Biochemical and Biophysical Study of Cannabinoid 1 and Cannabinoid 2 Receptors

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1. SUMMARY

Cannabinoid receptors are Class A G-protein coupled receptors (GPCRs) and are involved in the modulation of a diverse set of signaling pathways in the human body. The two subtypes of cannabinoid receptors are Cannabinoid 1 receptor (CB1) and Cannabinoid 2 receptor (CB2), which are expressed predominantly in the human brain and in the immune system, respectively. Both receptors are considered to be key drug targets with enormous therapeutic potential in disease conditions ranging from neuropathic pain and neurodegenerative disorders to cardio-metabolic and inflammatory disorders. In order to develop high-affinity, selective Cannabinergic ligands with specific downstream signaling effects, we need to better understand the structural aspects of the receptor in its native environment.

To study the ligand binding site(s) of CB2, we had previously developed an experimental approach termed as Ligand Assisted Protein Structure (LAPS). Using a combination of chemically functionalized Cannabinergic ligands and site-directed mutagenesis and pharmacological studies, we were able to ascertain that AM1336, a potent CB2-selective inverse agonist, attaches covalently to the receptor through critical interactions with the two cysteines on transmembrane helix 7 of CB2. To confirm directly the covalent attachment of AM1336 to amino acid(s) on CB2, we used a bottom-up proteomic approach using mass spectrometry. We used a baculovirus-based insect cell expression system to express and purify functional, full-length human CB2 receptor for
mass spectrometric studies. Purified CB2 was treated with AM1336 and analyzed using bottom-up proteomic analysis to identify the modified amino acid residue(s). Using high-resolution mass spectrometric analysis, we were able to confirm the covalent modification of C284 (7.38) of transmembrane helix 7, confirming the previous biochemical evidence. Using comprehensive modeling and docking techniques, the binding site of AM1336 was visualized and other receptor-ligand interactions were identified.

From previous reports on other GPCRs, following ligand binding, the receptor undergoes critical conformational changes, which determine the activation of various downstream signaling pathways. In order to study the receptor dynamics of CB2 in its native lipid environment, we developed a system for future structural studies. We incorporated purified functional CB2 into lipid bilayer mimics known as nanodiscs and further purified CB2-incorporated nanodiscs using size-exclusion and affinity chromatography. This work could provide us a system to study the changes in the structural conformation of CB2, in the presence of cannabinergic ligands, using hydrogen-deuterium exchange mass spectrometry. This system would not only enable us to determine the key conformational changes in CB2, following ligand binding, but also provide critical insight into the structural motifs involved in receptor function and downstream signaling pathway activation.

The LAPS approach-based study of CB1 has proven to be a difficult challenge owing to the lack of significant quantities of highly purified, functionally active protein
sample. Here, we used both an *E. coli*-based cell-free expression system containing nanodiscs or a bacterial expression system to express an engineered, human CB1 receptor. The expressed receptor was confirmed to be functionally active in radioligand binding assays and further purified using affinity chromatography. In order to ascertain the feasibility of this system to be part of our previously developed LAPS approach, we performed bottom-up mass spectrometric analysis of the purified receptor. We were able to achieve reasonable peptide coverage using both MALDI-TOF and LC-MS/MS analysis and this work could provide a new avenue for the ligand-assisted CB1 receptor binding site(s) studies using different cannabinergic ligands.
2. ACKNOWLEDGEMENTS

This dissertation would not have been possible without the guidance and the help of several individuals who have contributed and extended their valuable assistance in the preparation and completion of this study.

I would like to extend my heartfelt gratitude to my thesis Chair and advisor, Dr. Alexandros Makriyannis for extending me the opportunity to work on this project at the Center for Drug Discovery (CDD) and also for his continuous support and guidance.

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<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ABHD4</td>
<td>Abhydrolase domain-containing protein</td>
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<tr>
<td>AC</td>
<td>Adenyl cyclase</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AEA</td>
<td>Anandamide</td>
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<tr>
<td>2-AG</td>
<td>2-Arachidonoyl glycerol</td>
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<tr>
<td>CB</td>
<td>Cannabinoid</td>
</tr>
<tr>
<td>CBD</td>
<td>Cannabidiol</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DGL</td>
<td>Diacylglycerol-lipase</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
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<tr>
<td>ECL</td>
<td>Extracellular loop</td>
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<tr>
<td>ECS</td>
<td>Endocannabinoid system</td>
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<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
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<tr>
<td>GEFs</td>
<td>Guanine nucleotide exchange factors</td>
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<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
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<td>GRK</td>
<td>G-protein receptor kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>hMGL</td>
<td>Human monoacylglycerol lipase</td>
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<tr>
<td>ICL</td>
<td>Intracellular loop</td>
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<tr>
<td>IMAC</td>
<td>Immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionization time-of-flight</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
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<tr>
<td>MGL</td>
<td>Monoacylglycerol lipase</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>NAPE</td>
<td>N-Acylphosphatidylethanolamine</td>
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<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PLD</td>
<td>Phospholipase D</td>
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<tr>
<td>Q-TOF</td>
<td>Quadrupole time of flight</td>
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<td>rMGL</td>
<td>Rat monoacylglycerol lipase</td>
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<tr>
<td>THC</td>
<td>Tetrahydrocannabinol</td>
</tr>
<tr>
<td>Δ9-THC</td>
<td>(-)-Δ9-Tetrahydrocannabinol</td>
</tr>
<tr>
<td>TMH</td>
<td>Transmembrane helix</td>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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Chapter 1

Introduction

1.1 G-protein coupled receptors:

G-protein coupled receptor (GPCR) superfamily represents the largest family of integral membrane proteins in the human proteome and comprises of more than 800 proteins classified (GRAFS classification) into 5 families - glutamate, rhodopsin, adhesion, frizzled/taste, and secretin.\(^1\) The rhodopsin-family (Class A) of GPCRs is the largest group with about 670 full-length proteins.\(^2\) These receptors are characterized by seven transmembrane-spanning helices (H1-H7) with the extracellular N-terminus and intracellular C-terminus and the transmembrane interconnected by three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3).\(^3\) In general, the extracellular domain and the transmembrane domains are involved in orthosteric ligand binding, while the intracellular loops and the terminal tail are found to interact with G-proteins and other downstream signaling proteins. GPCRs exhibit the greatest homology within the transmembrane regions and the greatest diversity in the amino terminus.\(^3\) In spite of high structural similarity of GPCRs, the ligands show great diversity and range from subatomic particles (photon) to ions, small molecules, peptides and proteins.\(^3\)

The transmembrane helices upon ligand binding are subjected to conformational changes and transferring this information, from the ligand-binding site, to intracellular domains associated with the G-proteins to elicit a cascade of cellular signals and effects\(^3\). These activated GPCRs act similar to guanine nucleotide exchange factors (GEFs) by promoting exchange of the GDP in the G-protein, bound to the intracellular surface of the
receptor, with GTP, followed by proteins dissociation. GPCRs have been shown to mediate both G-protein dependent pathways (through four major G-protein sub-classes - Gs, Gi/o, Gq/11, and G12/13) and G-protein independent pathways (arrestins, GRKs, and SRCs).

GPCRs mediate a large number of vital cellular responses and therefore are of extreme importance as a drug target and for translational research. Currently about 50% of all small molecule drugs approved by the FDA target GPCRs and ion channels confirming their enormous pharmacotherapeutic importance. Therefore, it is vital that we better understand these complex proteins and their function in order to develop better pharmacological agents.

1.2 Endocannabinoid system:

*Cannabis sativa* is the illicit plant with the longest recorded history, of many millennia, as an herb for medication and therapy. Extracts from the plant were used for a range of indications ranging from pain relief to cancer. The mechanism of action of cannabis was not well understood until the 1970s and 1980s, until the major cannabinoids present in the plant were isolated and analogues were synthesized. In 1988, following some binding studies with mouse brain membranes, it was confirmed that a specific receptor in the brain is involved in the action of these molecules. By 1990, it was identified that this brain receptor was an orphan G-protein coupled receptor (GPCR) now known as Cannabinoid 1 receptor (CB1). This was quickly followed by the identification of a peripheral receptor, found in low concentration in immune cells, and now known as
Cannabinoid 2 receptor (CB2).\textsuperscript{10} Further studies over the past two decades have led to discovery of the endogenous cannabinoid system, also known as the endocannabinoid system that, in addition to the two GPCRs, encompasses a family of endogenous lipids, including anandamide\textsuperscript{11} (AEA) and 2- arachidonoylglycerol\textsuperscript{12} (2-AG), and related biosynthetic and metabolic enzymes. The two metabolic enzymes - fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL) hydrolyze AEA and 2-AG respectively.\textsuperscript{13, 14}

1.3. Cannabinoid receptors (CB):

CB receptors are members of the α group of class A (rhodopsin-like) subfamily of G-protein coupled receptors and are activated by endogenous, natural and synthetic cannbinergic ligands. CB receptors share high sequence similarity with eight other receptors; sphingosine-1-phosphate (S1P) receptors (S1P1, S1P2, S1P3, S1P4, S1P5) and LPA receptors (LPA1, LPA2, and LPA3) in the α group of Class A GPCRs.\textsuperscript{15} The α group also contains receptors for melanocortin peptides (MC1-MC5), adenosine receptors (A1, A2A, A2B, A3), and the orphan receptors GPR3, GPR6, and GPR12. Based on comparative analysis of genome sequence data, adenosine receptors have been identified to have the widest phylogenetic distribution in this group, indicating that the common ancestor of this group may have been an adenosine receptor.\textsuperscript{16} The presence of orthologs of CB receptors exclusively in the phylum Chordata also indicates the probable rise of CB receptors in a common ancestor of extant chordates.\textsuperscript{17}
Two subtypes of the cannabinoid receptors – cannabinoid 1 receptors (CB1) and cannabinoid 2 receptors (CB2) have well characterized. The CB1 receptor is predominantly found in the central nervous system (CNS) and is considered to be the most widely expressed receptor in the brain.$^{18}$ On the other hand, CB2 receptors are found in the peripheral tissues predominantly in the immune system, although recent reports have detected CB2 receptors in the CNS (microglial cells and neurons).$^{19}$ The two cannabinoid receptors share 44% of sequence homology and both mediate downstream pathways through $G_i$ or $G_o$ signaling. They are therefore negatively coupled to adenylyl cyclase and positively to mitogen-activated protein kinase (MAP Kinase).$^{20}$

Figure 1.1: Schematic detailing the retrograde signaling mechanism of endocannabinoid system.
The major endogenous cannabinoid ligands, AEA and 2-AG are potent agonists of the cannabinoid receptors. They are synthesized locally in the neuronal cells on demand from the membrane phospholipids by complex pathways which involve enzymes such as phospholipase C (PLC), diacylglycerol lipase (DGL), transacylase, etc. These ligands bind to the CB1 receptor on the surface of the pre-synaptic neurons and mediate numerous neurotransmitter-signaling networks such as dopaminergic, adrenergic, serotonergic, etc. through retrograde signaling (Figure 1.1). Apart from the neurotransmitter function, there is growing evidence that, in some disease states or disorders, these endocannabinoids have an 'autoprotective' role. It was found in in vivo experiments that increased amounts of endocannabinoid molecules were released both in response to skeletal muscle spasm or spasticity in multiple sclerosis and in response to inflammatory pain, and this resulted in the amelioration of such symptoms by their action through the cannabinoid receptors. Cannabinergic ligands are generally classified into five main groups based on their structures: Classical Cannabinoids such as Δ9-THC; Non-classical Cannabinoids such as CP55940; Endocannabinoids such as AEA and 2-AG; Aminoalkylindoles such as WIN55212-2; and Biarylpyrazoles such as SR141716A and SR144528 (Figure 1.2). Arrays of novel compounds of other structural classes have also been designed and studied for their pharmacotherapeutic value. [3H]CP55940 is the most commonly used radiolabeled synthetic cannabinoid ligand; found to bind to the orthosteric ligand-binding pocket of both CB1 and CB2 receptor with high affinity.
1.4 Cannabinoid receptor 1 (CB1):

The human CB1 receptor is a 472 amino acid long integral membrane protein expressed by the gene CNR1 located in the q14-q15 region of chromosome 6.²⁶ It is found predominantly in the central nervous system and to a smaller extent in the periphery.⁸ CB1 receptor plays an important role in the central and peripheral regulation of food intake, fat accumulation, lipid and glucose metabolism, through several signaling pathways.²⁷ It has also been identified to induce the dopaminergic reward pathway at the ventral tegmental area (VTA) and thus increase food and drug craving.²⁸ It is also heavily involved in pain modulation as it is extensively expressed in the dorsal root ganglion (DRG) and periaqueductal gray (PAG).²⁹ Experimental and clinical evidence suggest that in the CNS, endocannabinoid signaling through CB1 receptor helps control human motor, cognitive, emotional, and sensory functions and modulates pain perception, hormonal activity, thermoregulation, and cardiovascular functions.³⁰ In peripheral organs such as liver and pancreas, CB1 receptor regulates energy homeostasis and lipid metabolism and deposition.³¹ Thus, CB1 receptor is involved in the modulation of a diverse set of physiological pathways both in the CNS and in peripheral systems. The development of specific ligands modulating these effects could have therapeutic benefits in a variety of pathological conditions including obesity, cardio-metabolic diseases, drug dependence, pain, and neuro-degeneration.³²

Amino acid sequence analysis shows that human CB1 receptor shares 97.3% homology with rat CB1 receptor overall and 100% homology within the trans-membrane regions.³³ Due to this high similarity, rat CB1 and human CB1 receptors are used
interchangeably in \textit{in vitro} studies. The two major endocannabinoid ligands, 2-AG and AEA, both show good affinity to CB1 receptor. Both 2-AG and AEA behave as partial agonists, with some selectivity towards CB1 over CB2 receptors.\textsuperscript{16} The classical cannabinoids such as (−)-trans-$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and its synthetic analogues show very high affinity and potency (as agonists) at both CB1 and CB2 receptors. Similarly, non-classical group of synthetic cannabinoids, which contain bicyclic and tricyclic analogues of $\Delta^9$-THC and lack the pyran ring (e.g., CP55940), show very high non-selective activity at CB receptors. Owing to its high affinity and physicochemical properties, CP-55940 (Ki of 0.5-2.0 nM respectively) is the primarily used radiolabeled ligand in competitive binding experiments.\textsuperscript{34} The aminoalkylindole groups of cannabinoids, such as WIN55212, were found to bind CB1 receptor with high affinity but exhibit differences in signaling activity. WIN55212 is known to cause substantial CB1 receptor internalization\textsuperscript{35} but less receptor desensitization than $\Delta^9$-THC.\textsuperscript{36} Some of these effects maybe partly explained through evidence from mutation and chimera studies, which suggest that WIN55212 binds to CB1 receptor through distinct different binding pocket interactions, when compared to CP55940 and $\Delta^9$-THC.\textsuperscript{37}

Numerous homology models, constructed based on the known GPCR structures of rhodopsin and beta-adrenergic receptors, could be a useful tool in drug design but their use remains equivocal.\textsuperscript{38} This is partly due to the fact that CB1 receptor has very a low overall similarity with other class-A GPCRs. There is only a sequence identity of 23\%, 26\%, and 27\% between the trans-membrane helices of human CB1 receptor and the bovine rhodopsin, the human beta-2 adrenergic receptor, and the human A2A adenosine
receptor, respectively.\textsuperscript{39} Thus, direct biochemical and biophysical studies of CB1 receptor are a necessity to provide key structural insights for drug design and development.

![Snake plot diagram of human CB1 receptor](image)

\textbf{Figure 1.2 Snake plot diagram of human CB1 receptor}

Based on mutagenesis and homology modeling studies, the residues: F3.25(189), K3.28(192), C174, and C179 are considered to be critical for CP55940 binding to CB1 receptors.\textsuperscript{40} The key residues considered to be important for signal transduction are \textsuperscript{-}D2.50(163/164), F3.36(201), L6.34(341), A6.35(342), C6.47(355) and the C-terminus \textsuperscript{(401–417)}.\textsuperscript{40,41,42,43} Although CB1 binding sites for synthetic cannabinoid ligands, such as SR141716A, WIN55212 and CP55940, have been explored through extensive site-directed mutagenesis and homology modeling studies, very few studies have been
reported on endocannabinoid binding sites. It has been reported that K3.28A, F268W, P269A and I271A mutations have profound effect on AEA binding.

A 102 amino acid truncation at the N-terminal has been observed (unpublished results) to increase the expression of CB1 receptor in HEK293 cells, with no changes in the binding profile of CP-55940. Only two of the three potential glycosylation sites identified on the N-terminal end were found to be glycosylated and both sites are absent in the truncated CB1 receptor. Due to the lack of an extremely long, free-floating N-terminal end, N-terminal truncation can be hypothesized to help reduce the complexity in protein folding and membrane insertion. As full-length CB1 receptor over-expression has proven to be an extremely difficult proposition, this truncation might be useful approach for efficient expression of functional CB1 for biophysical characterization of receptor.

Numerous drug discovery studies are underway to develop CB1-selective agents to treat overweight/obesity, cardio-metabolic disorders, substance abuse, neuropathic pain, and multiple sclerosis. In order to efficiently develop better CB1-specific drugs, we need to better understand these complex proteins at a molecular-level, in their native membrane-like environment.

