild-type CB₁ receptor.

				
Entry	LDK	R ³	vs. [³ H] CP55940 nM	vs. [³ H] SR141716A nM
26	1212		1076 (643.2-1801)	-
27	1272 [^]		NB	NB
28	1273*		NB	NB
29	1274		NB	NB

[^]compound LDK1272 is the free base form of LDK1270.

NB: no detectable binding with up to 32 mM of LDK compound.

* designed to be peripherally active.

- : not performed.

CHAPTER 4

Site-specific Incorporation of the Unnatural, Photo-cross-linking Amino Acid *p*-benzoyl-L-phenylalanine (*p*Bpa) into the Cannabinoid Receptor CB2

4.1 Abstract

Many of the intracellular loops of GPCRs, including the carboxyl terminus, have been implicated in the regulation of receptor activation, signaling, and subcellular localization as well as the binding and dynamic recruitment of numerous proteins. However, studies thus far on interactions between the receptor and binding partners have mostly been restricted to peptides corresponding to regions of GPCRs and *in vitro* assays. Although we have learned extensively from these, predictions and extrapolation of data to the full-length receptor without really knowing how its presence may alter the structures, orientations and membrane interactions of the intracellular loops have been necessary. Incorporating *p*-benzoyl-L-phenylalanine (*p*Bpa), an unnatural amino acid with photo-cross-linking properties, into the full length CB2 receptor under its native cellular conditions will enable us to identify the binding partners of CB2 *in situ* either by mass spectrometry (MS) or by western blotting with antibodies to the putative binding partner. This technique has been used in bacterial cells but has only recently been applied to mammalian cells. In this chapter, we piloted and optimized the technique and show the successful incorporation of this unnatural amino acid into different sites on the CB2 receptor. The data presented here lays the foundation for the application of this technique to delineating the binding partners of CB2, and can also be used as a basis for other GPCR model systems. It will also aid in the

understanding of the ligand-dependent modulation of receptor activity. Different ligands produce different downstream signaling patterns and therefore recruit different binding partners for the different processes or pathways each ligand activates. We hope that this technique will help to delineate some of these details so that they can be used to ultimately design drugs that can fine-tune and regulate a GPCR and select the signaling pathway that produces the desired therapeutic effect while maintaining the least number of side effects.

4.2 Introduction

GPCRs associate with a plethora of interacting partners such as G proteins, GRKs, arrestins and many more accessory proteins (reviewed in (Brady and Limbird, 2002; Ritter and Hall, 2009)). In some cases these proteins can directly mediate receptor signaling, whereas in other cases they act as scaffolds to modulate G protein-mediated signaling.

G proteins. A myriad of studies involving chimeric substitutions, various mutational approaches, and the use of synthetic peptides have been performed to define the structural determinants of GPCRs in the interactions with G proteins. The studies have established the pivotal roles of the second (IC2) and the third intracellular (IC3) loops plus, in some receptors, the proximal part of the carboxyl-terminus in G protein coupling (reviewed in (Strader et al., 1994; Wess, 1998)). Moreover, numerous studies have established the pivotal role of IC2 in determining receptor and G protein coupling and interaction (Burstein et al., 1998; Fernandez and Puett, 1997; Wess et al., 1997). Replacement of the entire IC2 of the bradykinin B2 receptor with the E2 prostaglandin receptor resulted in a cAMP-generating receptor, which indicated the importance of this domain for Gs coupling and activation (Pal-Ghosh et al., 2003). For the AT1

angiotensin receptor (Gáborik et al., 2003), the IC2 domain appears to have a direct role in agonist-induced G protein coupling. The role of IC2 was further substantiated by Brann and co-workers (Burstein et al., 1998), who developed a random mutagenesis approach for their study of muscarinic receptor coupling. In IC2 of the M5 muscarinic receptor, they found that substitution of residues clustering on one side of a presumed IC2 α -helix extending from TM3 caused constitutive activation, while substitutions of residues clustering on the opposite side of the helix compromised G protein coupling. Taken together, the data suggest that the residues on the constitutively activating side were critical for maintaining the receptor in an inactive state, whereas the residues on the opposing side were important for G protein activation (Burstein et al., 1998). It was therefore inferred that IC2 could act as a switch that enables G protein coupling (Burstein et al., 1998).

In addition, the crystal structures of GPCRs provide us with information on the relationship between the structure and function of a GPCR. Recent crystallographic studies have suggested that G protein activation requires a conformation change in IC2 (Rasmussen et al., 2011; Scheerer et al., 2008a). The recent β 2AR-Gs crystal structure showed receptor residues Thr 68 and Asp 130 interacting with the IC2 helix of the β 2AR via Tyr 141, positioning the helix so that Phe 139 (IC2) of the receptor docks into a hydrophobic pocket on the G protein surface, thereby structurally linking receptor–G protein interactions with the highly conserved E(D)RY motif of the β 2AR (Rasmussen et al., 2011).

In a recent cannabinoid receptor study (Zheng et al., 2013), the substitution of IC2 of CB2 with the corresponding region of CB1 displayed a two-fold increase in basal activity as compared to the wild-type, suggesting the possible role of IC2 in the interaction of CB2 with G proteins. The chimeric mutant with a replacement of both the IC2 and carboxyl-terminal tail with the

corresponding regions of CB1 led to a switch of G protein coupling from Gi to Gs. These results indicate that the IC2 of CB2 is likely to play a key role in the specificity of G protein coupling in coordination with the carboxyl-terminal tail (Zheng et al., 2013).

GRKs and β -arrestins. At the receptor level, GRKs phosphorylate agonist-activated 7TM receptors on serine and threonine residues located in the carboxyl-terminal tail region or on the third cytoplasmic loop. β -arrestin is recruited with high affinity to agonist-occupied, GRK-phosphorylated receptors, and ultimately sterically inhibits G protein coupling (Shenoy and Lefkowitz, 2003). The carboxyl termini of GPCRs have been implicated in the binding and dynamic recruitment of numerous proteins as well as the regulation of receptor activation, signaling, and subcellular localization. For CB1, significant structure within its carboxyl-terminus has been elucidated (Ahn et al., 2009b) and interactions of accessory proteins with this region have been found to be critical for mediating key points of the receptor life cycle (Figure 4.1; reviewed in (Stadel et al., 2011)).

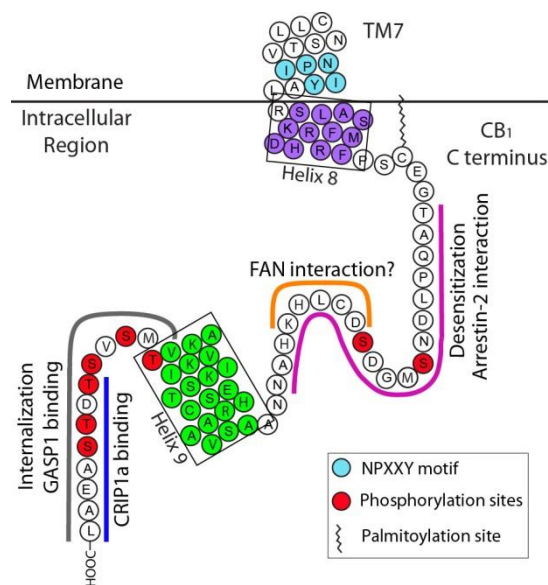


Figure 4.1. Schematic diagram of the carboxyl terminus of the human CB1 receptor showing the proposed structural and functional domains. The NPXXY motif, helix 8, and helix 9 are highlighted in cyan, purple and green respectively. Potential phosphorylation sites are indicated by red filled circles and the TM7 NPXXY motif by turquoise filled circles.