1.5 Cannabinoid receptor 2 (CB2):

The human CB2 receptor is a 360 amino acid-long (Figure 1.3) integral membrane protein that belongs to the Class A GPCR family and is encoded by the CNR2 gene. It has a modest sequence similarity of 44% overall (68% within the
transmembrane regions) with the CB1 receptor and is expressed predominantly in the periphery in the human body.\textsuperscript{10} It has been shown to express predominantly in immune cells, such as monocytes, macrophages, T-lymphocytes and B-lymphocytes,\textsuperscript{19} gastrointestinal system\textsuperscript{46} and to a lower extent in microglia in the brain. Therefore, CB2 receptor modulation have been proposed as a therapy for the treatment or management of a range of disease conditions including neuropathic pain, chronic inflammatory pain,\textsuperscript{47} multiple sclerosis\textsuperscript{24}, amyotrophic lateral sclerosis\textsuperscript{48}, huntington’s disease,\textsuperscript{49} stroke,\textsuperscript{50} inflammatory bowel diseases,\textsuperscript{51} liver cirrhosis,\textsuperscript{52} osteoporosis\textsuperscript{53} and cancer.\textsuperscript{54}

\textbf{Figure 1.3. Snake plot diagram of human CB2 receptor. The transmembrane cysteines are labelled in red circles.}
Unlike CB1 receptor, the CB2 receptor shows lower sequence homology between species; rat CB2 has an amino acid sequence homology of 93% and 81% with mouse and human CB2 receptors respectively. Although the majority of the differences are found in the C-terminal end of the receptor, a number of cannabinergic ligands show significant differences in affinities between species. As human CB2 receptor has a sequence identity of 68% with human CB1 receptor within the transmembrane region, they recognize the similar structural features of cannabinoid ligands as CB1 receptor, with some minor differences. While the endogenous ligand, 2-arachidonoyl glycerol (2-AG), binds to CB2 receptor with similar affinity as to CB1 receptor, anandamide (AEA) has lower affinity to CB2 receptor. Classical cannabinoids, such as Δ9-THC show similar affinities to both CB1 and CB2 receptor, but show selectivity to CB2 receptor upon etherification at the C-1 position. 5-(4-Chloro-3-methyl-phenyl)-1-(4-methyl-benzyl)-1H-pyrazole-3-carboxylic acid (1,3,3-trimethyl-bicyclo[2.2.1]hept-2-yl)-amide (SR144528) and 6-Iodopravadoline (AM630), biarylpyrazole-class inverse agonist/antagonists, were the first sub-nanomolar affinity CB2 selective ligands. Additional CB2-selective inverse agonists including JTE-907 and triaryl bisulfone compound SCH336 were later synthesized.

CB2 receptor couple primarily to G_i/o proteins to modulate a number of downstream signaling pathways (Figure 1.4) including adenylyl cyclase, mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), protein kinase B/phosphoinositide-3 kinase (Akt/PKB pathway), cAMP response element-binding protein/activating transcription factor, Janus kinase, inward rectifying potassium channels
and N- and P/Q-type calcium channels. CB2 receptor ligands are primarily characterized based on their effects on the modulation of adenylyl cyclase, activation of MAPK (ERK1/2), receptor internalization through beta-arrestin recruitment and ion channel function modulation.

**Figure 1.4. Schematic representation of Cannabinoid receptor signaling pathway**

Classical, aminoalkylindole, bicyclic and endogenous cannabinoids vary greatly in their signaling effects through CB2 receptor. Mackie and coworkers reported that CP55940 robustly promoted MAPK activation and voltage-gated calcium channel inhibition and β-arrestin2 recruitment followed by receptor internalization. But WIN55,212-2 did not promote CB2 receptor internalization or have any effect on
voltage-gated calcium channels yet activated MAPK pathway and recruited β-arrestin2 to the membrane. They also found evidence of functional selectivity in other CB2 receptor ligands, further emphasizing the need for careful evaluation of cannabinoid ligands in multiple signaling pathways for good therapeutic efficacy in clinical studies.

As the crystal structure of CB2 receptor has not been solved, a number of homology models, using other known GPCR structures, have been developed over the years. In 1999, Reggio and co-workers first reported the 3D homology model of CB2 receptor based on the α-helical periodicity in the CB2 sequence as detected by Fourier transform analysis. Following the elucidation of the first Class A GPCR’s crystal structure of bovine rhodopsin, a comparative 3D model of CB2 receptor was constructed in 2003. Later in 2012, models were generated based on adenosine 2A receptor (A2AR), which allowed for the construction of the previously, experimentally confirmed extracellular disulphide bond linkage (between cysteine 174 and cysteine 179). By 2014, at least 24 crystal structures of unique GPCRs have been published. Recently, Feng, et al. constructed comparative CB2 homology models based on the structures of 10 other GPCR structures. They performed pre-screen docking studies with over 1000 compounds and further validation studies with 100 CB2-selective compounds and 70 CB1-selective compounds using these models and found that the model constructed based on beta 1- adrenergic receptors (β1AR) had the best prediction of ligand binding affinity. Although such improved models are being constructed, the low receptor homology, even within species, indicates the necessity for experimental characterization of CB2 binding sites.
Despite favorable results in preclinical models, CB2 ligands have fared poorly in human clinical trials. A comprehensive strategy that encompasses both experimental evidence from biochemical and biophysical techniques and in-silico predictions would facilitate the design and optimization of therapeutically attractive CB2-selective ligands with minimal off-targets.

1.6 Pharmacological significance of cannabinoid receptors:

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<tr>
<th>Ligands</th>
<th>Pathological conditions</th>
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<td>CB1 Agonists</td>
<td>Neuropathic Pain</td>
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<td>Nausea</td>
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<td>Inflammatory bowel disease</td>
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<td>CB2 Antagonists</td>
<td>Rheumatoid arthritis</td>
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Table 1.1 Therapeutic potential of Cannabinoid receptors.
Cannabinoid receptors are widely expressed and therefore implicated in the regulation of a wide variety of central and peripheral physiological functions including neurogenesis, neuromodulation, energy balance and metabolism, immunomodulation, thermoregulation, as well as reproductive and cardiovascular functions. They are therefore considered to be key drug targets with enormous therapeutic potential in a number of disease conditions. CB1-selective agents are currently being developed to treat a number of disease conditions including overweight/obesity, cardio-metabolic disorders, substance abuse, neuropathic pain, and multiple sclerosis. CB2-selective agents have shown promise in the treatment of peripheral pain, neuroinflammatory diseases such as multiple sclerosis, and other cardio-metabolic and inflammatory disorders.66, 67

Although both EMA and FDA have approved a few drugs targeting cannabinoid receptors, a significant number of issues still remain. CB1 agonists that penetrate the CNS cause catalepsy, sedation and undesirable psychotropic effects; and with prolonged administration produce tolerance. Rimonabant, a CB1 antagonist/inverse agonist was approved in EU for the treatment of obesity in patients with associated risk factors, such as type 2 diabetes or dyslipidaemia. However, due to severe side effects such as depression and suicidality, the drug was withdrawn from the market. But other peripherally acting drugs such as AM6545 have shown great promise in preclinical studies for the treatment of obesity without CNS-mediated adverse effects.68 Similarly, clinical data with some CB2 agonists has not been promising. Cannabinor (Pharmos Corporation) did not show efficacy in acute pain (capsaicin) or postoperative
pain (molar extraction). Also, GW842166 (GSK) failed to reduce tooth extraction-induced postoperative pain in patients \(^6^9\), while AZD1940 (Astrazeneca), a peripherally acting CB(1)/CB(2) receptor agonist, did not show efficacy in the human capsaicin pain model \(^7^0\). However, using a structure-based drug design approach, AM1710\(^{7^1, 7^2}\) and AM1241\(^{7^3}\) have been synthesized and have shown good efficacy in neuropathic pain studies without any CNS adverse effects. Therefore, to develop more efficacious therapeutic agents with fewer side effects, we need to better understand the structure and function of cannabinoid receptors.

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Chapter 2

Cloning, Expression, Purification and Characterization of Human CB2 Receptor

2.1 Introduction:

Cannabinoid 2 receptor (CB2) is an integral membrane protein that belongs to Class A of G-protein coupled receptor (GPCR) superfamily. CB2 is expressed predominantly in the periphery, specifically in immune cells such as monocytes, macrophages, T- and B- lymphocytes. It is also found in microglia and in neurons under certain physiological conditions. Therefore, CB2 is considered to be a valuable drug target for a number of disease conditions such as neuropathic pain, chronic inflammation, multiple sclerosis, inflammatory bowel disease, osteoporosis and neurodegenerative diseases. When activated by endogenous or exogenous ligands, CB2 couples to G protein and inhibits the activation of adenylyl cyclase, thereby reducing production of cAMP production. Further, CB2 activates a myriad of downstream effector molecules including MAPK, JNK, Akt/PKB and, GIRK and P/Q-type Ca²⁺ ion channels. Despite favorable results in preclinical models, CB2 ligands have fared poorly in human clinical trials. We therefore need an effective mechanism to study the structural features of ligand binding to CB2 to develop better and safer drugs. Here, we report the cloning, expression, purification and characterization of CB2 to provide a platform for mass spectrometry-based ligand binding site characterization studies.
2.2 Recombinant expression of CB2 for proteomics studies:

Proteomics research involving the study of protein structure, modifications, localization, function or interactions requires a means of producing functional proteins of interest. Although immune cells have been show to express significant levels of CB2, larger quantities are required for detailed structural studies. For many decades, difficulty in developing high-level heterologous expression systems had hampered the progress in structural characterization of GPCRs. Although, in the past decade, tremendous progress has been made in this front, recombinant production of GPCRs still remains a matter of trial and error, encompassing myriad of challenges in extraction, purification and biophysical characterization trials. This phenomenon can partly be explained by the complex, not-yet-fully-understood GPCR folding and membrane insertion process and general instability of GPCRs. Multiple quality control systems and molecular chaperones that are involved in the correct folding and transport of GPCRs are difficult to control in heterologous systems and therefore prevents the use of a rational approach to heterologous expression. Owing to recent developments, numerous members of the GPCR family have been successfully expressed in different cell systems including *Escherichia coli*, yeast, baculovirus/insect and mammalian expression to produce adequate quantities for biochemical studies. Although other systems have shown some promise, baculovirus/insect cell expression remains the favored method for large-scale production of GPCRs for structural studies using mass spectrometry, NMR and x-ray crystallization.
2.3 Bac-to-Bac® Baculovirus Expression System:

The Bac-to-Bac® Baculovirus Expression System (Figure 2.1) has been shown to be a rapid and efficient method for the generation of recombinant baculoviruses.¹² This system is based on the site-specific transposition of an expression cassette from donor plasmid into a baculovirus shuttle vector known as Bacmid. First, the gene of interest is cloned into a pFastBac1 vector under the control of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) or p10 promoter and flanked by two Tn7 transposon sites. The pFastBac vector containing the gene of interest is then propagated in DH10Bac *Escherichia coli* strain, which contains a bacmid with mini-*attB*Tn7 target site and helper plasmid encoding transposase, which enables transposition of the gene of interest. Following the transposition reaction, the high-molecular weight recombinant bacmid DNA is isolated and transfected into insect cells to generate recombinant baculovirus. The highly amplified and titered baculovirus stock can then be used for large-scale expression of recombinant protein of interest.

**Figure 2.1 Schematic representation of protein expression using Bac-to-Bac® system**
2.4 Methods:

pFastBacFlaghCB2his6 plasmid DNA preparation for CB2 expression in Sf21 insect cells using baculovirus expression system:

a. Plasmid DNA preparation:

The pFastBac-based plasmid (Invitrogen) containing a full-length human CB2 gene with a N-terminal Flag-tag (DYKDDDDK) and a C-terminal His6-tag (HHHHHHH) (pFastBacFlaghCB2his6) was obtained from Dr. Nikolai Zvonok. To obtain larger quantities of plasmid DNA, pFastBacFlaghCB2his6 was transformed into XL1-Blue cells (Agilent Technologies) and colonies were selected on LB agar plates using antibiotic ampicillin. A single colony was inoculated in one liter of LB media containing appropriate antibiotic and after 18 h incubation plasmid DNA was purified using the GeneJET™ Plasmid Midiprep Kit, according to the manufacturer’s recommended method. The size and concentration of the plasmid preparation was evaluated by electrophoresis in 0.8% agarose gel.

b. Preparation of recombinant bacmid-FlaghCB2his6 DNA:

The bacmid-FlaghCB2his6 DNA was prepared using the Bac-to-Bac baculovirus expression kit’s (Invitrogen, Carlsbad, CA) recommended protocol. Briefly, MAX Efficiency DH10Bac chemically competent cells (Invitrogen) were transformed with pFastBacFlaghCB2his6 and plated onto an LB agar plate containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, and 40 µg/ml IPTG. The plates were incubated for 48 hours at 37 °C. 6 white colonies were picked and restreaked on fresh LC agar plates containing 50 µg/ml kanamycin, 7 µg/ml
gentamicin, 10 µg/ml tetracycline, 100 µg/ml Blu-gal, and 40 µg/ml IPTG. The plates were incubated overnight at 37 °C. Upon confirmation of white phenotype, a single colony from each plate was inoculated into 5 ml LB liquid culture containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline. Glycerol stocks of these colonies were prepared and stored at -80 °C.

Recombinant bacmid-FlaghCB2his6 DNA was isolated using the following procedure. 5 ml of bacterial cells were harvested by centrifugation at 4000 x g for 15 min at 4 °C. The cells were resuspended in 0.2 ml of resuspension buffer (50 mM Glucose, 25 mM Tris.Cl (pH 8.0), 10 mM EDTA (pH 8.0) and 100 µg/ml RNase A). The cells were lysed with 0.4 ml of lysis buffer (1% SDS in 0.2 M sodium hydroxide) by gently inverting the tube 3-4 times. Then 0.3 ml of neutralizing buffer (3 M potassium acetate, pH 5.5) and mixed gently. After centrifugation at 16000 x g for 10 min, the supernatant was collected in a new tube and plasmid DNA was precipitated with 0.7 volume of isopropanol. The DNA was collected by centrifugation at 16000 x g for 10 min at 4 °C. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was air-dried to remove any remnants of ethanol and dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA).

c. PCR analysis of recombinant bacmid-FlaghCB2his6 DNA:

The presence of FlaghCB2his6 gene in the recombinant bacmid was evaluated using PCR analysis. PCR reaction mixtures were prepared as follows: 1 µl Bacmid DNA sample, 0.3 µl 10 µM DPR primer 5’ – CCCAGTCACGACGTTGTAACG – 3’, 0.3 µl
10 μM RPR primer 5’ – AGCGGATAACAATTTCACACAGG – 3’ or 0.3 μl 10 μM RvCB2rt1 primer 5’ – GATGAACAGGAGCCAGCTCAG – 3’, 0.3 μl (10 mM total concentration) dNTPs, 1.0 μl 10x Advantage2 buffer (Clontech), 0.2 μl Advantage2 polymerase (Clontech) and 6.9 μl of sterile distilled water to give a total volume of 10 μl per reaction. Amplification cycles were then carried out using a MyCycler™ Thermal cycler (Biorad) as follows: A single denaturation step of 94 °C for 2 min was followed by thirty cycles of 94 °C for 10 s, 55 °C for 33 s and 72 °C for 1 min 33 s and completed with a final extension step of 72 °C for 10 min. The sizes of PCR products were evaluated by electrophoresis in 0.8% agarose gel.

**d. Large-scale purification of bacmid-FlaghCB2his6 DNA:**

DH10bac cells transformed with pFastBacFlaghCB2his6 were streaked onto LB agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline. A single colony was inoculated in 200 ml of LB media containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline and incubated for 18 h at 37 °C with 250 rpm shaking. The bacmid DNA was isolated using the PureLink HiPure Midiprep kit (Invitrogen) according to the manufacturer’s recommended method. Briefly, 200 ml bacterial cells were harvested by centrifuging at 4000x g for 10 min and the media was discarded. The cells were resuspended in resuspension buffer (R3) containing RNase A to the pellet until homogeneous. The cells were lysed with equal volume of lysis buffer (L7) and mixed gently by inverting tube 3-4 times and incubating for 5 min at RT. Then equal amount of precipitation buffer (N3) was added and mixed gently. The mixture was centrifuged at 15000x g at RT for 10 min. The supernatant was loaded onto the
equilibrated column and the solution was allowed to drain by gravity flow. The column was washed twice with wash buffer (W8) and bound DNA was eluted with 5 ml of elution buffer (E4) by gravity flow. To the flow through, 3.5 ml of isopropanol was added and the mixture was centrifuged at 12000 x g for 30 min at 4 °C. The supernatant was carefully discarded, and the pellet was resuspended in 3 ml of 70% ethanol. The mixture was centrifuged at 12000 x g for 5 min at 4 °C and the supernatant was discarded carefully. The pellet was air-dried for 10 min and resuspended in 200 ul TE Buffer (TE). The size and concentration of the DNA preparation was evaluated by electrophoresis in a 0.8% agarose gel.

**Expression of FlahgCB2his6 using baculovirus/insect cell system:**

*Sf21* insect cells were transfected with bacmid-FlaghCB2his6 DNA according to the Bac-to-Bac baculovirus expression kit’s (Invitrogen) recommended protocol. Briefly, *sf21* cells were cultured in Sf-900™ II SFM media containing 2% fetal bovine serum (FBS). The cells were verified to be in log phase (1.5-2.5 x 10⁶ cells/ml) with greater than 95% viability. 2 ml of Sf-900™ II SFM media was added to each well of a 6-well culture plate. 8 x 10⁵ cells were seeded into each of these wells and allowed to attach by incubating the plate in the hood, at RT, for 15 min. 8 ul of Cellfectin® II reagent and different amounts of recombinant bacmid-FlaghCB2his6 DNA (a, b, c ug) were mixed with 100 µl of Sf-900™ II SFM media separately and then combined together. Following 15 min incubation, the mixture was added dropwise onto the attached cells and the cells were incubated for 4 hours at 27 °C. The transfection media was then removed and replaced with 2 ml of Sf-900™ II SFM media containing 2% FBS and incubated at 27 °C.
for 5 days (until ~80% cell lysis). On day 5, the contents of each well were collected and centrifuged at 2000x g for 15 min. The supernatant (viral stock, P1) were collected and stored at 4 °C, protected from light. The cell pellets were used to test for the expression of FlaghCB2his6 receptor. The cells were resuspended in lysis buffer (300 mM NaCl, 50 mM sodium phosphate, 10% Glycerol, 1% dodecyl maltoside, pH 8.0) and lysed on ice by three, 33 s sonication cycles, each cycle consisting of 1 s bursts at 50 W separated by a 5 s interval. The lysate was centrifuged at 16000x g for 15 min and supernatant and pellets were analyzed separately using SDS-PAGE analysis in AnykD™ Mini-PROTEAN® TGX™ precast polyacrylamide gels followed by western blot analysis using His-tag or FLAG-tag based immunodetection as detailed above.

The P1 viral stock was filtered through a 0.22 µm Millex-GS syringe filter unit (Millipore, Billerica, MA) and used for further infection steps. Different quantities of filtered P1 viral stock was used for infecting sf21 cells in log phase and incubated at 27 °C for 5 days (until ~80% cell lysis).

**Anti-FLAG immunoaffinity purification of FlaghCB2his6:**

Cells pellets were suspended in lysis buffer (50 mm sodium phosphate, 300 mm NaCl, 10% glycerol, 1% dodecyl-β-d-maltoside, pH 7.4) and lysed on ice by three, 33 s sonication cycles, each cycle consisting of 1 s bursts at 50 W separated by a 5 s interval. The lysate was centrifuged at 16000x g for 15 min (to remove cell debris) and the supernatant was collected. An equal volume of purification buffer (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 7.4) was added to the supernatant. This mixture was then added to pre-equilibrated (in 50% lysis buffer, 50% purification buffer)
ANTI-FLAG® M2 affinity gel (200 ml) and incubated for 2 hours at 4 °C on a rotating wheel. The resin was washed twice with wash buffer (200 ml of 50 mm sodium phosphate, 300 mm NaCl, 10% glycerol, 0.2% dodecyl-β-d-maltoside, pH 7.4) and the protein was eluted 5 times using 100 ml of DB buffer containing 150 ug/ml of FLAG® peptide. SDS-PAGE and western blotting analysis were performed on the aliquot of samples taken during purification according to the procedure previously described.

**Insect cells membrane preparation with FlaghCB2his6 for saturation binding assays:**

Insect cell pellets were suspended in 20 ml of TME [25 mM Tris base, 5 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4]. Cells were lysed under nitrogen cavitation for 30 min at 1000 psi on ice, the cell lysate was centrifuged at 2000 g for 5 min, and the supernatant collected. The pellet was washed three times with 20 ml of TME and centrifuged. The combined supernatants from each wash were ultracentrifuged at 100,000 g for 45 min at 4 °C. The resultant membrane pellet was used immediately or stored at −80 °C. Membrane protein was quantified with a Bradford dye-binding method (Bio-Rad Laboratories).

**Saturation binding assay with FlaghCB2his6 membrane preparation:**

Saturation binding assays were performed in a 96-well format. About 25 mg of protein was added to each assay well. The [³H]CP-55,940 was diluted in TME-BSA buffer to yield ligand concentrations ranging from 0.625 to 20 nM. Nonspecific binding was assayed in the presence of 5 mM unlabeled CP-55,940. The assay was performed at
30 °C for 1 h with gentle agitation. The resultant material was transferred to Unifilter GF/B filter plates and unbound ligand was removed using a Packard Filtermate-196 Cell Harvester. Filter plates were washed four times with ice-cold wash buffer (50 mM Tris-base, 5 mM MgCl$_2$ containing 0.5% BSA, pH 7.4). 40 µl of Microscint$^\text{TM}$ 20 scintillation fluid was added to each well and the plates were counted using TopCount NXT$^\text{TM}$ Microplate Scintillation and Luminescence Counter. The data obtained was processed using Microsoft Excel and Prism 5. All concentration points were performed in triplicate and data points used for plotting are base-line corrected. $B_{\text{max}}$ and $K_d$ values were calculated by nonlinear regression using Graphpad Prism version 5.03 (one site-binding analysis equation $Y = B_{\text{max}} \times X/(K_d + X)$) on a Windows platform.

**Saturation binding assay with purified FlagCB2his6 receptor:**

96-well GF/B filtration plates were pre-treated with 0.5% polyethylenimine (PEI) for 3 hours at 4 °C. The plates were placed on vacuum manifold (Pall) and washed twice with 200 ml of binding buffer (BB - 25 mM Tris base, 5 mM MgCl$_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% BSA, pH 7.4). The radioligand-receptor binding were performed in 1.5 ml eppendorf tubes containing 10 ml of eluate from FLAG purification of FlagCB2his6 in BB at six radioligand [$^3$H]CP-55,940 concentrations ranging from 2.1875 to 70 nM. Nonspecific binding was assayed in the presence of 5 mM unlabeled CP-55,940. The assay was performed at 30 °C for 1 h with gentle agitation. The resultant material was transferred, as triplicates, to selected wells of a PEI-pre-treated-washed, 96-well GF/B filtration plate. The plate with the samples was placed on a vacuum manifold and washed with in BB (pH 7.4) buffer under 25 mm Hg vacuum. 40 µl
of Microscint™ 20 scintillation fluid was added to each well and the plates were counted using TopCount NXT™ Microplate Scintillation and Luminescence Counter. $B_{\text{max}}$ and $K_d$ values were calculated by nonlinear regression using Graphpad Prism version 5.03 (one site-binding analysis equation $Y = B_{\text{max}} \times X/(K_d + X)$) on a Windows platform. To ascertain change in CP55940 binding affinity upon covalent ligand treatment, 50 nM of AM1336 was added to all wells and incubated along with different concentrations of [3H]CP55,940 as described above.

**Mass spectrometric analysis of purified FlaghCB2his6 receptor:**

a. **Reduction, alkylation and in-solution trypsin digestion of purified FlaghCB2his6 receptor:**

The samples for MS analysis were prepared using the previously reported standard procedures. Briefly, 35 µl (0.5 µg) of purified FlagCB2his6 receptor was reduced using dithiothreitol (DTT, 20 mM) and alkylated using iodoacetamide (IAM, 50 mM) by incubating with each at RT for 1 h. The mixture was then desalted into 25 mM ammonium bicarbonate with 0.05% Cymal-5 using Micro BioSpin 6 columns (Biorad) using the manufacturer’s recommended protocol. The sample was then subjected to overnight digestion with Tryspin Gold, MS-grade (Promega, Madison, WI) at 37 °C. The digests were analyzed immediately or stored at -80 °C until further processing. Alternatively, tryptic peptide mixture was further concentrated using Zip-tip-based extraction using manufacturer recommended protocol. Briefly, the C4 Zip-tip was wetted with 50% ACN and equilibrated with 0.1% TFA. The peptide mixture was bound to the equilibrated zip-tip and washed with 0.1% TFA and eluted with 95% ACN in 0.1% TFA.
b. MALDI-TOF/TOF analysis of FlaghCB2his6 receptor tryptic peptides:

The samples were analyzed, directly or following Zip-tip-based extraction, using AB SCIEX MALDI TOF/TOF™ 4800 (AB SCIEX) and TripleTOF 5600 (AB SCIEX) instruments in both reflectron and linear modes using standard procedures. All MS spectra were externally calibrated using a mixture of peptide standards [des-Arg1-bradykinin at MH+ 904.4681; angiotensin I at MH+ 1296.6853; Glufibrino peptide at MH+ 1570.6774; ACTH (clip 1-17) at MH+ 2093.0867; ACTH (clip 18-39) at MH+ 2465.1989; and ACTH (clip 7-38) at MH+ 3657.9294]. MS/MS spectra were acquired on selected ions of interest. The instrument was calibrated in the MS/MS mode using five daughter ions (at m/z 175.119, 684.346, 813.389, 1056.475 and 1441.634) generated from the fragmentation of Glu-fibrino peptide (MH+ 1570.6774.) MS/MS spectra were acquired under the following conditions: precursor isolation resolution of 200; collision energy of 2 kV; cell pressure of 2 x 10⁻⁵ torr; air as collision gas. All spectral data points were accumulated following analysis in multiple locations on each sample spot. The theoretical molecular weights of expected peptides following reduction, alkylation and trypsin digestion were calculated using MS digest (UCSF MS facility, San Francisco, CA). The MS spectra was then be analyzed by comparing the monoisotopic m/z values obtained from MALDI-MS analysis with the theoretical molecular weights using FindPept software (Swiss Institute of Bioinformatics, Geneva, Switzerland).
2.5 Results and Discussion:

Cloning of FlaghCB2his6 receptor:

Figure 2.2 A) Agarose gel analysis of purified pFastBacFlaghCB2his6-Bacmid: Lane 1: DNA Ladder standard with molecular weights labeled on the left. Lane 2: Purified Bacmid DNA. B) Agarose gel analysis of pFastBacFlaghCB2his6-Bacmid PCR products: Lane 1: DNA Ladder standard with molecular weights labeled on the left. Lane 2-6: PCR products of 5 pFastBacFlaghCB2his6-Bacmid clones; clones 3-5 give a single product of ~3.5 kbp.

To generate the recombinant receptor in sufficient quantities for mass spectrometric analysis, the human CB2 receptor was overexpressed in *Spodoptera frugiperda* (*Sf21*) cells using the Bac-to-Bac baculovirus expression system (Invitrogen). We obtained the pFastBac plasmid construct of full-length human CB2 receptor with N-terminal Flag-tag and C-terminal His6 tag (FlaghCB2his6) from Dr. Nikolai Zvonok, and termed as pFastBacFlaghCB2his6. It was previously shown that the addition of N- and C-
terminal affinity tags such as Flag and His6 did not affect the ligand binding characteristics of CB2 receptor.\textsuperscript{13} Based on this knowledge, a double affinity-tag construct was used for CB2 expression. Following the transformation of pFastBacFlag\textsuperscript{h}CB2\textsuperscript{his6} into DH10bac cells, the clones with correct transposition of CB2 gene into the baculovirus shuttle vector (bacmid) was identified using the blue-white screen technique and isolated\textsuperscript{14}. The high molecular weight bacmid (>130 kbp) was purified and its concentration was determined using agarose gel analysis (Figure 2.2A).

The presence of Flag\textsuperscript{h}CB2\textsuperscript{his6} gene in the recombinant bacmid was also confirmed by PCR analysis using primers RPR and DPR as detailed in the methods section (Figure 2.2B). The PCR product was found to ~3500 bp, which corresponds to ~2300 bp from the transposed region of pFastBac1 vector and ~1200 bp of Flag\textsuperscript{h}CB2\textsuperscript{his6} insert, confirming the insertion of CB2 gene.

**Expression and characterization of CB2 receptor using baculovirus expression system:**

The pFastBacFlag\textsuperscript{h}CB2\textsuperscript{his6}-bacmid coding for full-length human CB2 receptor construct encoding a N-terminal FLAG-tag and a C-terminal 6His-tag (Flag\textsuperscript{h}CB2\textsuperscript{his6}) was used for baculovirus expression in sf21 cells. Following the transfection of sf21 cells using different amount of bacmid DNA (1, 2.5 and 5 µg), the cells were monitored closely for morphological changes and cell lysis. At about >80% cell lysis, the viral stock was collected and amplified according to standard protocols as detailed above. All steps of the receptor expression following Flag\textsuperscript{h}CB2\textsuperscript{his6} bacmid transfection into Sf21 cells
and subsequent virus amplification were monitored using anti-His western blot analysis. Under optimized receptor expression conditions, more than 80-90% of the FlagCB2his6 receptor was extracted with detergent into the soluble-fraction (Figure 2.3).

**Figure 2.3 Anti-His western Blot analysis of FlagCB2his6 receptor baculovirus expression:** **Lane 1:** Protein ladder with the molecular weights of each band mentioned on its left. **Lane 2, 3:** Detergent-solubilized fraction (S) and detergent-insoluble fraction (P) of untransfected sf21 cells. **Lane 4, 5:** Detergent-solubilized fraction (S) and detergent-insoluble fraction (P) of sf21 cells transfected with 1 µg of pFastBacFlagCB2his6 bacmid. **Lane 6, 7:** Detergent-solubilized fraction (S) and detergent-insoluble fraction (P) of sf21 cells transfected with 2.5 µg of pFastBacFlagCB2his6 bacmid. **Lane 8, 9:** Detergent-solubilized fraction (S) and detergent-insoluble fraction (P) of sf21 cells transfected with 5 µg of pFastBacFlagCB2his6 bacmid.
The radioligand binding experiment using \[^{3}\text{H}]\text{CP55,940},\) a classical cannabinergic ligand shown to be a highly potent agonist for CB2 receptor, with FlaghCB2his6-sf21 membrane preparation produced a curve (Figure 2.4) with a \(B_{\text{max}}\) of 538 pmol/g and a \(K_{d}\) of 0.62 nM. This results correlates well with the previously reported \(K_{d}\) values (0.7-3.7 nM) for the native CB2 receptor preparations from various cells, indicating the proper folding of FlaghCB2his6 receptor.

**Figure 2.4** Saturation binding assay with baculovirus/insect cell expressed FlaghCB2his6 receptor: The 6-point binding assay was performed with [3H]-CP55940 radioligand using the 96-well GF/B filtration plates. To determine the non-specific binding, cold CP-55940 (2 µM) was used. The plates were counted using TopCount NXT™ Microplate Scintillation and Luminescence Counter after the addition of 40 µl/well of scintillation fluid. The data obtained was processed using GraphPad Prism 5 as detailed above.
Purification of CB2 receptor into detergent micelles:

Figure 2.5 Anti-His western Blot (lanes 1-8) and coomassie-stained SDS-PAGE (lanes 9 and 10) analysis of FlaghCB2his6 receptor affinity purification: **Lane 1 and 9**: Protein ladder with the molecular weights of each band mentioned to the left of lane 9. **Lane 2**: The detergent-solubilized fraction of FlaghCB2his6-sf21 cells. **Lane 3**: The flow-through fraction after binding to FLAG M2 affinity resin. **Lane 4 and 5**: FLAG-resin wash 1 and wash 2 respectively. **Lane 6**: FlaghCB2his6 receptor eluate 1 from FLAG-tag based immunoaffinity purification (5 µl). **Lane 7**: FlaghCB2his6 receptor eluate 3 from FLAG-tag based immunoaffinity purification (5 µl). **Lane 8**: FLAG M2 affinity resin after final elution. **Lane 10**: FlaghCB2his6 receptor eluate 1 from FLAG-tag based immunoaffinity purification (35 µl).
We used the immobilized metal affinity chromatography on a Talon cobalt resin and immunoaffinity chromatography on an ANTI-FLAG M2 affinity gel as the two purification strategies. We found that direct solubilization of FlaghCB2his6 from the intact Sf21 cells rather than from the membrane preparation in buffer containing 1% dodecyl maltoside reduces receptor losses and eliminates time-consuming procedure of cell membrane preparation.

The FlaghCB2his6 receptor eluates from the both purification methods were analyzed using anti-His western blotting and coomassie staining of SDS-PAGE gel (Figure 2.5). IMAC purification was found to yield marginally higher amounts of the recombinant receptor, whereas Flag-tag based immunoaffinity purification resulted in eluates of higher purity. In accord with prior results, the eluate from FLAG-tag based immunoaffinity purification was judged, from SDS-PAGE coomassie-stained gels, to be >80% pure. Along with receptor monomer, dimer and higher order oligomeric states were also observed.

Further, a modified radioligand binding experiment of the purified FlaghCB2his6 receptor and $[^{3}\text{H}]$CP55,940, produced a saturation binding curve with a $B_{\text{max}}$ of 8130 pmol/g and a $K_d$ of 23.7 nM (Figure 2.6). This confirmed that the detergent-solubilized, purified, FlaghCB2his6 receptor was still functionally active and can be used for further characterization studies.
Figure 2.6  Saturation binding assay with purified FlaghCB2his6 receptor in DDM:
The 6-point binding assay was performed with [3H]CP55940 radioligand using the 0.5% PEI-coated 96-well GF/B filtration plates. To determine the non-specific binding, cold CP-55940 (5 µM) was used. The plates were counted using TopCount NXT™ Microplate Scintillation and Luminescence Counter after the addition of 40 µl/well of scintillation fluid. The data obtained was processed using GraphPad Prism 5 as detailed above.

In relation to our primary goal of using proteomics-based mass spectrometric analysis to study ligand binding pocket interactions, we performed a preliminary proteomics analysis of human CB2, following trypsin digested, using MALDI-TOF/TOF mass spectrometry (Figure 2.7).
Figure 2.7 MALDI-TOF/TOF analysis of FlagCB2his6 tryptic peptides in Reflectron mode: FlagCB2his6 peptides that were confirmed using MS/MS analysis are labeled in the spectrum (Hydrophilic intra- and extracellular peptides in blue, Hydrophobic transmembrane peptides in red).

Using preliminary MALDI-TOF/TOF analysis we were able to identify 11 peptides that correspond to specific cleavage of FlagCB2his6 upon trypsin digestion (Table 2.1). Out of 11 peptides, 9 were confirmed by MS/MS analysis with a score of >80 in DeNovo Explorer™. This corresponds to a sequence coverage of ~40%. Interestingly, most of the peptides identified belonged to the hydrophilic extracellular and intracellular domains of the receptor including the ECL, ICL, C-terminal and N-terminal
ends. Only one transmembrane peptide corresponding to Helix 7 and one membrane associated peptide corresponding to Helix 8 were identified. Owing to the amphipathic nature of the transmembrane proteins, GPCR proteins are highly hydrophobic and tend to irreversibly aggregate during purification and in-solution digest and aggressively adhere to surfaces. Further, CB2 receptor contains Lysine or Arginine outside the membrane region, leading to long (20-25 amino acids) tryptic peptides that are extremely hydrophobic; for example the peptide 158-223, corresponding to TM4 and TM5 has GRAVY 1.118). This leads to significant losses of transmembrane peptides and poor peptide identification using MALDI-TOF/TOF analysis.

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**Table 2.1** FlaghCB2his6 tryptic peptides identified from MALDI-TOF/TOF analysis: Peptides that were confirmed by MS/MS analysis are labeled in green, Peptides that could not be confirmed by MS/MS analysis are labeled in red. Modifications for each peptide are labeled as follows – MSO – Methionine oxidation, CYS-CAM – Carbamidomethylation of Cysteine.
In spite of constrains in MS analysis, we demonstrate a highly effective, rapid purification system for analyzing human CB2 receptor. Future steps might include the use of LC/MS analysis for better hydrophobic peptide recovery and identification, which could enable full mass spectrometric characterization of CB2. This work, in conjunction with covalent affinity labeling, could prove to be an important step towards development of experimental approach that will enable ligand binding pocket characterization of CB2.

2.6 References:


Chapter 3

Ligand Assisted Binding Site Characterization of Human CB2 using Mass Spectrometric Analysis

Parts of this chapter are in preparation for publication

(2015) Binding site characterization of AM1336, a novel covalent inverse agonist, at human cannabinoid 2 receptor using mass spectrometric analysis. (J Proteome Res)

3.1 Introduction:

Cannabinoid 2 receptor (CB2R) is a Class A G-protein coupled receptor and is an important drug target in a wide array of pathological conditions such as neuropathic pain\(^1\), neuroinflammatory disorders,\(^2\) inflammatory bowel syndrome\(^3\) and cancer\(^4\). Rational drug discovery has been hindered due to our poor understanding of the structural features involved in ligand binding. Owing to the high flexibility and very lipophilic transmembrane domains in GPCRs, three-dimensional structural analysis had proven to be difficult. But due the advances in lipid-cubic phases crystallization techniques and introduction of structural modifications, over 24 unique high-resolution crystal structures have been reported. However, the atomic structure of human CB2 receptor has remained elusive and calls for the use of alternative techniques to obtain direct structural information of fully intact CB2 receptor.