For CB2, very little information is known regarding the functional domains within IC2, IC3 and the carboxyl-terminus to which accessory proteins are recruited. Therefore, an understanding of the extent to which the CB2 cellular fate is governed by these domains is still in its infancy. It is important to elucidate the CB2 protein binding partners, domains they interact with, and under what circumstances. Knowledge of these binding partners opens up avenues by which receptor activity can be fine-tuned and regulated.

To explain the molecular mechanisms underlying cell activities it is important to analyze the network of interactions between molecules in their native cellular context. Methods involving co-immunoprecipitation with tagged proteins can isolate the protein complexes that actually form in cells. However, for these methods to be effective, the complexes must be stable and cannot dissociate during cell extraction. Moreover, it is possible to capture nonspecific interacting partners in immunoprecipitation complexes. Cross-linkers, which can be incorporated into proteins as amino acids with photo-reactive linkers in their side-chain moieties (Brunner, 1993) are therefore an important tool in stabilizing such complexes. One such photo-reactive amino acid is *p*-Benzoyl-L-phenylalanine (*p*Bpa) (Figure 4.2a). Upon excitation at 350-365nm, the benzophenone group of *p*Bpa reacts with nearby C-H bonds forming a covalent bond with its bound biomolecule (Figure 4.2b; (Dorman and Prestwich, 1994)). Because the *p*Bpa is incorporated into the amino acid sequence of the protein, its location within the protein is known

and therefore gives much information regarding the location of the bound cross-linked biomolecule.

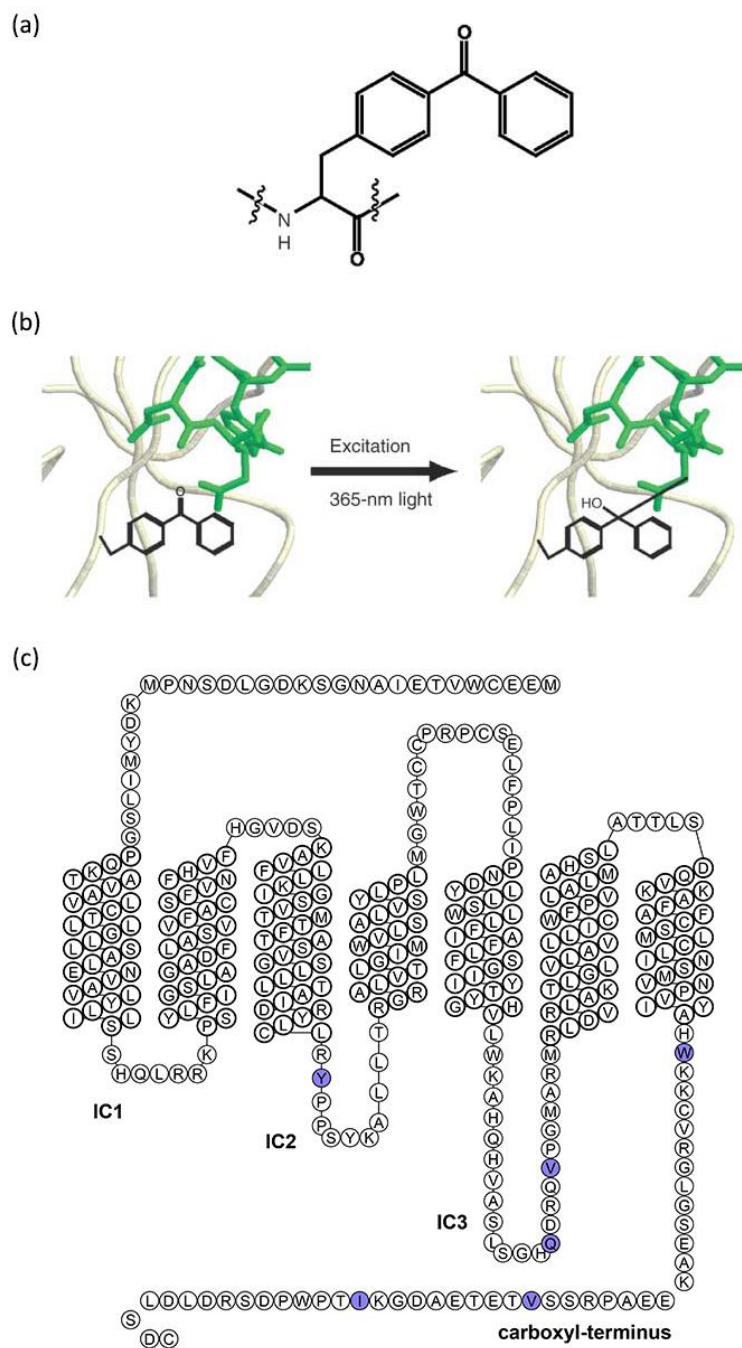


Figure 4.2. *pBpa* cross-linking (a) Chemical structure of *pBpa*; (b) Illustration of a photo-cross-link between *pBpa* and a bound protein upon excitation with 365nm light. (c) Snake diagram of

CB2 with the sites in IC2, IC3 and the carboxyl-terminus that were separately replaced with *p*Bpa in blue. SOURCE: images **(a)** and **(b)** are modified from (Hino et al., 2005).

The Kendall lab has previously used this photo-cross-linking method for purified proteins and in bacterial cells (Wang et al., 2004; Yu et al., 2013) and it has now been adapted to mammalian cells (Hino et al., 2005). In 2011, Sakmar and co-workers (Grunbeck et al., 2011) employed an *in situ* photocrosslinking approach by incorporating the unnatural amino acids *p*-Azido-*l*-phenylalanine (AzF) and *p*-acetyl-*l*-phenylalanine (AcF) to study the binding interactions of inhibitor T140 specific for CXC chemokine receptor 4 (CXCR4). This receptor is important in directed cell migration, cancer metastasis, and HIV entry. They tested eight amino acid positions at the receptor interface and mapped a unique UV-light-dependent crosslink *in vivo*—the site of specific interaction between CXCR4 residue 189 and T140 (HIV-1 co-receptor blocker). Similarly, Becker and co-workers (Huang et al., 2008) used *p*Bpa to probe the potential binding site of the pheromone receptor Ste2 (a yeast GPCR). They were able to identify positive crosslinks as potential binding sites for its natural peptide α -factor ligand (Huang et al., 2008). These first experiments fully confirmed that site-specific unnatural amino acid insertion within the receptor is an invaluable noninvasive tool to study the mechanisms and dynamics of GPCR ligand binding and signal transmission.

To delineate the sites on the CB2 receptor that interact with different proteins, *p*Bpa was incorporated into different sites on IC2, IC3 and the carboxyl-terminus of CB2 individually (Figure 4.2c) based on the importance of these regions in CB1 and other class A GPCRs. This technique enables us to observe the *in situ* crosslinking of the full-length CB2 in its native environment under defined conditions to identify its binding partners and their site of interaction. The genetic encoding of this non-natural amino acid in mammalian cells requires a bacterial pair

of an amber suppressor tRNA and an aminoacyl-tRNA synthetase (aaRS) specific to the non-natural *p*Bpa (Hino et al., 2006). We therefore used an exogenous tRNA-aaRS pair specific to *p*Bpa (Figure 4.3) to translate this amino acid in place of the amber codons. The tRNA used is only recognized by the *Eco-p*BpaRS (Figure 4.3a) but not recognized by any endogenous mammalian cell amino acyl-tRNAs (aaRS). The *Bst*-sup-tRNA (Figure 4.3b) used will suppress the phenotypic “stop” signal of the amber codons and become charged with *p*Bpa by the action of the *Eco-p*BpaRS. The pcDNA4/TO vector that incorporated this tRNA-aaRS pair uses the complete cytomegalovirus promoter (PCMV) to promote high expression levels as well as control elements from the bacterial tetracycline operon (TetO2) to give flexibility to transcription control. It also includes zeocin (ZeoR) and ampicillin (AmpR) antibiotic resistance genes that will allow for effective selection of the properly transfected HEK293 mammalian cells. Using site directed mutagenesis, we introduced an amber stop codon (UAG), one at a time, into several locations along IC2, IC3 and the carboxyl-terminus substituting for naturally occurring phenylalanine or other bulky hydrophobic residues when possible (Figure 4.2c). It is anticipated that substitutions at the heart of any binding site could disrupt interactions but regions immediately flanking binding sites would lead to successful cross-linking. We show here pilot and optimization studies of the successful incorporation of *p*Bpa into different sites on the CB2 receptor. The data presented here lays the foundation for the application of this technique to delineating the binding partners of CB2, and can also be used as a basis for other GPCR model systems.

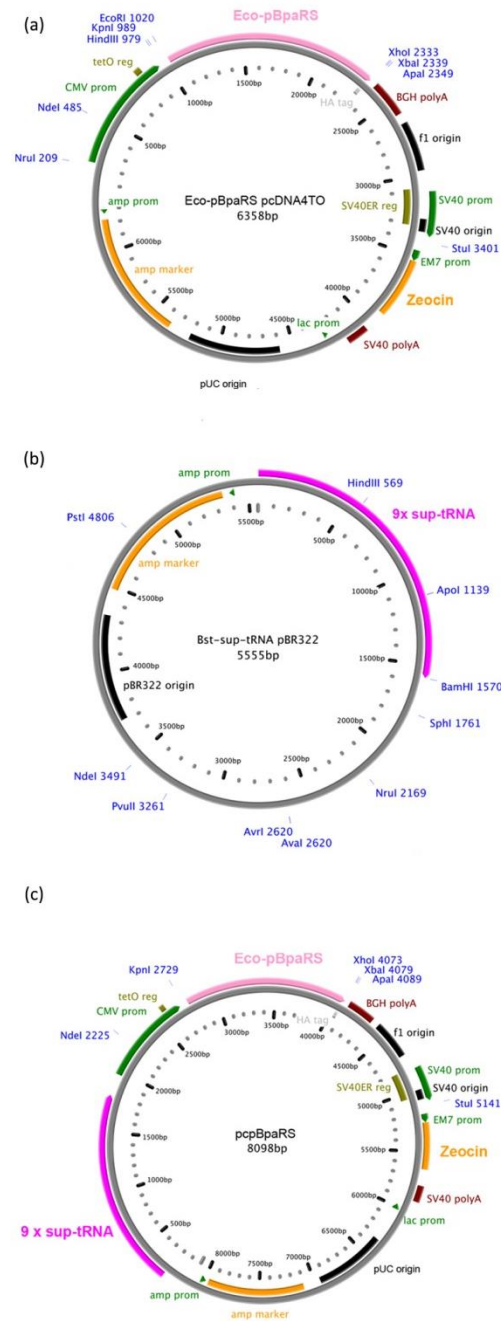


Figure 4.3. Map of the plasmids needed for *pBpa* incorporation. (a) Eco-*pBpaRS* plasmid encoding the *pBpa* synthetase; (b) *Bst*-suppressor tRNA pBR322 encoding the ‘stop’ codon suppressor tRNA; (c) pcBpaRS plasmid encoding both the suppressor tRNA and the *pBpa* synthetase

4.3 Materials and Methods

Materials. *pBpa* was purchased from Bachem AG (Bubendorf, Switzerland). The anti-hemagglutinin antibody was from Roche Diagnostics (Indianapolis, IN). The horse-radish peroxidase-conjugated anti-mouse IgG was from Genscript (Piscataway, NJ).

Plasmids. The tRNA-aaRS pairs necessary for the genetic encoding of non-natural amino acids have been developed from naturally occurring tRNA and aaRS species (Chin et al., 2003) and were a generous gift from the Yokoyama lab at RIKEN Genomic sciences center in Japan (Hino et al., 2006). The naturally occurring *Escherichia coli* tyrosyl-tRNA synthetase (*E.coli* TyrRS) was engineered to only recognize *pBpa* and is known as the *Eco-pBpa* tRNA synthetase (Kiga et al., 2002). This synthetase was cloned into the MCS site of a pcDNA4/TO plasmid (*Eco-pBpaRS*; Figure 4.3a) under the tetracycline-regulated CMV promoter, and the Zeocin resistant gene under the SV40 promoter.

The amber suppressor tRNA was derived from *Bacillus stearothermophilus* tRNA and nine tandem copies of it were cloned into the MCS site of pBR322. Each of the nine tRNA genes has the internal promoter that works in mammalian cells (*Bst-sup-tRNA* pBR322; Figure 4.3b) (Hino et al., 2006). This tRNA is only recognized by the *Eco-pBpaRS* but not recognized by any endogenous mammalian cell amino acyl-tRNAs (aaRS). In addition, another plasmid, *pcpBpaRS* (Figure 4.3c), was constructed and carries both the *pBpaRS* gene under the tetracycline-regulated CMV promoter, together with the nine tandem copies of the *sup-tRNA* gene and the Zeocin resistant gene under the SV40 promoter. Two versions of the *pBpaRS* synthetase were used, one with an HA tag and one without.

Site-directed mutagenesis. The wild-type CB2 gene in a pcDNA3.1 vector was mutagenized using the QuickChange site-directed mutagenesis kit, to generate a series of clones with individual amber codons in CB2 at position 137 in IC2, positions 231 and 227 in IC3 and positions 317, 337 and 346 of the CB2 carboxyl terminus.