To address this issue, we developed a comprehensive strategy - Ligand Assisted Protein Structure (LAPS) that encompasses both experimental evidence from biochemical and
biophysical techniques and *in-silico* predictions would facilitate the design and optimization of therapeutically attractive CB2-selective ligands with minimal off-targets.\(^5\) LAPS experimental approach involves the integration of four elements - functionalized covalent ligand-probe development, point mutation-based pharmacological analysis, proteomic mass spectrometric analysis and computer modeling of probe-receptor complex was developed and reported. Using the LAPS approach, we have previously shown that (−)-7′-isothiocyanato-11-hydroxy-1′,1′-dimethylheptylhexahydro-cannabinol (AM841), a high-affinity CB2 agonist, interacts covalently to C6.47(257) of the conserved CWxP motif in transmembrane helix 6 (H6).\(^5\) This approach proved to be a powerful technique for the characterization of critical receptor residues that interact with a chemically and functionally diverse set of ligands, at the amino-acid level.

Over the years, a large number of distinct classes of synthetic cannabinoid ligands, including Classical cannabinoids (\(\Delta^9\)-THC), Non-classical cannabinoids (CP55940), aminoalkylindoles (WIN-55212-A) and biarylpyrazoles (SR144528), have been developed and reported\(^6\). Rimonabant, a CB1-selective biarylpyrazole analogue, was approved for treatment of obesity in patients with associated risk factors, such as type 2 diabetes or dyslipidaemia\(^7\) but was later was withdrawn from the market due to severe side effects (depression and suicidality)\(^8\). SR144528, a rimonabant analogue, was the first reported sub-nanomolar affinity CB2 selective ligand and functions as an inverse agonist.\(^9\) In the present study, we strategically functionalized the aliphatic side chain of SR144528 with an electrophilic isothiocyanate (-NCS) chemical group to generate novel covalently interacting ligand (AM1336).\(^5\) At the physiological pH of 7.4, ligands functionalized with isothiocyanate have been shown previously to selectively and covalently modify proteins at nucleophilic thiol groups of cysteines.\(^10\) Using systematic covalent
binding studies on multiple-site cysteine mutants of human CB2 receptor overexpressed in HEK293 cells, we identified that the two cysteines - C7.38(284) and C7.42(288) were critical for the optimal covalent interaction of AM1336 with human CB2 receptor.\(^5\)

![Chemical structures of few biarylpyrazole analogues and their binding affinities to human CB2.](image)

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This table is generated from competitive binding experiments using radioligand \(^3\)HCP55940.

**Figure 3.1 Chemical structures of few biarylpyrazole analogues and their binding affinities to human CB2.**

In this study, we use proteomic mass spectrometric analysis to provide direct evidence of this covalent interaction between AM1336 and H7 of human CB2 receptor. To further understand and visualize the other interactions of AM1336 in the binding pocket of CB2 receptor, we performed receptor docking studies and molecular dynamics simulations in a lipid bilayer system.
3.2 Reagents and Materials

Standard laboratory chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Chemical (Pittsburgh, PA) if not otherwise specified. Coomassie G-250 stain, Laemmli electrophoresis sample buffer, PVDF membrane, molecular weight markers and 1D SDS-PAGE gels were from Bio-Rad (Hercules, CA). Trypsin Gold, MS grade, was purchased from Promega (Madison, WI). n-Dodecyl-β-d-maltoside (DDM) and 5-cyclohexyl-1-pentyl-β-d-maltoside (CYMAL5) were purchased from Anatrace (Maumee, OH). [3H]CP-55940 was provided by the National Institute on Drug Abuse, National Institutes of Health (Bethesda, MD). AM-1336 was synthesized at the Center for Drug Discovery, Northeastern University (Boston, MA). A 27-mer peptide corresponding to human CB2 receptor transmembrane H7 (KKAFACSLCLINSMVPNVIYALRAAG) with 23 amino acids in common with the helix 7 tryptic peptide (no missed cleavages) was synthesized by standard methods (GenScript; Piscataway, NJ) to >95% purity according to liquid-chromatography (LC) and MS analyses.

3.3 Methods:

3.3.1 Baculovirus/Insect Cell Expression of Flaghcb2his6:

Sf21 insect cells were transfected with bacmid-FlaghCB2his6 DNA according to the Bacto-Bac baculovirus expression kit’s (Invitrogen) recommended protocol. Briefly, sf21 cells cultured in Sf-900™ II SFM media containing 2% fetal bovine serum (FBS), when in log phase (1.5-2.5 x 10^6 cells/ml) with greater than 95% viability, were transfected with different amounts of recombinant bacmid-FlaghCB2his6 DNA using Cellfectin® II reagent. The supernatant (viral stock, P1) were collected and stored at 4 °C, protected from light. The cell pellets were used to test for the expression of FlaghCB2his6 receptor. The cells were resuspended in lysis buffer (300
mM NaCl, 50 mM sodium phosphate, 10% Glycerol, 1% dodecyl maltoside, pH 8.0) and lysed on ice by three, 33 s sonication cycles, each cycle consisting of 1 s bursts at 50 W separated by a 5 s interval. The lysate was centrifuged at 16000x g for 15 min and supernatant and pellets were analyzed separately using SDS-PAGE analysis or western blot analysis as detailed below.

The P1 viral stock was filtered through a 0.22 µm Millex-GS syringe filter unit (Millipore, Billerica, MA) and used for further infection steps. Different quantities of filtered P1 viral stock was used for infecting sf21 cells in log phase and incubated at 27 °C for 5 days (until ~80% cell lysis).

3.3.2 SDS-PAGE and Western Blot Analysis

Samples were preincubated at room temperature for 10 min in Laemmli sample buffer containing 5% β-mercaptoethanol. Protein samples were resolved on AnykD™ Mini-PROTEAN® TGX™ precast polyacrylamide gels. Proteins were transferred to PVDF membranes, and the membranes were prepared for immunodetection following the procedures outlined in the QIAexpress Detection and Assay Handbook (QIAGEN, Valencia, CA). Membranes were incubated with a 1:10 000 dilution of Penta-His antibody horseradish peroxidase (HRP) conjugate (Sigma) or a 1:10000 dilution of monoclonal mouse ANTI-FLAG® M2 antibody (Sigma) followed by incubation in a 1:10000 dilution of goat anti-mouse IgG-HRP (Sigma). Protein bands were visualized using the ECL Western blotting analysis system (GE Healthcare, Piscataway, NJ). The image was captured using the Fluorchem SP™ (Alpha Innotech Santa Clara, California) system.
3.3.3 FlaghCB2his6 Membrane Preparation for Saturation Binding Assays

Insect cell pellets were suspended in 20 ml of TME [25 mM Tris base, 5 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4]. Cells were lysed under nitrogen cavitation for 30 min at 1000 psi on ice, the cell lysate was centrifuged at 2000 g for 5 min, and the supernatant collected. The pellet was washed three times with 20 ml of TME and centrifuged. The combined supernatants from each wash were ultracentrifuged at 100,000 g for 45 min at 4 °C. The resultant membrane pellet was used immediately or stored at −80 °C. Membrane protein was quantified with a Bradford dye-binding method (Bio-Rad Laboratories).

3.3.4 Saturation Binding Assay with Flaghcb2his6:

Saturation binding assays were performed in a 96-well format. About 25 µg of protein was added to each assay well. The [3H]CP-55,940 was diluted in TME-BSA buffer to yield ligand concentrations ranging from 0.625 to 20 nM. Nonspecific binding was assayed in the presence of 5 µM unlabeled CP-55,940. The assay was performed at 30 °C for 1 h with gentle agitation. The resultant material was transferred to Unifilter GF/B filter plates and unbound ligand was removed using a Packard Filtermate-196 Cell Harvester. Filter plates were washed four times with ice-cold wash buffer (50 mM Tris-base, 5 mM MgCl₂ containing 0.5% BSA, pH 7.4). 40 µl of MicroscintTM 20 scintillation fluid was added to each well and the plates were counted using TopCount NXT™ Microplate Scintillation and Luminescence Counter. The data obtained was processed using Microsoft Excel and Prism 5. All concentration points were performed in triplicate and data points used for plotting are base-line corrected. Bmax and Kd values were calculated by nonlinear regression using Graphpad Prism version 5.03 (one site-binding analysis equation \( Y = \frac{B_{\text{max}} \times X}{(K_d + X)} \)) on a Windows platform.
For the saturation binding assays with purified FlaghCB2his6, 96-well GF/B filtration plates were pre-treated with 0.5% polyethylenimine (PEI) for 3 hours at 4 °C and washed twice with 200 ml of binding buffer (BB - 25 mM Tris base, 5 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% BSA, pH 7.4).

3.3.5 Anti-FLAG Immunoaffinity Purification of Flaghcb2his6

Cells pellets were suspended in lysis buffer (50 mm sodium phosphate, 300 mm NaCl, 10% glycerol, 1% dodecyl-β-d-maltoside, pH 7.4) and lysed on ice by three, 33 s sonication cycles, each cycle consisting of 1 s bursts at 50 W separated by a 5 s interval. The lysate was centrifuged at 16000x g for 15 min (to remove cell debris) and the supernatant was collected. An equal volume of purification buffer (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 7.4) was added to the supernatant. This mixture was then added to pre-equilibrated (in 50% lysis buffer, 50% purification buffer) ANTI-FLAG® M2 affinity gel and incubated for 2 hours at 4 °C on a rotating wheel. The resin was washed twice with 5 column volumes of wash buffer (purification buffer with 0.2% dodecyl-β-d-maltoside) and FlaghCB2his6 was eluted wash buffer containing 150 µg/ml of FLAG® peptide. SDS-PAGE and western blotting analysis were performed on the aliquot of samples taken during purification according to the procedure previously described.
3.3.6 MALDI-TOF/TOF analysis of human CB2 H7 model peptide covalently modified by AM1336:

The human CB2 H7 model peptide was treated with AM1336 at a molar ratio of 1: 1.33 in 0.4 mM ammonium bicarbonate containing 20% methanol and 40% DMSO for 30 min at 37°C. The reaction mixture was mixed with equal volume of α-cyano-4-hydroxycinnaminic acid matrix solution (5 mg/ml dissolved in 50% acetonitrile, 50% water, and 0.1% trifluoroacetic acid) and spotted onto an Opti-TOF 384-well plate insert. MALDI-TOF MS spectra were acquired on a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA) fitted with a 200-Hz solid state UV laser (wavelength 355 nm). Spectra of the peptides were acquired in both reflectron and linear modes. All MS spectra of the tryptic digests were externally calibrated using a mixture of peptide standards [des-Arg1-bradykinin at MH+ 904.4681, angiotensin I at MH+ 1296.6853, Glu-fibrino peptide at MH+ 1570.6774, ACTH (clip 1–17) at MH+ 2093.0867, ACTH (clip 18–39) at MH+ 2465.1989 and ACTH (clip 7–38) at MH+ 3657.9294]. MS/MS spectra were acquired on select ions of interest using conditions as described by Zvonok et al.

3.3.7 Reduction, alkylation and in-solution trypsin digestion of purified FlaghCB2his6 receptor:

The samples for LC/MS/MS analysis were prepared using the previously reported standard procedures. Briefly, 0.5 µg of purified FlagCB2his6 receptor was reduced using 20 mM dithiothreitol (DTT) and alkylated using 50 mM iodoacetamide (IAM) by incubating with each at RT for 1 h. The mixture was then desalted into 25 mM ammonium bicarbonate with 0.05% Cymal-5 using Micro BioSpin 6 columns (Biorad) using the manufacturer’s recommended
protocol. The sample was then subjected to overnight digestion with Tryspin Gold, MS-grade (Promega, Madison, WI) at 37 °C. The digests were analyzed immediately or stored at -80 °C until further processing.

3.3.8 LC/MS/MS analysis of FlaghCB2his6 receptor tryptic peptides:

The tryptic digest samples were analyzed by nanoLC-MS using the Eksigent NanoLC Ultra2D coupled to a LTQ Orbitrap XL with ETD (Thermo Scientific) for peptides identification. Briefly, 5 µl of FlaghCB2his6 tryptic digest was loaded onto a 50 micron ID in-house polymerized polystyrene-divinyl benzene C10 derivitized monolithic (10 cm) trap column with a flow rate of 600nl/min using a loading solvent (mobile phase A). The mobile Phase A consisted of 0.1% formic acid in 5% acetonitrile and mobile phase B consisted of 0.1% formic acid in 95% acetonitrile. Peptide separation was performed on a 40 cm long, 50 µm ID, in-house packed column with Magic C18 3 µm beads and 150 angstrom pores, using a linear gradient from 100% A: 0% B to 5% A: 95% B over 60 min at a flow rate of 100 nl/min. Electrospray ionization was carried out using a distal coated 20 µm ID capillary pulled to a 5 um tip on a new objective source. The peptide spectra was obtained in positive-ion mode and analyzed using the PEAKS software. Additional searches were performed using Proteome Discoverer 1.4 software.

3.3.9 In-silico modeling studies with human CB2 receptor:

The human CB2 receptor homology model, created based on the mutation-stabilized Turkey beta-1 adrenergic receptor (PDB: 2y00) structure bound by partial agonist dobutamine, was obtained from the GPCRDB. The homology model was energy minimized by the steepest-
descent and conjugate-gradient algorithms and then subjected to refinement (relaxation) by all-
atom molecular dynamics (MD) simulation using AMBER03 force field in phosphatidyl-
ethanolamine (PEA) lipid bilayer using YASARA software. The ligands for docking were
energy minimized and cleaned for right bond orders and bond lengths. The ligands were docked
into 10 nm² cell covering the predicted GPCR binding pocket in the energy minimized human
CB2 model using Autodock VINA algorithm. The poses with the highest binding energies were
further analyzed. The docked AM1336 was covalently attached to either helix 7 cysteines and
energy minimized using YASARA force field. The covalently docked AM1336-hCB2R model
was subjected to MD simulation for >2 ns in phosphatidyl-ethanolamine (PEA) lipid bilayer as
described previously. Additionally, the Calpha atoms of the transmembrane domains were fixed,
while the residues - Phe87, Phe91, Phe94, Asp101, Phe106, Lys109, Ile110, Val113, Phe117,
Glu181, Leu182, Phe183, Trp194, Phe197, Trp258, Val261, Met265, Phe281 and Ser285 and all
residue side chains were allowed to be flexible. The 2D protein-ligand interaction diagrams were
generated using the LIGPLOT⁶ v1.4 as previously reported.²⁰

3.4 Results and Discussion

3.4.1 Expression and purification of human CB2 receptor:

To generate the recombinant receptor in sufficient quantities for proteomic mass
spectrometric studies, the human CB2 receptor was overexpressed in Spodoptera frugiperda
(Sf21) cells using the Bac-to-Bac baculovirus expression system (Invitrogen). Consistent with
previously published work¹¹, a full-length human CB2 receptor construct encoding a N-terminal
FLAG-tag and a C-terminal 6His-tag (FlaghCB2his6) was chosen for recombinant receptor
expression (Figure 3.2). All steps of the receptor expression following FlaghCB2his6 bacmid
transfection into Sf21 cells and subsequent virus amplification were monitored using anti-His
western blot analysis. Based on prior empirical detergent screen,\textsuperscript{11} FlaghCB2his6 was solubilized in buffer containing mild, non-ionic, MS-compatible detergent dodecyl maltoside (DDM), previously shown to be suitable for efficient extraction of functional GPCRs.

**Figure 3.2 Snake plot of FlaghCB2his6.**

Under optimized receptor expression conditions, more than 70-80\% of the FlaghCB2his6 receptor was extracted with detergent into the soluble-fraction (Figure 3.3A). Direct solubilization of FlaghCB2his6 from the intact S/2I cells, rather than from membrane preparation, resulted in reduced receptor losses and eliminated time-consuming procedure of cell membrane preparation. FlaghCB2his6 was purified using immunoaffinity chromatography on ANTI-FLAG M2 affinity gel and analyzed using anti-His western blotting and coomassie staining of SDS-PAGE gel (Figure 3.3B). In accord with prior results, the eluate was judged to be >80\% pure. Upon storage, along with receptor monomer, dimer and higher order oligomeric states were observed, indicating time-dependent protein instability in detergent, as observed in the case of other GPCRs.
**Figure 3.3: FlaghCB2his6 expression and purification:** (A) Anti-His western blot analysis of solubilized sf21 cells untransfected (lanes 2 and 3) and transfected with FlaghCB2his6 bacmid (Lanes 4 and 5); S and P indicate the supernatant and pellet fractions following high-speed centrifugation of solubilized cells; L indicates the protein ladder and molecular weights (kDa) are labeled (left). (B) Anti-His western blot (lanes 1-8) and coomassie-stained SDS-PAGE (lanes 9 and 10) analysis of FlaghCB2his6 receptor affinity purification: **Lane 1 and 9:** Protein ladder with the molecular weights (kDa) of each band mentioned to the left of lane 9. **Lane 2:** The detergent-solubilized fraction of FlaghCB2his6-sf21 cells. **Lane 3:** The flow-through fraction after binding to FLAG M2 affinity resin. **Lane 4 and 5:** FLAG-resin wash 1 and wash 2 respectively. **Lane 6:** FlaghCB2his6 receptor eluate 1 from FLAG-tag based immunoaffinity purification (5 µl). **Lane 7:** FlaghCB2his6 receptor eluate 3 from FLAG-tag based immunoaffinity purification (5 µl). **Lane 8:** FLAG M2 affinity resin after final elution. **Lane 10:** FlaghCB2his6 receptor eluate 1 from FLAG-tag based immunoaffinity purification (25 µl).
3.4.2 Ligand binding competency of FlaghCB2his6:

To ascertain the functional activity of the expressed recombinant protein, saturation radioligand binding experiment using \[^{3}H\]CP55,940, a highly potent cannabinoid radioligand, was performed with FlaghCB2his6-sf21 membrane preparation. The assay revealed the level of FlaghCB2his6 receptor to be 0.5 pmol/mg of total protein in membrane preparation (Bmax) (Figure 3.4A). The K_d of 0.62 nM also correlated well with the previously reported values (0.7-3.7 nM) for the wild-type human CB2 receptor preparations from native and overexpression systems, indicating the proper folding of FlaghCB2his6 receptor.

**Figure 3.4: Saturation binding of \[^{3}H\]CP55940 ligand to (A) FlaghCB2his6-sf21 membrane preparation and (B) purified FlaghCB2his6 in DDM detergent micelles in the presence of 50 nM AM1336 or DMSO. The experimental conditions for radioligand binding assay are detailed in the methods section.**

The ligand binding competency of the purified FlaghCB2his6 receptor was ascertained using a modified radioligand binding experiment where the filter plates were pre-treated with
polyethelenimine to help retain solubilized receptor.\textsuperscript{12} The assay showed the presence of 8.1 pmol of FlagCB2his6 per mg of total eluate (Figure 3.4B). This confirmed that the detergent-solubilized, purified, FlagCB2his6 receptor was still functionally active and can be used for further characterization studies. Further, when purified receptor was tested in saturation radioligand binding assay in the presence of 50 nM AM1336 (\textasciitilde 10 times the $K_i$ value obtained from radioligand displacement assay using CP55,940 with mammalian cell derived human CB2 receptor membrane preparation), the $B_{\text{max}}$ decreased to 2.2 pmol/mg (Figure 3.4B). This decrease in $B_{\text{max}}$ indicates 73\% receptor labeling with AM1336 and is consistent with the previously reported data for human CB2 receptor labeling by ligand. This experiment confirmed that purified FlagCB2his6 receptor could be efficiently labeled with AM1336 for mass spectroscopic proteomic studies.

3.4.3 Bottom-up Proteomic Analysis of FlaghCB2his6 and FlaghCB2his6-AM1336 Using LC/MS

We used previously optimized methods of hCB2R sample preparation for proteomic analysis.\textsuperscript{17} The FlaghCB2his6 and FlaghCB2his6-AM1336 samples were reduced and alkylated using dithiothreitol (DTT) and iodoacetamide (IAM), respectively, desalted on Micro Biospin columns into 25 mM ammonium bicarbonate, pH 8.0 containing 0.05\% CYMAL5 and digested overnight with trypsin. Although the desalting columns led to some protein losses, we found that they efficiently removed salts and excess of detergent. The MS-compatible detergent, CYMAL5 was added to exchange buffer to prevent protein aggregation and precipitation. The MS analysis of this samples, performed using the above detailed nanoLC/MS/MS procedure, yielded a protein sequence identity of \textasciitilde 90\% to full-length wild-type hCB2R (Figure 3.5). The H7 peptides with
different modifications including carbamidomethylation of the cysteines, deamidation of the asparagines and oxidation of the methionines, with the exception of AM1336 attachment were identified in FlaghCB2his6 and FlaghCB2his6-AM1336 digested samples. We assumed that the presence of covalently attached ligand in already hydrophobic peptides significantly decrease their solubility and concentration to the level below of detection limit used for standard analysis.

Figure 3.5: Bottom-up proteomic analysis of FlaghCB2his6 receptor tryptic digest and peptide coverage using LTQ Orbitrap MS. The peptides identified using high mass accuracy MS1 measurements and MS/MS analysis are indicated in grey and blue, respectively. The
protein modifications – carboximethyl (e), deamidation (+0.98) (d) and oxidation (+15.99) (o) are also indicated.

3.4.4 Targeted AM1336-H7 Peptides Analysis of Trypsin Digested FlaghCB2his6 and FlaghCB2his6-AM1336 Using NanoLC/MS:

Compounds containing isothiocyanate functional group reacted at neutral pH exclusively with cysteine sulfhydryl group through nucleophilic addition. Indirect evidence from the mutation-based ligand binding studies indicated that both cysteines C284(7.38) and C288(7.42) in H7 of the hCB2R are critical for the covalent interaction with AM1336.\textsuperscript{16} To define directly the amino acid residues in receptor that modified with AM1336 we used LTQ Orbitrap MS/MS for analysis of the FlaghCB2his6 receptor tryptic peptides following treatment with DMSO or AM1336. Since the hydrophobic peptides in receptor tryptic digests are prone to precipitation, up to 30% of trifluoroethanol was added to samples before injection to enhance solubilization of the preparation. NanoLC separations were performed on a shorter (20 versus 40 cm), less hydrophobic (C8 versus C18) column with a two-step acetonitrile gradient (2-40%, 10 min; 40-95%, 30 min versus 5-95%, 60 min). Due to poor separation of the highly hydrophobic peptides from transmembrane helices, particularly those having AM1336 attached, targeted analysis was used to identify ligand modified peptides. The high-resolution MS spectra of H7 (AFAFCSMLCLINSMVNPVIYALR) tryptic peptide in DMSO-treated FlaghCB2his6 receptor sample is shown in Figure 4A. The monoisotopic mass of +3 charge peak corresponding to naïve H7 peptide where both cysteines are carboximethylated, was observed at \textit{m/z} 897.4497 Th (observed and predicted monoisotopic masses are 2689.3491 and 2689.3253 Da, mass error of 8.8 ppm). In AM1336-treated samples, the monoisotopic mass of +3 charge peak provisionally assigned to AM1336-H7 peptide was observed at \textit{m/z} 1032.5238 Th (Figure 4B; observed and predicted monoisotopic masses are 3094.5714 and 3094.5491 Da; mass error 7.2 ppm). The AM1336 modified H7 peptide was shown to have a ligand at one cysteine and the second cysteine was carboximethylated, while both cysteines are carboximethylated in peptide without
ligand. The observed and calculated mass differences between these two peptides are 405.2223 and 405.2238 Da (462.2453-57.0215 Da; error -3.7 ppm), respectively that strongly indicated addition of AM1336 to one of the two cysteines in H7. The signal intensity of H7 peptide modified with AM1336 ($3.91 \times 10^3$) was considerably lower than that from the unmodified peptide ($2.89 \times 10^4$), This is attributed to increase in the hydrophobicity of the modified peptide due to the addition of lipophilic ligand thus resulting in significant losses during sample preparation and nanoLC/MS analysis (Figure 4A-B).

**Figure 3.6:** High-resolution LC-MS/MS spectra of the hCB2R transmembrane helix 7 peptide (AFAFCSMLCLINSMVPNVIYALR) obtained from analysis of tryptic digests of purified FlaghCB2his6 treated with DMSO (monoisotopic $m/z = 897.4497$ Th, z=3) (A) or purified FlaghCB2his6 treated with AM1336 (monoisotopic $m/z = 1032.5238$ Th, z=3) (B). The mass difference is equivalent to the mass of AM1336.

MS/MS fragmentation of triple charged ions of H7 peptide $m/z 897.4497$ Th (both cysteines carbamidomethylated) and $m/z 1032.5238$ Th (one cysteine carbamidomethylated and one modified with AM1336) was used for identification of amino acid residue with attached AM1336. The mass difference
between the y19 fragments from naïve H7 (2252.5738 Da) (y19^{2+}, m/z 1127.2869 Th) and AM1336-H7 (2659.3245 Da) (y19^{3+}, m/z 887.4415 Th) peptides indicated the presence of ligand on either C284(7.38) or C288(7.42) (Figure 5). However, no difference in mass between b10 and b8 fragments from unlabeled H7 (m/z +273.1853 Th) or AM1336-H7 (m/z +273.0725 Th) peptides excluded attachment of ligand to C288(7.42). Further, the mass difference of m/z +273.1853 Th and m/z +273.0725 Th, between b8^{+} and b10^{+} fragments in both DMSO-treated and AM1336-treated H7 peptides, respectively, corresponds to the combined mass of L287(7.41) and carbamidomethylated C288(7.42). Similarly, no significant shift was observed in mass difference between b8^{+} and b11^{+} fragments in both DMSO-treated and AM1336-treated H7 peptides and it corresponded to the L287(7.41), carbaniminomethylated C288(7.42) and I289(7.43). The bottom-up proteomics analysis of the hCB2R-AM1336 directly indicated that AM1336 covalently attached to the C284(7.38) in transmembrane H7. These results are not fully congruent with our previous indirect radioligand binding studies in which hCB2 cysteine mutants treated with AM1336 suggested that both cysteines C284(7.38) and C288(7.42) in H7 are involved in covalent binding of AM1336 with each cysteine having approximately equal probability for ligand attachment. This discrepancy of AM1336 attachment between the data obtained from our LC-MS/MS work when compared to our mutation based data may have any of the following explanations: (1). The preparations from recombinant hCB2Rs expressed in insect and mammalian cells probably have membrane related differences that affect the ligand binding motifs; (2). Moreover, for the mutation-based experiments AM1336 covalent attachment to hCB2R was performed with CB2R incorporated in the full membrane, while in our MS studies the reaction was conducted in solubilized receptor preparations.
3.4.5 Molecular Modeling of AM1336 Binding to hCB2R:

Collective data from other GPCRs, based on biochemical and modeling studies, have proposed that full cannabinoid agonists such as CP55490 interact to transmembrane helix 3-6-7 of hCB2R and activate intracellular receptor signaling. Using the LAPS approach, we previously reported that AM841, a classical cannabinoid agonist with isothiocyanate moiety at the end of its alkyl tail, covalently attached to C257(6.47) of the CWxP hinge motif in transmembrane helix 6. Similarly, previous reports predicted that in case of SR144528, a CB2-selective inverse agonist, the amide moiety interacts with transmembrane helix 3/7 and the aromatic rings interact near...
transmembrane helix 3-5-6 region of the receptor.\textsuperscript{13} The X-ray crystal structure of GPCR with greater sequence identity to CB2R - turkey beta-1 adrenergic receptor (PDB 2y00) was used to create a CB2R homology model. Figure 3.8A presents the docking results for AM1336 ligand when flexibly docked into the predicted hCB2R binding pocket. As predicted from biochemical data, the AM1336 binds similarly to hCB2R as SR144528 with pi-pi interactions between the aromatic rings and W258(6.48) and F197(5.46) with the adamantyl moiety packed against H7 and the alkyl tail ending with isothiocyanate group pointing towards C284(7.38).

We then attached the docked AM1336 through the sulfhydryl group of C284(7.38) and energy minimized the AM1336-hCB2R complex in a solvated lipid bilayer membrane as described in the methods. The final structure revealed that AM1336 showed similar hydrophobic interactions to that of SR144528 with residues I110(3.29), S193(5.42), W194(5.43), M265(6.55) and S285(7.39) (Figure 3.8B). Additionally, the movement of the adamantyl group into the pocket between H2, H7 and ECL2 indicating that large bulky groups can be accommodated at this position without significant changes to ligand affinity. It has been suggested that I110(3.29), E181 (ECL2), L182 (ECL2) and V261(6.51) are critical for CB2 selectivity in SR144528\textsuperscript{14} but were not found to be directly interacting with AM1336, consistent with its poor CBR subtype selectivity. S285(7.39) has previously been shown to be critical for binding of classical CB agonists such as HU243 in both CB1 and CB2 receptors.\textsuperscript{15} Interestingly, it is found to make critical interactions with both SR144528 and AM1336 in our modeling experiments. The 2D ligand plot reveals that residues F87(2.57), V113(3.32), W258(6.58), V261(6.51) and F281(7.35), consistent with previous reports, are involved in hydrophobic interactions with AM1336 and part of the binding pocket for cannabinergic ligands at hCB2R.\textsuperscript{13}
**Figure 3.8: In-silico modeling results of AM1336-hCB2R complex.**

**A)** AM1336 (in elemental colors) covalently attached to C284(7.38) in human CB2R following ~2 ns of energy minimization with a molecular dynamics simulation in PE lipid bilayer membrane. The helices – H6, H7, H2 and H4 of hCB2R and C7.38(284) and C7.42(288) are labeled accordingly. H1, 4 and 5 are hidden for better visualization of the binding pocket. Residues that make critical interactions with AM1336 - K109(3.28), I110(3.29) and F281(7.35) are labeled in magenta. 

**B)** Ligand binding site of hCB2R with AM1336 attached at C284(7.38) respectively as predicted by molecular modeling when depicted in 2D. The conserved residues between both models are labeled (red circles) and the site of hydrophobic interactions are labeled at each atom on the ligand structure. The hydrogen bonding interactions are shown as green dotted lines and distance is indicated in angstrom.
3.5 Conclusion:

The hCB2R agonists have generated significant interest as candidates for the treatment of neuropathic and inflammatory pain and for neuroprotective properties in neurodegenerative disorders. By contrast, CB2 antagonists have shown promise in the treatment of bone disorders, however this remains controversial. To investigate these avenues further, a better understanding of the receptor structure is necessary for novel ligand design and development.

The past decade has been very successful for X-ray structure determination of different classes of GPCRs in their active or inactive states. From the active-state structures, certain critical changes have been observed that are consistent among most class A GPCRs; this includes the breakage of H3-H6 salt bridge, the outward movement of H6, the inward movement of H7 and decrease and increase in the orthosteric binding pocket and G-protein binding pocket volumes respectively. The recent hCB1R crystal structures provide further insight into the specific structural features of receptors in the cannabinoid family. However, since the 3D structure of the hCB2R is not available, alternate biophysical techniques could be a useful starting point for the design of new drugs with predictable functions.

Interestingly, the pharmacological profiling of cysteine to serine or alanine substituted hCB2R mutants in mammalian cell lines indirectly revealed that both cysteines C284(7.38) and C288(7.42) in transmembrane H7 are critical for the covalent attachment of AM1336, a potent biarylpyrazole CB2 inverse agonist. In this work MS analysis of the hCB2R treated with AM1336 directly identified ligand attachment to cysteine C284(7.38) of the H7, the important component of the ligand binding motif at the hCB2R. Together with the computational modeling data, this could prove to be a useful guide to our structure-based cannabinoid drug design platform.

Using our LAPS approach we have been able to identify the direct attachment of AM841, a CB2 agonist, to C257(6.47) in H6 and AM1336, a CB2 inverse agonist, to C284(7.38) in H7, respectively.
Further investigation may be required to completely understand the significance of these covalent ligands attachments on the functional profile of the CB2R. An interesting possibility maybe the design of selective hCB2R ligands with required functional profile (agonist or antagonist) by targeting specific helices of the cannabinoid receptor.

3.6 References:


Chapter 4

Reconstitution of Human CB2 into Nanodiscs

4.1 Introduction:

The Cannabinoid 2 receptor (CB2) belongs to the Class A family of G-protein coupled receptors and functions through the modulation of numerous cell signaling molecules.\(^1\) This multiplicity of signaling transduction pathways supports the modulation of a diverse set of physiological functions throughout the human body.\(^2\) They therefore have been identified as valuable therapeutic targets for the treatment of a myriad of pathological conditions including neuropathic pain\(^3\), inflammation\(^4\), metabolic dysfunction\(^5\), cancer\(^6\) and drug seeking behaviors\(^7\). However, a number of clinical studies performed using Cannabinergic ligands have failed due to lack of efficacy.\(^2\) Understanding the dynamic structural changes that occur upon ligand binding will enable use to design and develop ligands targeting specific signaling pathways, which could prove to be a viable approach for better therapeutic success.

Owing to the exponential progress in structure determination methods of membrane proteins in the last few years, over 119 different GPCR crystal structures of 22 unique GPCRs have been reported.\(^8\) Based on these structures, we now have a better understanding of the general features of GPCR structural changes upon ligand binding. The large outward displacement of transmembrane helix VI and inner movement of helix VII, upon agonist binding, generally characterize GPCR activation. Other changes include the rearrangements in the side chains of residues in conserved D(E)RY and
NPxxY motifs in transmembrane helix III and VII respectively. These changes in the transmembrane helices have been shown to cause significant changes in the interactions with intracellular effector molecules such as G-proteins, G-protein receptor kinases (GRKs) and β-arrestin. However, very few studies have provided direct evidence of receptor conformational changes that occur upon ligand binding.

Due to the lack of x-ray crystal structures of CB2 receptors, most of our knowledge of structural features of CB2 activation is from signaling assays and mutational studies often in conjunction with in-silico modeling studies. In order to directly study conformational changes of CB2 receptor in its native environment, we need to develop a better model system for the study of protein dynamics using biophysical methods such as NMR, hydrogen deuterium exchange mass spectrometry (HDX-MS), small-angle x-ray scattering, electron paramagnetic resonance (EPR), and Fourier transform infrared spectroscopy (FTIR).

4.2 Hydrogen deuterium exchange mass spectrometry (HDX MS):

Hydrogen deuterium exchange (HDX) is a widely used technique for exploring and understanding protein conformation and dynamics in solution. The method relies on the exchange of hydrogens of the protein backbone amide groups with deuterium in the solution at the rate determined by the local structure and environment. The backbone amide hydrogens that are either located at the surface of the protein or involved in weak hydrogen bonds, can exchange rapidly. The backbone amide hydrogens that are located in the interior of the protein or involved in highly stabilizing hydrogen bonds exchange...
more slowly.\textsuperscript{16} Measuring and analyzing the rates of amide hydrogen exchange can provide valuable information on protein conformation and dynamics. The deuterium exchanged in the protein can be detected using several analytical techniques such as mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy\textsuperscript{17} and fourier transform infrared (FTIR) spectroscopy\textsuperscript{18}.

Over the past two decades, HDX coupled with specific enzymatic proteolysis and mass spectrometry has become a powerful technique to study higher order structure of peptides, proteins and proteins complexes. Mass spectrometry is considered to be ideally suited for HDX studies due to its high accuracy, sensitivity and degree of automation without limitations on protein size, unlike other analytical techniques.\textsuperscript{19} Vast and rapid advances in the HDX methodology and MS instrumentation have enabled its use in a wide range of biopharmaceutical applications, including the study of protein-ligand interactions\textsuperscript{20}, protein refolding\textsuperscript{21}, protein aggregation\textsuperscript{22}, conformational state alterations\textsuperscript{23}, etc. However, HDX studies have primarily been focused on soluble proteins and due to numerous technical challenges in analyzing membrane proteins, very few studies have been reported on GPCRs.

Following careful optimization of both digestion and MS analytical methods, Zhang, Xi, et al.\textsuperscript{24} demonstrated the methodology for HDX MS study of detergent-solubilized beta 2-adrenergic receptor ($\beta_2$AR) bound with inverse agonist, carazolol. It was observed that the highest degree of protection was seen toward the core of the seven transmembrane helices. The peptides representative of the intracellular loop 3 (ICL3),
previously known to be involved in G-protein interaction, demonstrated the most rapid exchange kinetics. It was also observed that the extracellular loop 2 displayed intermediate exchange rate (faster than transmembrane helices but slower than intrahelical loops), indicating that the ordered, short, helical loop may act as a lid domain over the ligand-binding site in different conformational states.