Transfection. HEK293 cells were seeded into 6-well plates with 1×10^5 cells/well on the day prior to transfection. The cells were maintained in about 2 ml/well of DMEM growth medium supplemented with 10% FBS and 3.5 mg/ml glucose at 37°C in 5% CO₂. Transfection was carried out using the Lipofectamine 2000 reagent (Invitrogen; according to manufacturer's protocol) in the Gibco OptiMEM growth medium (Invitrogen). The HA tagged CB2 receptor plasmid was used at a concentration of 0.2 µg/well (whether it was wild-type CB2 or it contained an amber mutant codon). The CB2 amber mutant gene plasmid was mixed with the suppresser tRNA plasmid (Bst-sup-tRNA/pBR322) and the synthetase plasmid (EcopBpaRS (no HA tag)) in one condition (3 plasmids total) and with the combination plasmid (pcpBpaRS (no HA tag)) in another condition (2 plasmids total). In the pilot studies, the plasmids coding for the tRNA-aaRS pair were used at concentrations ranging from 0.2-5 µg. Four hours after transfection, the transfection medium was replaced with DMEM (supplemented with 10% FBS and 3.5 mg/ml glucose) containing *pBpa* at a concentration of 0.5 mM and was incubated at 37°C for 36-40h for proper *pBpa* incorporation. To prepare 15 ml of the DMEM medium containing *pBpa* at the final concentration of 0.5 mM, 2 mg of *pBpa* were dissolved in 300 µl 1 M HCl, and immediately added to 13.5 ml of the DMEM pre-warmed at 37 °C. The pH of this medium was adjusted to 7.2 with 1 M NaOH and the final volume to 15 ml with sterile water. Finally, the medium was filtered through a 0.22 µm filter connected to a syringe for sterilization. Because *pBpa* is light

sensitive, most experimental steps involving the preparation or following the addition of *pBpa* to the media were carried out in low to no light conditions.

During the 4h transfection incubation, the mutant CB2 gene, the gene encoding *pBpaRS* and the suppressor tRNA will begin to be expressed. Because *pBpaRS* does not recognize any amino acid except *pBpa*, if the medium is not supplemented with *pBpa*, only the gene products truncated at the amber position will be expressed (see results).

UV Photo-cross-linking. Approximately 30 h after *pBpa* was added to the media (during the 36-40h *pBpa* incorporation incubation), the media was supplemented with 10 μ M SR144528, a CB2 inverse agonist. After 8 h, the cells were washed with ice-cold PBS and treated with CP55,940 for 1min, 5min and 20min and washed again. The culture dishes containing these cells in PBS were then irradiated with a Spectroline Built-in-Ballast UV lamp (model BIB-150P, 365nm) for 30sec, 1min, 5min and 15min.

Western Blotting. Following the 36-40h incubation in *pBpa*-containing media (and after treatment with the CB2 inverse agonist SR144528 and/or the agonist CP55940 for some of the samples), the cells were solubilized and lysed in 200 μ l/well of ice-cold Cell Lytic M reagent (Sigma) (plus 1% Triton X-100, and 1% protease inhibitor cocktail). Samples were subjected to SDS-polyacrylamide gel electrophoresis in 7.5% gels, and proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 5% nonfat milk (Bio-Rad), the membrane was incubated for 1 h with the anti-hemagglutinin (anti-HA) primary antibody (1:4000). The membranes were washed with PBS, and then they were incubated with the horseradish peroxidase anti-mouse secondary antibody (1:6000) for 1 h at room temperature. The specific immunoreactive proteins were detected using the SuperSignal West Femto

Chemiluminescent Substrate System (Thermo Fisher Scientific, Rockford, IL) following the manufacturer's protocol and visualized on x-ray film.

Initial Membrane Solubilization. Crude cell membranes expressing CB1 (tagged with c-myc, Flag or HA) were used. Briefly, HEK293 cells were plated with 1×10^6 cells/100-mm dish on the day prior to transfection. The cells were maintained in DMEM growth medium supplemented with 10% FBS and 3.5 mg/ml glucose at 37°C in 5% CO₂. 24 hours after transfection (refer to Chapters 2 and 3 for calcium phosphate transfection method) cells were harvested and resuspended in PBS. The cells were then centrifuged at 500 x g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 1ml PBS and centrifuged again in a swinging bucket microcentrifuge at 500 x g for 5 min. The supernatant was once again discarded and the cell pellet was resuspended in 250 µl of 1x KHD lysis buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 150 mM NaCl, 10% protease inhibitor cocktail) per 100-mm dish. The samples were then sonicated and centrifuged at 21,000 x g for 30 min in a refrigerated microcentrifuge. The supernatant was removed and the pellet was resuspended in 1 of 5 different detergents: RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), 1% SDS (dissolved in 1x KHD buffer), 1.2% digitonin (dissolved in 1x KHD buffer), 8 mM CHAPS or 1% Triton X-100. The pellet was left to solubilize for 1 hour at 4°C and centrifuged at 21,000xg for 30 min in a refrigerated microcentrifuge. The supernatant was removed and placed into pre-chilled 1.5 ml Eppendorf tubes. Solubilization efficiency was checked by running both 7.5% SDS-PAGE gels or running 8 M Urea 7.5% SDS-PAGE gels. Solubilization of the CB receptors was also initially tested after extraction of the plasma membrane from HEK293 cells that were transfected with the receptors. The solubilization from the plasma membrane preparations proved to be inefficient.

2.4 Results and Discussion

Cannabinoid receptor solubilization. Despite their critical importance, our current understanding of the structure and function of the cannabinoid receptors is limited because of their low natural abundance and the challenge of obtaining large quantities of purified protein. The same is true for other membrane proteins. Solubilization and stabilization of membrane proteins using detergents are the first critical steps in purifying membrane proteins, and constitute a bottleneck for the structural biology of membrane proteins (Hjelmeland and Chrambach, 1984; van Renswoude and Kempf, 1984). Membrane proteins require a membrane-like environment to maintain their correctly folded structures and functions during and after purification. Detergent micelles provide such environments surrounding the hydrophobic domains of membrane proteins and keeping them soluble in an aqueous environment, and thus are widely used for their solubilization and purification (le Maire et al., 2000). Generally, the suitable detergent or detergent mixture should effectively solubilize the target protein, keep it stable, and prevent its self-aggregation. However, due to individual differences between membrane proteins, the choice of detergent or detergent mixture for a particular protein cannot be predicted. Therefore, a systematic approach is required to select the optimal detergents to achieve solubilization and stabilization of each target protein. It is also important to be able to isolate the target protein on a gel into a distinct band that is free of other contaminating proteins so that it can be excised in a semi-pure form from the gel for further analysis, namely mass spectrometry.