Following up on this study, West GM., et al.\textsuperscript{12} used differential HDX MS studies to observe ligand-specific alterations in the exchange rates of $\beta_2$AR peptides. They found significant differences in hydrogen deuterium exchange in the intracellular and extracellular regions across all receptor-ligand complexes. Both inverse agonists and antagonists produced pronounced stabilizing effects on ECL2 and ECL3 through direct interactions with these domains. Similarly, significant protection against HDX was detected in ICL2 region upon carazolol binding. Further, a significant increase in protection to exchange was detected both in ICL3 and flanking regions of the transmembrane helices (V and VI) in the presence of inverse agonist and antagonist. But, presence of full and partial agonist resulted in increased exchange suggesting destabilization of ICL3 region. Thus, this study revealed distinct conformational changes in $\beta_2$AR when bound to functionally selective ligands.

In 2013, Parker CH., et al.\textsuperscript{25} demonstrated the use of a nanodisc-HDX MS platform to map the dynamic rearrangements of structural domains, upon ligand binding, in membrane proteins. They reported the study of the structural configuration of gamma ($\gamma$)-glutamyl carboxylase (GGCX), an integral membrane protein, in a nanodisc
phospholipid environment using HDX MS. GGCX is known to catalyze the conversion of glutamic acid residues to γ-carboxy glutamic acid in vitamin K-dependent proteins and therefore a valuable therapeutic target for blood coagulation disorders. Upon binding of a high-affinity consensus propeptide (pCON), pronounced structural rearrangements were observed in both propeptide binding site and the catalytic glutamate binding sites indicating a direct or allosteric mechanism that connects the two domains.

Although several hurdles remain to be resolved, HDX MS remains one of the key analytical techniques for epitope mapping and comparability studies in protein therapeutics discovery. Now, with advances in MS instrumentation, data analytical tools and development of novel platforms such as the use of nanodiscs, proteolysis-based HDX MS experiments of integral membrane proteins and protein complexes is possible. HDX MS experiments using CB2-incorporated nanodiscs would provide significant insight into the conformational changes that occur upon ligand binding in cannabinoid receptor in its native environment.

4.3 Nanodisc:

Most integral membrane proteins (IMP) are fully active when incorporated in cell membrane. Over the years, a number of alternatives such as ionic and non-ionic detergents, liposomes, monolayers, sugar-based polymers, synthetic polymers, etc. have been used to provide a native membrane-like environment for IMP. In 2002, Sligar et al. developed a novel technology using amphipathic alpha-helical proteins, known as membrane scaffolding protein (MSP) to form discoidal phospholipid bilayers, of defined
size, and provide native membrane-like environment for IMP. The MSP protein is amphipathic protein derived from apolipoprotein A-1 (apoA-1) known to hold together 100-150 molecules of phospholipids to form nanodiscs. Unlike most other lipid membrane alternatives, nanodiscs are easily prepared by mixing MSP with detergent-solublized phospholipid mixture and gradual removal of detergent results in the self-assembly of nanodiscs. Further, owing to their small size, solubility and monodispersity, this model bilayer system has become an effective strategy for characterization studies of membrane proteins.

![Figure 4.1: Schematic representation of empty nanodisc and GPCR-nanodisc complex.](image)

Over the past decade, a diverse set of membrane proteins, such as cholera toxin receptor ganglioside GM1\(^\text{26}\), cytochrome P450s\(^\text{27}\), cyclooxygenase-2\(^\text{28}\), NADPH-dependent cytochrome P450 reductase\(^\text{29}\), bacteriorhodopsin\(^\text{13}\), G-protein coupled receptors\(^\text{30}\), transporters\(^\text{31}\) and bacterial chemoreceptors\(^\text{32}\), have been successfully studied
upon reconstitution into nanodiscs. These lipid bilayer-mimics are a controlled system and facilitates the precise and critical assessment of the local membrane environment. Using this advantage, the redox potential changes in cytochrome P450 enzymes, upon ligand binding, were characterized following their reconstitution into nanodiscs. This platform also provides a unique opportunity to study protein-protein interactions. The study of UDPG glycosyltransferase and acyltransferase interactions with membrane-anchored proteins was performed using the nanodisc system. A single-molecule FRET and EPR analysis of the multiple conformations of a single SNAREpin formed between two nanodiscs revealed a diverse set of pre-fusion conformational states. The nanodisc system has also enabled the study of stoichiometry in protein-protein interactions. Using this system, it was confirmed that activated rhodopsin forms a complex with transducin at a stoichiometry of 1:1.

Traditionally, the presence of membrane protein embedded in detergent micelles or liposomes has hampered their study using biophysical methods such as NMR and mass spectroscopy. Using the nanodisc technology, structural features of functionally active CYP3A4, CYP3A4 and CYP2B4 enzymes were characterized using magic-angle spinning solid-state NMR (MAS SSNMR), small-angle x-ray scattering (SAXS) and atomic force microscopy (AFM) respectively. Apart from IMP, the nanodisc system has also been to study membrane-associated proteins. The biochemical and structural characterization of monoacylglycerol lipase, a membrane-associated enzyme, interacting with nanodiscs was performed using hydrogen deuterium exchange mass spectroscopy.
Apart from rhodopsin very few GPCRs have been extensively studied following receptor incorporation into nanodiscs. Whorton, et al.\textsuperscript{38} were able to demonstrate using single-molecule fluorescence imaging and FRET analysis that monomeric beta 2-adrenergic receptors (\(\beta_2\)AR) were reconstituted into HDL particles and they efficiently activate G-proteins and exhibit GTP-sensitive allosteric ligand-binding effects. In a following study, these \(\beta_2\)AR incorporated HDL particles were used as a bait to pull-down cytosolic-interacting proteins, which were then identified using a mass spectrometry-based proteomic analysis.\textsuperscript{39} Yoshiura, et al.\textsuperscript{40} reported a NMR study of the chemokine peptide ligand (MIP-1\(\alpha\)) interaction with chemokine receptor 5 (CCR5) reconstituted into HDL particles. They were able to identify the two key residues in the peptide involved in receptor interaction, thereby confirming that the protective role of SNP in MIP-1\(\alpha\) on HIV infection, due to change in affinity for CCR5. Proverbio, et al.\textsuperscript{41} demonstrated the use of nanodisc platform in the study of endothelin A and endothelin B receptors. They performed both post-translational and co-translational reconstitution of cell-free expressed endothelin receptors into nanodiscs. Using radioligand binding and surface plasma resonance (SPR) experiments they ascertained the ligand binding function of these reconstituted receptors. Inagaki, et al.,\textsuperscript{42} demonstrated that functional activity of the neurotensin receptor 1 (NTS1), reconstituted into nanodiscs, in G-protein activation assay is affected by the composition of the lipids.

Through the study of a diverse set of membrane proteins embedded in nanodiscs, it is well established now that, upon proper reconstitution of proteins into nanodiscs, they gain the stability and functional activity provided by native membrane-like environments.
Owing to the physicochemical properties of nanodiscs, they are well suited for biochemical and biophysical studies and provide a unique opportunity to study membrane proteins in their native environment. Here, we developed a method for efficient reconstitution of human CB2 into nanodiscs and its purification, to provide a system for future structural studies.

4.4 Reagents and Materials

Standard laboratory chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Chemical (Pittsburgh, PA) if not otherwise specified. Coomassie G-250 stain, Laemmli electrophoresis sample buffer, PVDF membrane, molecular weight markers and 1D SDS-PAGE gels were from Bio-Rad (Hercules, CA). n-Dodecyl-β-d-maltoside (DDM) and Triton-X100 were purchased from Anatrace (Maumee, OH). AM-1336 was synthesized at the Center for Drug Discovery, Northeastern University (Boston, MA). 1-palmitoyl-2-oleoyl- phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, Alabama).

4.5 Methods:

a) Baculovirus/Insect Cell Expression of Flaghcb2his6:

Sf21 insect cells were transfected with bacmid-FlaghCB2his6 DNA according to the Bac-to-Bac baculovirus expression kit’s (Invitrogen) recommended protocol. Briefly, sf21 cells cultured in Sf-900™ II SFM media containing 2% fetal bovine serum (FBS), when in log phase (1.5-2.5 x 10^6 cells/ml) with greater than 95% viability, were
transfected with different amounts of recombinant bacmid-FlaghCB2his6 DNA using Cellfectin® II reagent. The supernatant (viral stock, P1) were collected and stored at 4 °C, protected from light. The cell pellets were used to test for the expression of FlaghCB2his6 receptor. The cells were resuspended in lysis buffer (300 mM NaCl, 50 mM sodium phosphate, 10% Glycerol, 1% dodecyl maltoside, pH 8.0) and lysed on ice by three, 33 s sonication cycles, each cycle consisting of 1 s bursts at 50 W separated by a 5 s interval. The lysate was centrifuged at 16000x g for 15 min and supernatant and pellets were analyzed separately using SDS-PAGE analysis or western blot analysis as detailed below.

The P1 viral stock was filtered through a 0.22 μm Millex-GS syringe filter unit (Millipore, Billerica, MA) and used for further infection steps. Different quantities of filtered P1 viral stock was used for infecting sf21 cells in log phase and incubated at 27 °C for 5 days (until ~80% cell lysis).

b) SDS-PAGE and Western Blot Analysis

Samples were preincubated at room temperature for 10 min in Laemmli sample buffer containing 5% β-mercaptoethanol. Protein samples were resolved on AnykD™ Mini-PROTEAN® TGX™ precast polyacrylamide gels. Proteins were transferred to PVDF membranes, and the membranes were prepared for immunodetection following the procedures outlined in the QIAexpress Detection and Assay Handbook (QIAGEN, Valencia, CA). Membranes were incubated with a 1:10 000 dilution of Penta-His antibody horseradish peroxidase (HRP) conjugate (Sigma) or a 1:10000 dilution of
monoclonal mouse ANTI-FLAG® M2 antibody (Sigma) followed by incubation in a 1:10000 dilution of goat anti-mouse IgG-HRP (Sigma). Protein bands were visualized using the ECL Western blotting analysis system (GE Healthcare, Piscataway, NJ). The image was captured using the Fluorchem SP™ (Alpha Innotech Santa Clara, California) system.

c) Anti-FLAG Immunoaffinity Purification of Flaghcb2his6

Cells pellets were suspended in lysis buffer (50 mm sodium phosphate, 300 mm NaCl, 10% glycerol, 1% dodecyl-β-d-maltoside, pH 7.4) and lysed on ice by three, 33 s sonication cycles, each cycle consisting of 1 s bursts at 50 W separated by a 5 s interval. The lysate was centrifuged at 16000x g for 15 min (to remove cell debris) and the supernatant was collected. An equal volume of purification buffer (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 7.4) was added to the supernatant. This mixture was then added to pre-equilibrated (in 50% lysis buffer, 50% purification buffer) ANTI-FLAG® M2 affinity gel and incubated for 2 hours at 4 °C on a rotating wheel. The resin was washed twice with 5 column volumes of wash buffer (purification buffer with 0.2% dodecyl-β-d-maltoside) and FlaghCB2his6 was eluted wash buffer containing 150 µg/ml of FLAG® peptide. SDS-PAGE and western blotting analysis were performed on the aliquot of samples taken during purification according to the procedure previously described.
d) Saturation Binding Assay with FlaghCB2his6:

Saturation binding assays were performed in a 96-well format. About 25 µg of protein was added to each assay well. The [3H]CP-55,940 was diluted in TME-BSA buffer to yield ligand concentrations ranging from 0.625 to 20 nM. Nonspecific binding was assayed in the presence of 5 µM unlabeled CP-55,940. The assay was performed at 30 °C for 1 h with gentle agitation. The resultant material was transferred to Unifilter GF/B filter plates and unbound ligand was removed using a Packard Filtermate-196 Cell Harvester. Filter plates were washed four times with ice-cold wash buffer (50 mM Tris-base, 5 mM MgCl2 containing 0.5% BSA, pH 7.4). 40 µl of MicroscintTM 20 scintillation fluid was added to each well and the plates were counted using TopCount NXT™ Microplate Scintillation and Luminescence Counter. The data obtained was processed using Microsoft Excel and Prism 5. All concentration points were performed in triplicate and data points used for plotting are base-line corrected. Bmax and Kd values were calculated by nonlinear regression using Graphpad Prism version 5.03 (one site-binding analysis equation \( Y = \frac{B_{\text{max}} \times X}{K_d + X} \)) on a Windows platform.

For the saturation binding assays with purified FlaghCB2his6, 96-well GF/B filtration plates were pre-treated with 0.5% polyethylenimine (PEI) for 3 hours at 4 °C and washed twice with 200 ml of binding buffer (BB - 25 mM Tris base, 5 mM MgCl2, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% BSA, pH 7.4).
f) Cloning and expression of MSP1D1:

The membrane scaffolding protein (MSP1D1) was expressed in BL21 *E. coli* cells as previously described. Briefly, the pET28aMSP1D1his6 plasmid obtained from Dr. Mahmoud Nasr (Harvard Medical School, Boston, MA) was transformed into BL21(DE3) cells using manufacturers recommended procedure and colonies selected on agar plate with kanamycin (25 µg/ml). A single colony was inoculated into 5 ml LB media containing kanamycin (25 µg/ml) and grown at 37 °C overnight, with shaking at 250 rpm. This overnight culture was then be added to 1L of LB media containing kanamycin and allowed to grow to an O.D. of ~3, at 37 °C and shaking at 250 rpm. The expression of MSP1D1 was induced using 1 mM IPTG and incubation of cells for 4 h at 33 °C with shaking at 250 rpm.

g) Purification of MSP1D1:

MSP1D1 was purified using Immobilized metal affinity chromatography (IMAC) using with manufacturers recommended protocol. Briefly, the collected *BL21* cells expressing MSP1D1 were resuspended using 20 mM NaH₂PO₄ (monobasic) with 1% Triton X-100 and lysed by repeated (5-7 times with 3 min pauses) probe sonication (55 sec duration, 36% amplitude, 1 sec on/5 sec off pulse). The cell lysis mixture is bound to pre-equilibrated Cobalt-based Talon metal affinity resin and washed twice with 10 column volumes of wash buffer (40 mM Tris/HCl, 300 mM NaCl and 50 mM sodium cholate, pH 8.0) and once with wash buffer containing 25 mM imidazole. MSP1D1 was then eluted using wash buffer containing 300 mM imidazole.
The purified MSP1D1 was analyzed using SDS-PAGE analysis and buffer exchanged into 20 mM Tris/HCl, 100 mM NaCl, 0.5 mM EDTA, pH 8.0 by dialysis using cellulose membrane with molecular weight cutoff at 6 kDa. The buffer exchanged samples would be concentrated using Amicon Ultra centrifugal filters (Millipore, Billerica, MA) with molecular weight cut-off at 10 kDa and stored at -80 °C until further use. The concentration of purified MSP1D1 would be determined using Quick Start™ Bradford Protein Assay kit (Biorad, Hercules, CA) according to the manufacturer’s recommended protocol and absorbance values measured at 595 nm using EnVision™ Multilabel Plate Reader (PE).

**h) Reconstitution of FlaghCB2his6 into POPC/POPG nanodisc:**

Purified recombinant FlaghCB2his6 expressed using baculovirus expression was produced as a stock of approximately 2 µM solubilized in 0.2% DDM. FlaghCB2his6 was reconstituted with MSP1D1 and lipid mixture (3:2 ratio of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG)) at ratios designed to result in an excess of target-free Nanodiscs. POPC was solubilized as a 25 mM solution in nanodisc buffer (20 mM sodium phosphate, 100 mM NaCl, 0.5 mM EDTA, pH 7.4) containing 50 mM sodium cholate. Purified MSP1D1, typically in concentrations of 0.5–1.0 mM, prepared as previously described, in nanodisc buffer, was added to solubilized FlaghCB2his6 and lipid mixture in a ratio of 25:1:1700. DDM was added from a 10% stock to make a final detergent concentration of 3 times molar concentration of lipid mixture to a final volume of 0.5-1.0 ml depending on the concentration of the MSP1D1 stock. As previously reported, final concentration of lipid
mixture was typically greater than 5 mM for efficient formation of Nanodisc complexes. The reconstitution mixtures were incubated on ice for 2 h followed by addition of 0.4 g of Bio-Beads® SM-2 wetted according to manufacturer recommended protocol, to initiate the self-assembly process by removal of detergents. Following incubation with Bio-Beads for 4 hours with gentle agitation at 4°C, the Bio-Beads were removed by centrifugation at 16,000× g for 30 s. The reconstituted nanodisc mixture was stored at 4°C until further analysis.

i) Size-exclusion analysis of nanodisc mixture:

Nanodisc were separated and isolated using size-exclusion chromatography as previously detailed. Briefly, high molecular weight globular protein standards (Biorad) were separated on a Superdex™ 200 10/30 column using Fast Protein Liquid Chromatography (FPLC) to calibrate the column elution times. The column is pre-equilibrated with 5 column volumes of nanodisc buffer (20 mM sodium phosphate, 100 mM NaCl, 0.5 mM EDTA, pH 7.4) at a flow-rate of 0.5 ml/min at 4°C. The FlaghCB2his6-nanodisc reconstitution mixture was filtered through 0.4 µm cellulose filter (Millipore) and injected onto the column and 1 ml fractions were collected at 4°C. The fractions corresponding to stokes radius of ~10 nm were pooled and stored at -80°C for further analysis.

j) Immunoaffinity purification of FlaghCB2his6-nanodisc complex:

FlaghCB2his6-incorporated nanodiscs were purified from empty nanodiscs using the N-terminal Flag affinity tag on human CB2 in batch mode. Briefly, the size-purified
nanodisc mixture mixture was added to pre-equilibrated (in nanodisc buffer) ANTI-FLAG® M2 affinity gel and incubated for 2 hours at 4°C on a rotating wheel and centrifuged at 500×g for 5 min. The resin was washed twice with 5 column volumes of nanodisc buffer and FlaghCB2his6-nanodisc complex was eluted using nanodisc buffer containing 150 µg/ml of FLAG® peptide with repeated centrifugation at 500×g for 5 min. SDS-PAGE and western blotting analysis were performed on the aliquot of samples taken during purification according to the procedure previously described.

k) cAMP assay:

HEK293 cells stably expressing human CB2 receptor were used for the studies. The cAMP assay was carried out using PerkinElmer’s Lance ultra cAMP kit according to manufacturer’s recommended procedure. Briefly, the assays were carried out in 384-well plates using ~1500 cells/well. The cells were harvested with Versene, washed twice with HBSS, and resuspended in the stimulation buffer. Various concentrations of the test compound in forskolin (2 µM final concentration) containing stimulation buffer were added to the plate followed by the cell suspension. The cells were stimulated for 30 min at room temperature. Then Eu-cAMP tracer working solution (5 µl) and Ulight-anti-cAMP working solution (5 µl) were added to the plate and incubated at room temperature for 60 minutes. The data were collected on a Perkin-Elmer Envision. The EC50 values were determined by non-linear regression analysis using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).
4.6. Results and discussion:

In order to investigate the structural dynamics of human CB2 receptors in the native membrane-like environment using hydrogen exchange mass spectrometry (HDX-MS), we developed a method for the reconstitution of purified FlaghCB2his6 receptors into nanodiscs. Consistent with previously published work\textsuperscript{44}, a full-length human CB2 receptor construct encoding a N-terminal FLAG-tag and a C-terminal 6His-tag (FlaghCB2his6) was chosen for recombinant receptor expression. The recombinant human CB2 receptor was overexpressed in \textit{Spodoptera frugiperda} (Sf21) cells using the Bac-to-Bac baculovirus expression system (Invitrogen) using standard protocol. Based on prior empirical detergent screen,\textsuperscript{44} FlaghCB2his6 was solubilized in buffer containing mild, non-ionic, MS-compatible detergent dodecyl maltoside (DDM), previously shown to be suitable for efficient extraction of functional GPCRs. FlaghCB2his6 was purified using immunoaffinity chromatography on ANTI-FLAG M2 affinity gel and analyzed using coomassie staining of SDS-PAGE gel (Figure 4.2A). In accord with prior results, the eluate was judged to be >80% pure.