For the cannabinoid receptors, five different detergents were tested based on their success in the solubilization of other GPCRs: CHAPS, 1.2% digitonin, 1% SDS, RIPA, and 1% Triton X-100. Figure 4.4a and 4.4b show four out of the five detergents used to solubilize the different tagged-

CB1 receptors. These are 8 mM CHAPS and 1.2% digitonin in Figure 4.4a and RIPA and 1% SDS in Figure 4.4b. In these four cases the solubilization was unsuccessful for the CB receptors even after several conditions, such as temperature, incubation time, buffer sample preparation, membrane preparation extractions versus crude cell lysate, were changed.

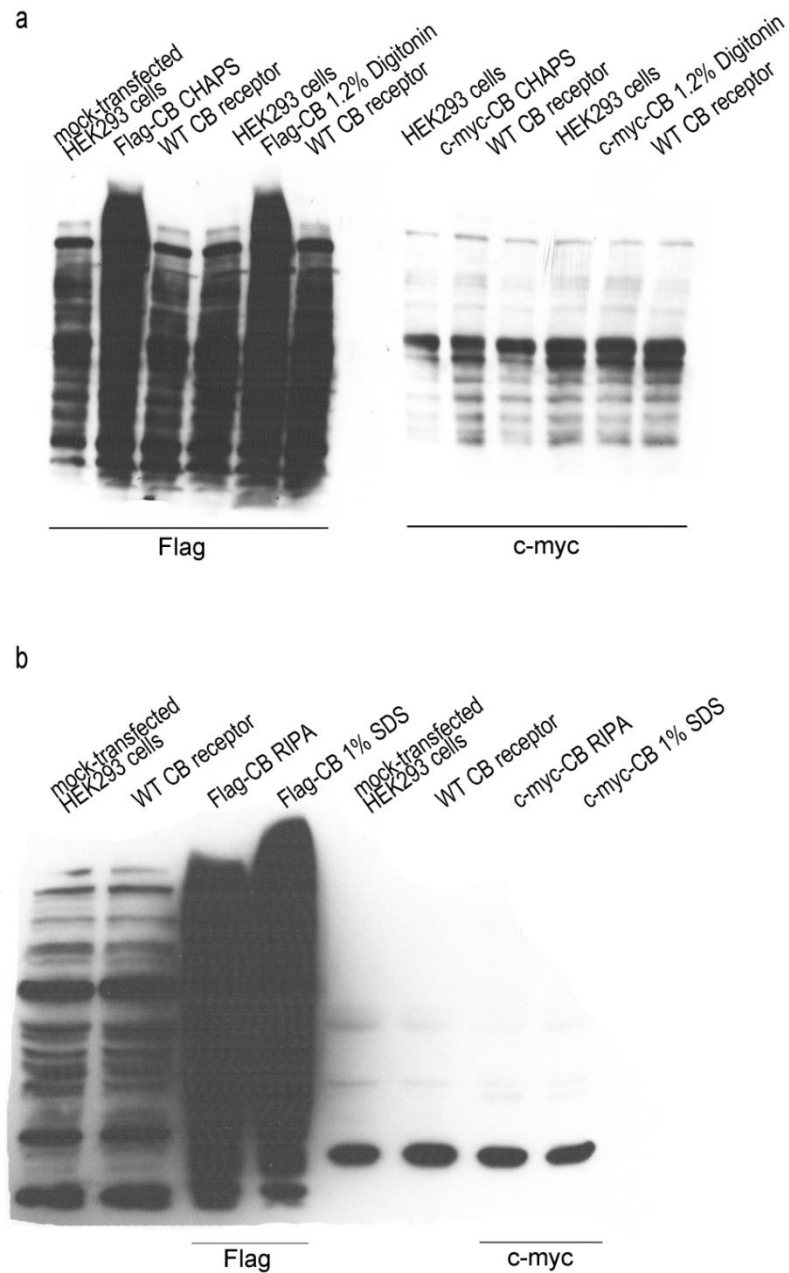


Figure 4.4 Initial solubilization of the CB receptor with CHAPS, 1.2% digitonin, RIPA or 1% SDS. Panels a and b show western blots of Flag- and c-myc CB tagged receptors under treatment with different detergents.

In Figure 4.5a, the use of Triton X-100 (plus 1% protease inhibitor cocktail) to solubilize and lyse HA tagged CB1 or CB2 receptors proved to be successful. Two bands corresponding to the molecular weights (MW) of HA-CB1 (53-60 kDa) and HA-CB2 (42-46 kDa) were visualized. However, the HA tagged CB2 receptor was chosen to be used in further experiments due in part to its higher level of expression in HEK293 cells, but mostly due to its slightly lower MW that produces a clear band that is not adjacent to any non-specific bands (see Figure 4.5a and 4.5b).

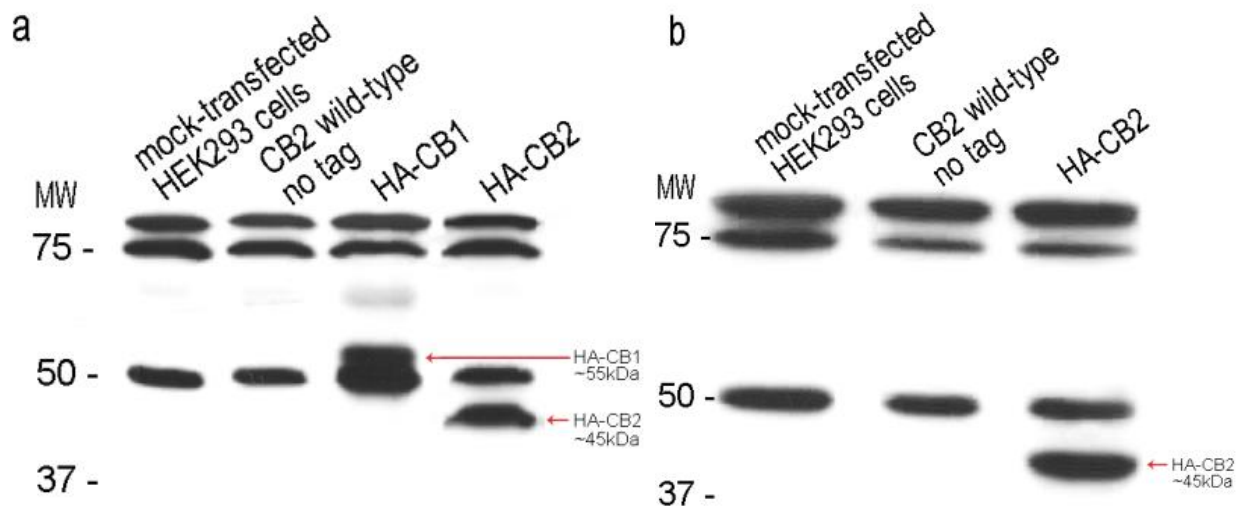


Figure 4.5 Solubilization of the CB receptors using 1% Triton X-100. (a) two bands corresponding to the molecular weights (MW) of HA-CB1 (53-60 kDa) and HA-CB2 (42-46 kDa). (b) The HA-CB2 band (42-46 kDa) is clear of any non-specific bands.

Amber codons and truncated CB2 receptors. As mentioned earlier, *pBpaRS* was engineered to only recognize *pBpa* and no other amino acids. It follows then, that if the cell growth medium is not supplemented with *pBpa*, that only the gene products truncated at the amber positions will be expressed. Figure 4.6 shows a shift in CB2 MW due to the successful incorporation of the stop codon and its truncation at positions Tyr137 in IC2 of CB2, positions Glu227 and Val231 in IC3 and positions Trp317, Val337 and Ile346 of the CB2 carboxyl terminus when no *pBpa* was added to the growth medium.

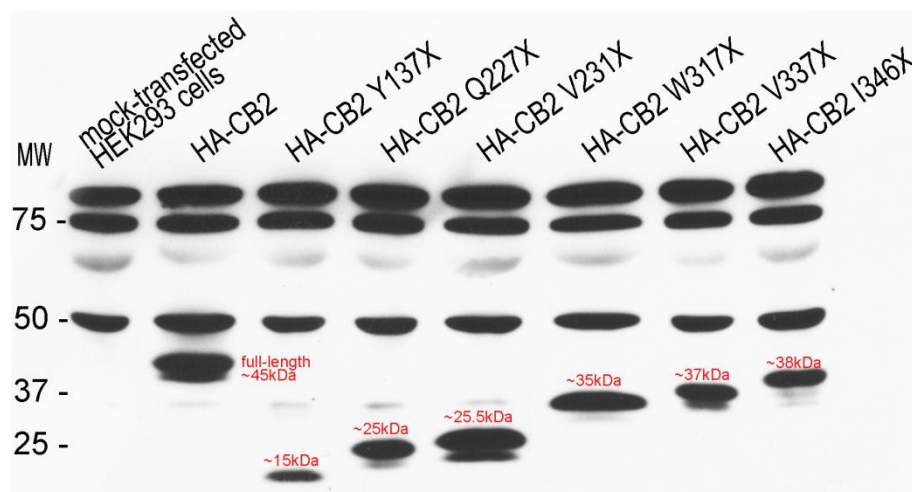


Figure 4.6 Incorporation of the stop codon at different positions in HA-CB2 evident by a molecular weight shift. Western blots using anti-HA antibodies to detect the truncated HA-CB2 receptors.

Truncated to full length CB2 receptors. Upon the addition of 0.5 mM of *pBpa* (concentrations ranging from 0.5 mM to 2 mM were tested; 2mM was found to be slightly toxic to the cells) to the cell growth medium, *pBpaRS* could now recognize the *pBpa* and incorporate the unnatural

amino acid in place of the stop codon. We also varied the concentrations of the pcBpaRS plasmid, which contained both the synthetase and the suppressor tRNA, and found that 2-3 μ g of this plasmid produced the full-length CB2 receptor in the cell from its truncated Y137X receptor (Figure 4.7a) and its W317X receptor (Figure 4.7b). Increasing the concentration of the plasmid to 5 μ g resulted in no bands on the blot (see Figure 4.8a lanes 6-8). In general, even after the addition of *pBpa* to the medium, the gene product truncated at the amber position is also expressed because the efficiency of amber suppression by the suppressor tRNA is not 100%. In the case that this truncated gene product is stable and exhibits some activity in the cell, future experimental results could be affected by the expression of this product and it may therefore need to be removed or taken into consideration. In Figure 4.7b, the amber suppression for the W317X receptor was not as efficient as the suppression for the Y137X receptor (Figure 4.7a). Because of this difference in amber suppression efficiency, the Y137X was used for future experiment optimization.

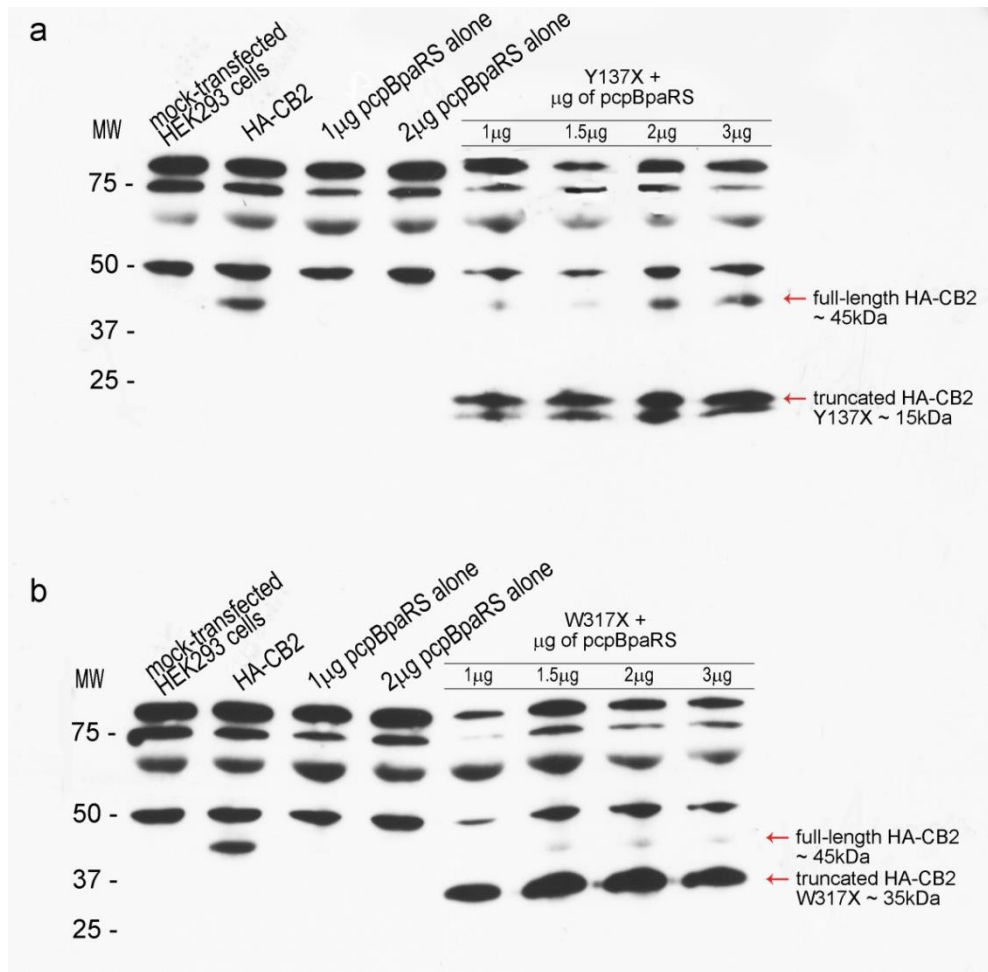


Figure 4.7 Truncated to full-length CB2 receptors. Concentrations of the pcpBpaRS plasmid, which contains both the synthetase and the suppressor tRNA, were varied. **(a)** The suppression of the amber codon in position Y137X was most effective when 2 or 3 µg of pcpBpaRS were used. This resulted in the formation of the full-length HA-CB2 receptor. **(b)** The suppression of the amber codon in position W317X was not as efficient as the suppression in a. This is evident by the faint band representing the full-length HA-CB2 receptor in b at approximately 45kDa. Control lanes 3 and 4 in both panels a and b show different concentrations of the plasmid alone and that the bands in other lanes are not due to the addition of the pcpBpaRS plasmid.