The ligand binding competency of the purified FlaghCB2his6 receptor was ascertained using a modified radioligand binding experiment where the filter plates were pre-treated with polyethilenimine to help retain solubilized receptor.\textsuperscript{45} The assay showed the presence of 8.1 pmol of FlaghCB2his6 per mg of total eluate (Figure 4.2B). This confirmed that the detergent-solubilized, purified, FlaghCB2his6 receptor was still functionally active and can be used for reconstitution into nanodiscs.
**Figure 4.2: Purification and characterization of FlaghCB2his6.** A) SDS-PAGE gel analysis of Flag-tag based immunoaffinity purified FlaghCB2his6 (Lane 2). B) Saturation binding of [³H]CP55940 ligand to purified FlaghCB2his6 in DDM detergent micelles. The experimental conditions for radioligand binding assay are detailed in the methods section.

Nanodiscs are made of phospholipids that are held together by membrane scaffolding protein (MSP). The length of the membrane scaffolding protein determines the diameter of the nanodisc. MSP1D1 contains 10 helices from Apo-lipoprotein A-1 resulting in ~9.7 nm nanodiscs, which are considered to be adequate for incorporation of G-protein coupled receptors. The FlaghCB2his6-nanodiscs would be prepared using previously described method with minor modifications (Figure 4.3). 

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Figure 4.3: Workflow of FlaghCB2his6-nanodisc complex.
Figure 4.4: Size exclusion chromatographic analysis of FlaghCB2his6-Nanodisc mixture. The plot indicates the absorbance ($A_{280}$) values of elution from size exclusion chromatography with the fractions (0.5 ml each) labeled in red from 1-28. The peaks identified are labeled as Peak 1-4 with their respective retention volume. The Stokes diameter (S.D) is indicated at the bottom based on gel filtration standards from Bio-Rad: Thyroglobulin (S.D = 19.2 nm), Bovine gamma globulin (S.D = 10.2 nm) and Chicken ovalbumin (S.D = 5.6 nm). The inset shows the coomassie-stained SDS-PAGE analysis of fractions from size exclusion chromatography.
FlaghCB2his6-nanodiscs were prepared using a 1:25:1700(3:2) ratio of FlaghCB2his6/MSP1D1/POPC-POPG in the presence of excess dodecyl maltoside. A large excess of scaffold protein and lipids compared with CB2 receptor was added to minimize nonspecific receptor loss during experimental manipulations as well as to favor incorporation of CB2 receptor as a monomer. The neutrally charged POPC and negatively charged POPG were chosen as they both share transition melting temperatures of ~2 to -5°C which is compatible with GPCRs. The nanodiscs were assembled by rapid removal of excess detergent by incubating the mixture with excess of SM2 Biobeads. Following the self-assembly of nanodiscs and the incorporation of FlaghCB2his6 receptor the nanodisc mixture was evaluated by size exclusion chromatography (SEC) (Figure 4.3). A clear peak (12.78ml retention volume) was identified around stokes diameter of ~10 nm indicating assembly of nanodiscs. Additional peaks were identified at 1.47, 7.13 and 19.87 ml and collected for SDS-PAGE analysis (Figure 4.4). The coomassie-stained SDS-PAGE analysis confirmed the incorporation of human CB2 into nanodiscs in peak fraction 2. The FlaghCB2his6 incorporated nanodiscs were purified from empty nanodiscs using the Flag-tag on human CB2 receptor. The immunoaffinity chromatography was performed in batch mode as detailed in the methods section. The fractions from each step in the purification process were analyzed by western blot and SDS-PAGE analysis (Figure 4.5). The eluate from immunoaffinity purification revealed two distinct bands corresponding to MSP1D1 and human CB2 at ~25 kDa and ~40 kDa, respectively in both western blot and SDS-PAGE analysis. Although, MSP1D1 can only incorporate GPCR monomer, higher order CB2 oligomers are observed. This might be
explained due to aggregation during SDS-PAGE analysis when the CB2-nanodisc complex is disassembled.

Figure 4.5: Anti-His western blot and coomassie-stained SDS-PAGE analysis of FlaghCB2his6-nanodisc complex purification. **Lane 1 and 7:** Protein ladder with molecular weights labeled on the left of lane 7; **Lane 2:** Flow through from immunoaffinity purification; **Lane 3 and 4:** Immunoaffinity purification washes 1 and 2, respectively; **Lane 5 and 8:** FlaghCB2his6-nanodisc complex eluate 1 from Flag-tag based immunoaffinity purification; **Lane 6:** Flag M2 affinity resin after final elution. **Lane 9:** Purified MSP1D1.

The incorporation of membrane proteins like GPCRs into nanoscale phospholipid bilayer-mimics have been shown to stabilize the protein and enables the use of labeling methods such as hydrogen-deuterium exchange in the protein’s native membrane-like environment. The GPCR-incorporated nanodiscs are homogenous population of highly
stable nanostructures with high aqueous solubility. In addition, the use of a nanodisc model system also prevents a complicated structural analysis, due to receptor oligomerization and aggregation, as it has been shown previously that nanodiscs prepared using MSP1D1 incorporate single molecules of integral membrane proteins.\textsuperscript{47}

4.7 Future directions:

Previous studies with GPCRs have shown that high hydrophobicity of the transmembrane domain, receptor aggregation and presence of detergents result in low digestion efficiency and peptide detection, under conditions generally optimized for HDX MS studies. HDX MS analysis of GPCRs is further complicated by the presence of a number of post-translational modifications such as glycosylation, phosphorylation, ubiquitination, palmitoylation and cysteine disulfide bonds.\textsuperscript{24} Using an optimized protocol for enhanced sequence coverage of membrane proteins in their native environment, under HDX compatible conditions, might enable better sequence coverage.\textsuperscript{48} Briefly, the purified FlaghCB2his6-nanodisc sample, labeled with deuteriated water (D$_2$O), would be quenched at times ranging from 10 s to 4 h after the introduction of D$_2$O by the addition of formic acid and maintained at pH 2.5 on ice. The nanodiscs would be immediately disassembled with the addition of excess dodecyl maltoside detergent and enzymatic digestion performed using porcine pepsin-immobilized resin. The excess phospholipids would be removed by incubation of peptic-digest samples with zirconium oxide-coated silica beads, filtered and analyzed using high-performance LC-MS/MS analysis. Following triplicate injections, all relative deuterium uptake curves for
each peptide fragment would be identified in all replicates and visualized with the help of previously generated homology models of the human CB2 receptor.

![Figure 4.6: LANCE cAMP accumulation assay with AM1336, AM841 and 2-AG in HEK293 cells overexpressing wild-type human CB2 receptor. The inset indicates the EC50 values (with percent change in Forskolin (FSK) stimulated cAMP) of each ligand.](image)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EC50 (nM)</th>
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<tbody>
<tr>
<td>AM1336</td>
<td>15.2 (-87%)</td>
</tr>
<tr>
<td>AM841</td>
<td>2.22 (77%)</td>
</tr>
<tr>
<td>2-AG</td>
<td>8.38 (55%)</td>
</tr>
</tbody>
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AM841, a classical cannabinoid (THC-analogue) ligand, and AM1336, a biarylpypyrazole (SR144528-analogue) ligand, both bind covalently to human CB2 with a binding affinity of 1.51 and 0.54 nM respectively. From previous LAPS studies with novel Cannabinergic ligands using bottom-up proteomic mass spectrometric analysis, we have successfully identified that AM841 covalently interacts with C6.47(257) and AM1336 interacts covalently with C7.38(284) residues. Moreover, FRET-based cell signaling studies using both ligands with human CB2 receptor overexpressed in mammalian expression system have revealed that AM841 and AM1336 is a potent agonist (EC50 = 2.2 nM) and inverse agonist (EC50 = 15.2 nM) respectively (Figure 4.6). Hydrogen deuterium exchange mass spectrometry (HDX-MS) following covalent binding with AM1336 and AM841, separately, could enable us to identify critical
conformational changes that occur upon binding of ligands with different functional profiles. In the future, development of covalent ligands that exhibit signaling bias towards one signaling pathway could help us understand the structural features involved in functional selectivity.

4.8 References:


Chapter 5

Cell-free and bacterial expression, purification and characterization of CB1 for mass spectrometric analysis

5.1 Introduction:

The human CB1 receptor is a integral membrane protein expressed that belongs to the Class A subtype of GPCR superfamily.\(^1\) It is found predominantly in the central nervous system (CNS) and to a smaller extent in the periphery.\(^2\) CB1 receptor play an important role in the central and peripheral regulation of food intake, fat accumulation, lipid and glucose metabolism, through several signaling pathways.\(^3\) Experimental and clinical evidence suggest that in the CNS, endocannabinoid signaling through CB1 receptor helps control human motor, cognitive, emotional, and sensory functions and modulates pain perception, hormonal activity, thermoregulation, and cardiovascular functions.\(^4\) In peripheral organs such as liver and pancreas, CB1 receptor regulate energy homeostasis and lipid metabolism and deposition.\(^5\) Thus, CB1 receptor is involved in the modulation of a diverse set of physiological pathways both in the CNS and in peripheral systems. The development of specific ligands modulating these effects could have therapeutic benefits in a variety of pathological conditions including obesity, cardio-metabolic diseases, drug dependence, pain, and neuro-degeneration.\(^6\) Due to the lack of high-resolution structures of CB1 receptor, most of our knowledge is based of mutational analysis and \textit{in-silico} modeling. In order to perform direct structural studies, large quantities of stable, pure and functionally active human CB1 receptor is required. Here
we report a novel method of expression, purification and characterization of CB1 receptor for future structural studies.

**5.2 Bacterial expression of GPCR:**

Owing to the simplicity, scalability, and homogeneity of the bacterial recombinant protein expression, it remains the most popular choice for studies in structural biology. However, due to the lack of machinery for post-translational modifications, poor incorporation of membrane proteins into the bacterial inner wall and significant differences in membrane lipid composition, bacterial expression is not well suited for GPCR expression. Moreover, Lundstrom et al. found that when 100 different class A GPCRs were expressed in *Escherichia coli*, only 18 receptors were expressed in significantly high quantities. In addition, most of the expressed receptors were found to be inclusion bodies and were functionally inactive.

In spite of these difficulties a number of GPCRs have been successfully expressed using bacterial expression system, such as Adenosine 2A receptor (A2A)\textsuperscript{7}, Cannabinoid 2 receptor (CB2)\textsuperscript{8}, Neurotensin 1 receptor (NT1)\textsuperscript{9}, Vasopressin 2 receptor (V2)\textsuperscript{10}, thyroid stimulating hormone receptor (TSH)\textsuperscript{11} and Serotonin 4A receptor (5-HT4A)\textsuperscript{12}. However, unmodified receptors usually exhibit poor stability and rapidly become inactive aggregates. Significant protein modifications such as addition of optimized fusion tags, low expression temperatures and prolonged induction times are required for efficient expression of functionally active receptors. Maltose binding protein (MBP) has been
found to function as a general molecular chaperone preventing self-association of their fusion partners. The addition of 43 kDa, N-terminal MBP-fusion tag proved useful for the large scale expression of functional Cannabinoid 2 receptor. Addition of a C-terminal thioredoxin (TrxA) was found to enable successful expression of soluble and functional rat neurotensin 1 receptor.

Alternatively, few GPCRs were expressed as inactive inclusion bodies and partially refolded into functional proteins. Leukotriene receptor (BLT1) was expressed as inclusion bodies using a short T7 tag and 30% of purified receptor was refolded as a functional protein in a mixture of lauryldimethylamine oxide (LDOA) and lipids (e.g. asolectin). Olfactory receptor (OR5) was expressed with a GST-fusion tag and almost 80% of folded receptor in lipid vesicles bound its lilial ligand. More recently, SDS-solubilized parathyroid hormone receptor 1 (PTH-1) and CB1 receptor were refolded using a mixture of the non-ionic detergents DDM and Cymal 6, resulting in almost 30% of functional CB1 receptor.

CB1 was expressed with N-terminal fusion tag of maltose binding protein (MBP) in significant quantities but was found to inactive in radioligand binding assays. Similarly, when CB1 was expressed with N-terminal GFP fusion tag, it was found to be active in ligand binding assay but unlike CB2, CB1-GFP expression did not increase upon co-expression with AAA+ protease FtSH. Our previous attempts to express functional receptors using optimized expression conditions found the expressed protein to
be active (unpublished results). Upon storage in -80°C, the protein rapidly lost its ligand binding activity due to protein instability and aggregation. For the efficient expression of functional CB1 receptor, significant protein engineering for receptor stabilization and exhaustive studies for optimization of protein expression conditions might be required.

5.3 Cell-free expression of GPCRs:

Over the past decade, cell-free expression has become a powerful tool to express various proteins with high efficiency. Traditional cell-free systems are composed of a crude cell lysate mixture containing the transcriptional and translational machinery, tRNAs, enzymes and to start expression required a DNA/mRNA template, amino acids and energy supply.\textsuperscript{15} Cell-free expression systems can be prepared from mammalian (rabbit reticulate lysate, porcine and human), bacterial (\textit{E. coli}), insect, plant (wheat germ extract) or yeast cells. The \textit{E. coli} cell-free systems are considered to be the most robust in protein expression, yielding up to 1 mg of target protein per ml of reaction mixture. Although the mammalian \textit{in vitro} expression systems are more expensive and much less efficient (yields usually up to 20 mg per ml of reaction mixture), their ability to express post-translationally modified, properly-folded proteins in the presence of selected molecules, added in the cell lysate, make them an attractive expression system to study the downstream signaling pathways.\textsuperscript{16}

The key advantage of the cell-free expression over \textit{in vivo} expression is the access to the in-vitro reaction during expression thus enabling easy labeling strategies for NMR
studies. It also eliminates a number of other complications of \textit{in vivo} expression systems such as large downtime, protein toxicity towards cells, and cumbersome membrane protein extraction steps. Furthermore, \textit{in vitro} expression does not require complex transfection and virus-amplification steps as in the case of mammalian cell culture and baculovirus/insect cell expression respectively.\textsuperscript{15} Recent development of tools, such as detergent mixtures, nano-lipid bilayers, amphipols, proteomicelles, peptide surfactants and liposomes, has enabled the expression of GPCRs and other membrane proteins. They provide the expressed receptors a membrane-like environment to fold properly and decrease formation of inclusion bodies and precipitation.\textsuperscript{17}

Klammt et al.\textsuperscript{18} reported the successful use of an \textit{E.coli}-based cell-free expression system to express several GPCRs: the human and porcine vasopressin type 2 receptors (hV2R, pV2R), the human endothelin B receptor (ETB), the human neuropeptide Y4 receptor (NPY), the human melatonin 1B receptor (MTN) and the rat corticotropin releasing factor (CRF). They were expressed in the presence of Brij78 detergent and reconstituted into proteoliposomes. They confirmed the ligand-binding affinity of ETB by using surface plasma resonance technique. Ishihara et al.\textsuperscript{19} showed that the yield of a functional GPCR (beta-2-adrenergic receptor) could be considerably increased by the introduction of a thioredoxin-fusion tag. Sansuk et al.\textsuperscript{20} expressed histamine (H1) receptor with a similar thioredoxin-fusion construct using \textit{E.coli}-based cell-free system and reconstituted it into asolectin liposomes. They were also able to perform a MALDI-TOF and LC/MS/MS analysis of the reconstituted protein.
Recently, Proverbio, et al.\textsuperscript{21} demonstrated the use of nanodisc platform for reconstitution of endothelin A and endothelin B receptors expressed in \textit{E.coli}-based cell-free system. They performed both post-translational and co-translational reconstitution of cell-free expressed endothelin receptors into nanodiscs and using both radioligand binding and surface plasma resonance (SPR) experiments ascertained the ligand-binding competency of reconstituted endothelin receptors. Katzen et al.\textsuperscript{22} also demonstrated that the presence of nanolipid bilayers in cell-free expression reactions assisted in the formation of soluble membrane proteins including GPCRs such as beta-2 adrenergic receptor, dopamine D1 receptor, histamine H2 receptor, melanocortin 5 receptor, and rhodopsin. But, none of these \textit{in vitro} expressed wild-type receptors show any ligand binding activity. However, cell-free expression of a re-engineered beta-adrenergic receptor with the incorporation of a mutant T4Lysozyme into intracellular loop 3 yielded competent receptor. Similarly, Wang and co workers reported the use of cell-free expression system for efficient expression of properly folded, T4Lysozyme modified human trace amine-associated receptor 5 (hTAAR-5).\textsuperscript{23}

In some situations, the current techniques of \textit{in vivo} expression of GPCR have been unable to produce pure, functional receptors in large enough quantities for structural studies such as X-ray crystallography, NMR, etc. Cell-free expression systems have been used to overcome some of these problems and could prove to be the right tool in our quest to understand these complex proteins in their native state.
5.4 Recombinant CB1 expression:

We have been unable to efficiently express and purify functional human CB1 receptor from recombinant expression systems. Purification of CB1 receptor from rat brain tissues or receptor overexpressed in cell lines is very inefficient and therefore does not provide sufficient amount of protein for structural studies, a key necessity for target-based drug design. Numerous homology models based on the known GPCR structures of rhodopsin and beta-adrenergic receptors have been published and could be a useful tool in drug design, but their use remains equivocal.\textsuperscript{24} This is partly due to the fact that CB1 receptor has very a low sequence homology with other well-characterized class-A GPCRs that are used for developing homology models. There is only a sequence identity of 23\%, 26\%, and 27\% between the trans-membrane helices of human CB1 receptor and the bovine rhodopsin, the human beta-2 adrenergic receptor, and the human A2A adenosine receptor, respectively.\textsuperscript{25} Therefore, it is imperative to develop a new expression system to produce significant quantities of purified native human CB1 receptors for studying key structural features of these complex proteins and finally using this knowledge for rational drug design and development.