UV photo-cross-linking. Once the *p*Bpa was successfully incorporated into the CB2 receptor, in order to see what the receptor could capture and what it interacts with, cross-linking requires exposure to 365 nm light. Most of the publications on the use of *p*Bpa and the incorporation of unnatural amino acids have exposed their samples to UV light for 15 minutes while the sample was on ice (4°C) to avoid heating up of the cells and the drying up of the liquid medium. However, it has been shown that at 4°C, the internalization of the receptors does not occur (Burke et al., 2001). We also know that many of the critical cellular events within a cell occur within a short period of time after the receptors are activated by a ligand. For example, our lab has shown that at 5 minutes of treatment with the CB1 agonist CP55,940, the phosphorylation of ERK1/2 peaks (Ahn et al., 2012; Ahn et al., 2013). This means that some of the key cellular processes, such as G protein coupling or phosphorylation by kinases at the cell surface or the recruitment of signaling complexes such as β -arrestin may occur rapidly at the plasma membrane before endocytosis. Our purpose for studying the receptors using *p*Bpa is to see some of these critical cellular processes and delineate the different proteins that are recruited at different receptor regions and at different time duration after treatment with different ligands. We therefore chose shorter UV exposure times and kept the cells at room temperature while being irradiated.

To reduce the diversity in the activation states and localizations of the CB2 receptors, we pre-treated the cells with 10 μ M SR144528, a CB2 inverse agonist, for 8 h. This treatment is intended to bring the CB2 receptors to the cell surface and standardize their starting point so that they all begin at the same location. After 8 hours, (the minimum amount of time determined experimentally using confocal microscopy needed for more than 85% of the CB2 receptors to be expressed on the cell surface) the cells were washed to remove excess SR144528 and treated

with 10 μ M CP55,940 for 1 min, 5 min or 20 min and then washed to remove excess CP55,940 after each time point. The culture dishes containing these cells in PBS were then irradiated with 365 nm light for 30sec, 1 min, 5 min and 15 min (Figure 4.8).

Figure 4.8a (lanes 5 and 8) show that irradiation with 365 nm light for 15 min produced no bands on the blot. This may be due to several factors including the denaturation of the receptors. Lanes 3 – 4 in Figure 4.8a, that were irradiated for a shorter time period, show the truncated CB2-Y137X receptors and the successful amber suppression resulting in full length receptors. Lanes 6-8 show that using more of the pcpBpaRS plasmid actually does not improve amber suppression efficiency but has the opposite effect. In addition, it is possible that some of the bands that are marked by a red asterisk are cross-linked CB2 complexes. Their apparent molecular weight is approximately 67kDa which very roughly estimates the molecular weight of a CB2-G protein complex (G protein kDa ~ 23kDa).

Figure 4.8b shows irradiation with 365 nm light for 30sec, 1 min, and 5 min without CP55,940 treatment (lanes 4 – 6) and with 10 μ M of CP55,940 treatment for 5 minutes (lanes 7 – 9) of the truncated CB2-V231X receptor. With no CP55,940 treatment, a possible CB2-G protein complex band at ~67kDa is visible (lanes 4 – 5) when irradiated with 1 minute or less of UV, consistent with the results in Figure 4.7a using the truncated CB2-Y137X receptor. 5 minutes irradiation with no CP55,940 treatment showed no complex formation.

After 5 minutes treatment with CP55,940 (lanes 7 – 9) and over 1 minute irradiation with UV (lanes 8 – 9), a new band appeared at approximately 130kDa, which roughly estimates the molecular weight of a CB2-kinase complex (kinase kDa ~ 80-85kDa).

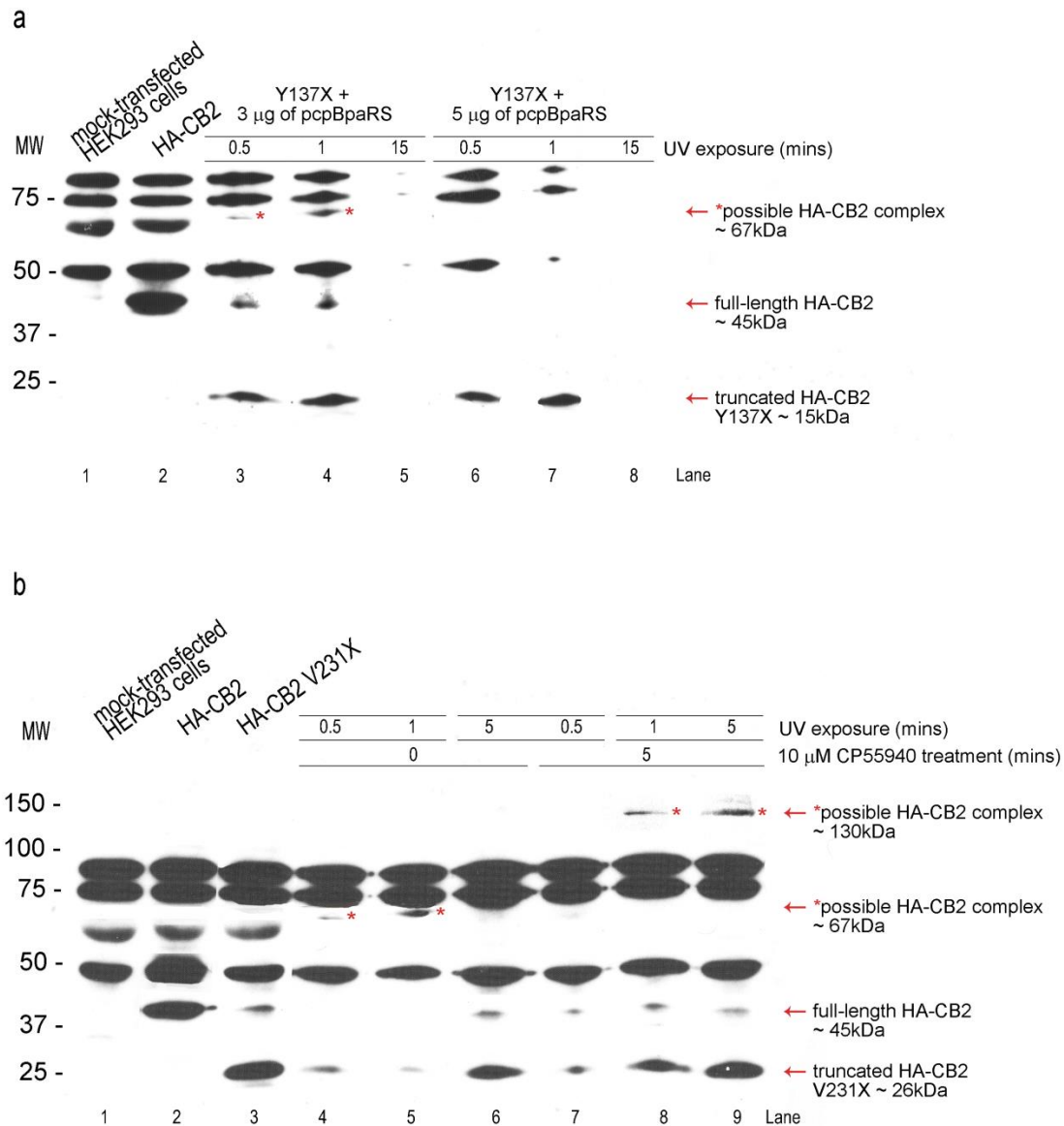


Figure 4.8 Western blots showing the formation of possible CB2-binding partner complexes upon irradiation with UV and treatment with 10 µM of the inverse agonist SR144528. **(a)** Irradiating the Y137X HA-CB2 mutant receptor with UV for 15 minutes resulted in no bands. Using 5 µg of the pcBpaRS plasmid resulted in no bands corresponding to the full-length HA-CB2 receptor at 45kDa. **(b)** HA-CB2 V231X receptor using 3 µg of pcBpaRS and treatment

with 10 μ M of the agonist CP55,940 at the designated time points. Possible HA-CB2 complexes are shown using a red asterisk.

4.5 Summary and Future Considerations

The main focus of this chapter was to optimize the incorporation of the photo-crosslinkable amino acid *p*Bpa into the intact receptor in live cells in order to identify and reveal the CB2 binding partners in IC2, IC3 and the carboxyl-terminus regions of the receptor. Literature on the receptor strongly argues that G proteins, kinases and arrestins bind these regions. This chapter shows the successful application of this technique and may lead the way to the investigation of a plethora of other molecules believed to interact with the CB2 receptor and regulate its function.

One of the major problems that I was faced with is the efficient suppression of the amber codon and the formation of a large enough amount of full-length CB2 receptor with incorporated *p*Bpa. The literature discusses this low yield as being normal since the suppression of the amber codon will never truly be 100%. It is also mentioned that the amber suppression and formation of full-length receptor in the presence of *p*Bpa may entirely depend on the location in which the amber codon is incorporated. Some residues on the protein may be in locations that are sterically inaccessible to the suppressor tRNA or the synthetase compared to other residues and thus result in less efficient amber suppression. This is evident in my own results in Figure 4.7b where the suppression of the amber codon in position W317X on the CB2 receptor was not as efficient as the suppression in Figure 4.7a of the amber codon in position Y137X. The only solution suggested was to just keep trying other residues in different locations.

One of the key tests that will need to be done on the HA-CB2 mutant receptors, is to ensure proper receptor folding and activity after the *p*Bpa is incorporated into the amber positions. This

can be done using the ligand binding and G protein coupling activities of the receptor. Ligand binding assays are used to determine receptor affinities for ligands and can be indicative of proper receptor folding and activity compared to the wild-type receptor. The G protein coupling assay is an indicator of receptor activation and therefore, indirectly, the extent of desensitization.

Once receptor activity is established, there are two possible ways by which the binding partners can be identified once they are cross-linked to the CB2 receptor. The first of these methods is to probe the same samples that resulted in possible complexes with antibodies specific to the receptor or to the putative cross-linked partner (e.g. kinase(s) or G protein(s)). Possible kinase candidates include G protein receptor kinases (GRKs), protein kinase A (PKA), and protein kinase C (PKC). This will serve to identify the locations that lead to successful crosslinking and under what *in situ* conditions.

The second avenue for identifying receptor binding partners is by performing quantitative MS. Bands that are suspected to represent a CB2 complex will be isolated and proteolytically digested to generate a mixture of peptides that can be identified by MS. The proteins present in the sample can be deduced by recombining the sequences of the identified peptides using suitable informatics tools. Western blotting, as described above, with antibodies specific for the identified binding partners from MS can be used to confirm this interaction. A third confirmatory method can involve the co-localization of the CB2 receptor with its binding partners using confocal microscopy. This technique will also allow us to vary the amount of time the receptor is exposed to ligand and pinpoint the time when CB2 reacts with its partner. It will also enable us to observe the intracellular location and compartment at which this partnership takes place.

Another point of consideration is the differentiation between surface and internal CB2 receptors and the ability of the CB2 receptor to associate with specific signaling molecules after endocytosis. Initially, we treated the receptor with the inverse agonist SR144528 to bring all of the receptors to the cell surface and limit the variation in receptor location as well as activation state. However, the use of a reversibly biotinylated antibody against the CB2 receptor that would not interfere with ligand binding or receptor function can also be used to differentiate between surface and internal CB2 receptors to avoid any residual effects of inverse agonist treatment. The antibody would theoretically be biotinylated with sulfo-NHS-SS-biotin. The disulfide bond in sulfo-NHS-SS-biotin allows the release of the biotin without denaturing the antibody upon treatment with 50 mM of glutathione. Sulfo-NHS-SS-biotin has been used previously to nonspecifically label cell surface proteins and to follow their internalization (Le Bivic et al., 1990; Schmidt et al., 1997). To isolate the CB2 receptor at different points in the endocytic pathway, the receptor can be treated with an agonist that will occupy the biotin-tagged CB2 receptor and induce its endocytic trafficking as a synchronized pool. After glutathione treatment, only the surface CB2 receptor should be associated with nonbiotinylated antibody while the internalized receptors will be biotinylated. Streptavidin agarose followed by secondary antibodies coupled to protein A Sepharose can then be used to sequentially separate the two populations of receptors.

This technique provides a lot of potential and a great avenue by which binding partners of the CB2 receptor can be captured. The idea of being able to engineer the unnatural amino acid, *p*Bpa, into specific locations on the receptor and then being able to covalently bind any proteins that come within 3 Å opens up doors to engineering specific proteins that capture specific binding partners at specific locations on the protein. Taking that together with time points upon treatment

with different ligands would give us enormous insight into how the cell works and would help us understand the ligand-based modulation of receptor activity. We can ask: (1) which kinases are recruited upon treatment with a certain agonist? (2) which adapter proteins help receptor internalization upon this treatment? (3) what differentiates an inverse agonist signaling pathway from an agonist signaling pathway? (4) what are the time points at which these interactions occur? The kinetics as well as the identity of these binding partners is essential in being able to fine-tune receptor activity. With the increase in the development of new ligands and drugs for GPCRs in general, such as was the case for the research presented in Chapters 2 and 3, understanding their downstream effect on receptor activity is crucial to the design and optimization of drugs. These optimized drugs will ultimately be used for the therapeutic treatment of many diseases and will abolish severe side effects. Future studies of these mechanisms of GPCR modulation may also lead to important new insights relevant to neuropsychiatric disease and may identify exciting new targets for the development of novel therapeutic drugs.

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