5.5 Materials and reagents:

Standard laboratory chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Chemical (Pittsburgh, PA) if not otherwise specified. Coomassie G-250 stain, Laemmli electrophoresis sample buffer, PVDF membrane, molecular weight markers and 1D SDS-PAGE gels were from Bio-Rad (Hercules, CA). Trypsin Gold, MS
grade, was purchased from Promega (Madison, WI). MembraneMax™ Protein Expression Kits were purchased from Invitrogen (Carlsbad, CA). n-Dodecyl-β-d-maltoside (DDM) and 5-cyclohexyl-1-pentyl-β-d-maltoside (CYMAL5) were purchased from Anatrace (Maumee, OH). [3H]CP-55940 was provided by the National Institute on Drug Abuse, National Institutes of Health (Bethesda, MD). 96-well assays plates and GF-B filter plates for radioactive binding assays were purchased from Perkin Elmer (Waltham, MA).

5.6 Methods:

DNA preparation for in vitro expression:

a. Preparation of pET15kzDhCB1his6 and pET26kzsDhCB1his6 plasmid DNA:

Two pET-based plasmids (pET15kzDhCB1his6 and pET26kzsDhCB1his6) containing a truncated hCB1 gene were obtained from Dr. Nikolai Zvonok. To obtain larger quantities of plasmid DNA, pET15kzDhCB1his6 and pET26kzsDhCB1his6 were transformed into XL1-Blue cells (Agilent Technologies, Santa Clara, CA) and colonies were selected on LB agar plates using antibiotic ampicillin or kanamycin respectively. For each construct, a single colony was inoculated in one liter of LB media containing appropriate antibiotic and after 18 h incubation plasmid DNA was purified using the GeneJETTM Plasmid Midiprep Kit (Fermentas, Maryland), according to the manufacturer’s recommended method.
b. Construction of plasmid DNA for in vitro expression of ΔhCB1T4L_his6:

i. Preparation of T4 lysozyme DNA by PCR:

    The T4 lysozyme DNA was amplified from the pFastbacCB2T4L template using the following primers designed for overlap extension PCR cloning – Forward (FwCB1T4L):

    **GTATATTCTCTGGAGGCTCACAGCCACAAATAT-ATTTGAAATGTTCAG** and Reverse (RvCB1T4L):

    **AACCTAATGTCCATGCGGGCTTTGGTCAATACCGGTCCCAAGT-GCC** (in bold primer sequences complemented to CB1 gene; in italic primer sequences complemented to T4 lysozyme DNA). The PCR reaction mixtures were prepared as follows: 1 µl pFastbacCB2T4L (10 ng/µl), 0.5 µl 10 µM Forward primer (FwT4LCB1long), 0.5 µl 10 µM Reverse primer (RvT4LCb1long), 1 µl (10 mM total concentration) dNTPs, 5.0 µl 10x Advantage2 buffer, 41.5 µl of sterile distilled water to give a total volume of 50 µl per reaction and 0.5 µl Advantage2 polymerase (Clontech, Mountain View, CA). Amplification cycles were then carried out using a MyCycler™ Thermal cycler (Biorad, Hercules, CA) as follows: A single denaturation step of 94 °C for 2 minutes was followed by 25 cycles of 94 °C for 10 seconds, 50 °C for 33 seconds and 72 °C for 1 minute 33 seconds and completed with a final extension step of 72 °C for 10 minutes. The quality and quantity of PCR products, before and after purification using the GeneJET™ PCR Purification (Mini) Kit according to the manufacturer’s protocol, were evaluated by electrophoresis in 0.8% agarose gel.
ii. Construction of pET26kzsΔhCB1T4Lhis6 by extension of T4 lysozyme DNA:

The overlap extension reaction mixtures were prepared as follows – 100 ng of pET26kzsΔhCB1his6, 1.9 µl of 10x Advantage2 buffer, 0.67 µl (10 mM total concentration) dNTPs, 150 ng of amplified T4L lysozyme DNA, 8.6 µl of sterile distilled water to give a total volume of 20µl per reaction and 0.43 µl Advantage2 polymerase. The overlap extension cycles were carried out in a MyCyclerTM Thermal cycler using the following PCR protocol - A single denaturation step of 95 °C for 2 minutes was followed by 20 cycles of 94 °C for 10 seconds, 50 °C for 33 seconds and 68 °C for 7 minutes and completed with a final extension step of 68 °C for 10 minutes. 10 µl of the DpnI digestion mixture (1µl of 10x Advantage2 buffer, 8.7 µl of sterile distilled water and 0.3 µl of DpnI (10 U/µl) (Agilent Technologies) was added to the overlap extension product and incubated for 2.5 hours at 37 °C. The size and concentration of the pET26kzsΔhCB1T4Lhis6 PCR were evaluated by electrophoresis in 0.8% agarose gel.

iii. pET26kzsΔhCB1T4Lhis6 plasmids preparation, PCR amplification and DNA sequencing:

The DNA of pET26kzsΔhCB1T4Lhis6 PCR obtained from overlap extension using the above mentioned procedure was transformed into XL1-Blue competent cells (Agilent Technologies) using the manufacture’s recommended protocol and were plated on LB agar plates containing antibiotic kanamycin and incubated overnight at 37 °C. Next morning, 6 colonies from the plate were inoculated in separate 10 ml culture tubes containing 5 ml LB media with 25 µg/ml of Kanamycin. After overnight incubated at 33
°C and shaking at 230 rpm, 400 µl aliquot of each of the samples were taken for glycerol stock preparation. 25 µl of Chloramphenicol in ethanol (35 mg/ml) was added to each of the tubes and incubated for 6 hours at 37 °C and shaking at 230 rpm. The plasmid DNA of all 6 colonies were purified using the GeneJET™ Plasmid Miniprep Kit, according to the manufacturer’s recommended method and their sizes and concentration were evaluated by electrophoresis in 0.8% agarose gel. The ΔhCB1_{T4L, his6} DNA fragments for all purified plasmids were amplified using the following PCR protocol – 0.2 µl plasmid DNA sample (60-75 ng/µl), 0.4 µl 10 µM Forward T7 primer CGCGAAATTACGACTCACTATAG, 0.4 µl 10 µM Reverse primer GGCTTGTTAGCAGCGGATCTC, 0.4 µl (10 mM total concentration) dNTPs, 2.2 µl 10x Advantage2 buffer, 16.4 µl of sterile distilled water to give a total volume of 20 µl per reaction and 0.2 µl Advantage2 polymerase. Amplification cycles were then carried out using a MyCycler™ Thermal cycler as follows: A single denaturation step of 94 °C for 2 minutes was followed by 28 cycles of 94 °C for 10 seconds, 55 °C for 33 seconds and 72 °C for 1 minute and completed with a final extension step of 72 °C for 10 minutes. The quality and quantity of PCR products, before and after purification using the GeneJET™ PCR Purification (Mini) Kit according to the manufacturer’s protocol, were evaluated by electrophoresis in 0.8% agarose gel. The purified kzsΔhCB1_{T4L, his6} PCR products were sequenced to confirm the correct insertion of T4 lysozyme and the absence of unwanted mutations by SeqWright DNA technology services (Houston, Texas).
iv. **Large-scale purification of pET26kzsΔhCB1T4L_his6 plasmid DNA:**

To obtain larger quantities of plasmid DNA, glycerol stock of pET26kzsΔhCB1T4L_his6 (2) in XL1-Blue cells was applied to LB agar plates containing antibiotic kanamycin (25 µg/ml). 1-5 single colonies were picked and inoculated in separate culture tubes with 4 ml of LB media containing kanamycin (25 µg/ml). After 6-8 hours incubation at 33 °C and shaking at 230 rpm, the cells were used to inoculate 1 L of LB media with antibiotic kanamycin (25 µg/ml). After overnight incubation at 33 °C and 230 rpm shaking, plasmid amplification was performed by adding chloramphenicol to a final concentration of 175 µg/ml and further incubated for 6 hours at 33 °C and 230 rpm shaking. The plasmid DNA was purified using the GeneJET™ Plasmid Midiprep Kit, according to the manufacturer’s recommended method.

b. **Construction of plasmid DNA for in vitro expression of FlagΔhCB1T4L_his6:**

i. **Restriction digestion of pET26kzsΔhCB1T4L_his6:**

The plasmid pET26kzsΔhCB1T4L_his6 was digested using *Nde*I and *Nco*I restriction enzymes as follows – 15 µl of pET26kzsΔhCB1T4L_his6 plasmid (3.0 µg) was mixed with 7 µl of 10x Restriction buffer, 0.7 µl of 100x bovine serum albumin (BSA) (New England Biolabs, Massachusetts), 41.3 µl of sterile distilled water to make up the volume to 70 µl, 3 µl each of *Nde*I and *Nco*I enzymes (Fermentas) and the mixture was incubated for 2.5 hours at 37 °C. After 2.5 hours, 8.0 µl of 10x Antarctic phosphatase buffer (New England Biolabs) and 3 µl of Antarctic phosphatase (New England Biolabs) were added and incubated further for 30 min at 37 °C. The digested plasmid DNA was
evaluated using agarose gel-electrophoresis. The band of digested plasmid was purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI) according to the manufacturer’s protocol.

**ii. Phosphorylation of oligonucleotides coding a FLAG-tag:**

The oligonucleotides, FwhCB1flag - 5’ TATGGATTATAAAGATGACGATG-ACAAAGC and RvhCB1flag - 5’ CATGGCTTTGTCATCGTCATCTTTATAATCCA were phosphorylated separately using the following protocol. 1 µl of the oligonucleotides (FwhCB1flag or RvhCB1flag, 10 µM) were mixed with 1.5 µl of 10x Tango buffer (New England Biolabs) sterile distilled water to make up the volume to 15 µl and 0.35 µl of T4 polynucleotide kinase (10 u/µl) (New England Biolabs). The mixture was incubated at 37 °C for 30 min and then the kinase was heat inactivated by incubation at 80 °C for 10 min.

**iii. Insertion of phosphorylated oligonucleotides coding a FLAG-tag into pET26kzsΔhCB1T4Lhis6 plasmid:**

Both the restriction digested and dephosphorylated pET26kzsΔhCB1T4Lhis6 plasmid DNA (final concentration of 0.05 pmol/µl) and each of the phosphorylated oligonucleotides coding a FLAG-tag (final concentration of 0.67 pmol/µl) were mixed to 2 µl of 10x T4 DNA ligase buffer, sterile water to a final volume of 20 µl and 1 µl of T4 DNA ligase (New England Biolabs). The mixture was incubated at room temperature for and then placed on ice.

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iv. Analysis and purification of pET26kzflagΔhCB1T4Lhis6 plasmid:

The DNA ligation mix was transformed into XL-1 blue cells and the colonies were selected on LB agar plates using antibiotic kanamycin. 16 selected colonies were analyzed using PCR and DNA sequencing, with the protocol mentioned previously, to confirm the presence of oligonucleotide insertion in pET26kzflagΔhCB1T4Lhis6 plasmid. To obtain larger quantities of plasmid DNA, single colony was inoculated in one liter of LB media containing kanamycin (final concentration of 24 µg/ml) and after 18 h incubation plasmid DNA was purified using the GeneJETTM Plasmid Midiprep Kit, according to the manufacturer’s recommended method.

c. Protein extraction from purified plasmid DNA using phenol-chloroform mixture:

Equal volumes of purified plasmid DNA in aqueous solution and phenol-chloroform mixture were mixed, vortexed for 1 min and then centrifuged at 13,500 rpm for 2 minutes. The aqueous (upper layer) was collected and the extraction was repeated. To the collected aqueous solution, 1/10 volume of sodium acetate (3M, pH 5.2) and 2 volumes of 100% ethanol were added to precipitate the DNA. After 15 min incubation at 4 °C and centrifugation at 13,500 rpm for 5 min, the precipitate was collected and washed twice with 70% ethanol to remove salts. The pellet was air-dried and dissolved in RNase/DNase-free water. The size and concentration of DNA were confirmed using agarose gel-electrophoresis.
Cell-free expression of ΔhCB1T4L.his6 or FlagΔhCB1T4L.his6 receptor:

The cell-free MembraneMax™ Protein Expression Kit (Invitrogen, Carlsbad, CA) and modified CB1 receptor DNA templates in pET plasmid were used for expression of CB1 receptor according to the manufacturer’s recommended protocol with minor modifications. To inhibit traces of RNases, Porcine Optizyme RNase Inhibitor (Fisher Scientific, Pittsburgh, PA) 4 units/μl of reaction mixture were added. The plasmid DNA (1 mg) in reaction buffer mixture (final volume of 100 ml) was incubated at 30 °C for 2 hours mixing at 300 rpm on a plate shaker. 100 ml of the feed buffer mixture was added 30 minutes after the start of the reaction according to the manufacturer’s recommendation. The expression of ΔhCB1T4L.his6 protein was confirmed by SDS-PAGE analysis in AnykD™ Mini-PROTEAN® TGX™ precast polyacrylamide gels (Biorad), followed by western blot analysis using His-tag based immunodetection as detailed below. The total protein concentration was determined using Quick Start™ Bradford Protein Assay kit (Biorad) according to the manufacturer’s recommended protocol and the absorbance values were measured at 595nm using the EnVision™ Multilabel Plate Reader (Perkin Elmer, Waltham Massachusetts).

Purification of CB1 receptor:

a. Immobilized metal affinity chromatography (IMAC) purification of solubilized ΔhCB1T4L.his6 receptor or nanodisc-ΔhCB1T4L.his6 receptor complex:

The ΔhCB1T4L.his6 receptor was purified either as solubilized ΔhCB1T4L.his6 receptor or as nanodisc-ΔhCB1T4L.his6 receptor complex. To purify the solubilized
receptor, ΔhCB1_{T4L}his6 \textit{in vitro} expression reactions (200 ml) were diluted with 2 volumes of dilution buffer (DB; 300 mM NaCl, 50 mM sodium phosphate, 10% Glycerol, pH 8.0) (200 ml) containing 1% dodecyl maltoside and the receptor was solubilized for 1 hour at 4 °C on a rotating wheel. The samples were then added to DB-equilibrated BD Talon\textsuperscript{TM} (IMAC) resin (Clontech) (100 ml) and incubated for 2 hours at 4 °C on a rotating wheel. The resin was washed twice with DB (100 ml) and the protein was eluted 5 times using 150 mM Imidazole in DB (5 x 50 ml). The aliquot of samples taken during purification were analyzed by western blot according to the procedure given below. To purify the nanodisc-ΔhCB1_{T4L}his6 receptor complex, the ΔhCB1_{T4L}his6 \textit{in vitro} expression reactions were not solubilized with detergent and directly diluted with DB.

\textbf{b. Anti-FLAG immunoaffinity purification of nanodisc-FlagΔhCB1_{T4L}his6 receptor complex:}

The \textit{in vitro} expression reactions (200 ml) of flagΔhCB1_{T4L}his6 were diluted with the DB (200 ml). The samples were then added to DB-equilibrated ANTI-FLAG\textsuperscript{®} M2 affinity gel (Sigma, St. Louis, MO) (100 ml) and incubated for 2 hours at 4 °C on a rotating wheel. The resin was washed twice with DB (100 ml) and the protein was eluted 5 times using 150 ug/ml of FLAG\textsuperscript{®} peptide (Sigma) in DB (5 x 100 ml). The aliquot of samples taken during purification were analyzed by western blot according to the procedure given below.
**Western blotting:**

The samples were analyzed using AnykD™ Mini-PROTEAN® TGX™ precast polyacrylamide gels and run at 150 V for 10 minutes followed by 200 V for 30 minutes. The blotting transfer was performed using the Trans-Blot SD semi-dry electrophoretic transfer cell (Biorad), according to the manufacturer’s recommended protocol. Towbin buffer (25 mM Tris, 192 mM glycine, 10% methanol and 0.1% SDS) was used to soak the filter papers, and to wash the gel and membrane pre-wetted with methanol. The transfer was performed for 10 minutes at 10 V followed by for 20 minutes at 15 V. The membrane was washed with 1x TBS with 0.25% Tween-20, twice for 10 minutes and then incubated in blocking buffer (Qiagen, Valencia, CA) for 1 hour. The blocked membrane was again washed twice with 1x TBS with 0.25% Tween-20 and 0.2% Triton, followed by another wash with 1x TBS with 0.25% Tween-20 for 10 minutes each and incubated with the Penta-His antibody horseradish peroxidase conjugate in blocking buffer (Qiagen) for 1 hour on a gel rocker. The washing steps were repeated and the proteins were visualized using the ECL Western Blotting Analysis System (GE Healthcare, Piscataway, NJ). The image was captured using the Fluorchem SB™ (Alpha Innotech Santa Clara, California) system.

**Saturation binding assays with nanodisc-ΔhCB1<sub>T4L</sub>his6 receptor complex:**

The radioactive binding assays were performed with [³H]-CP55,940 radioligand (obtained through the National Institute of Drug Abuse Drug Supply Program (NDSP)). 96-well GF/B filtration plates (Perkin Elmer) were pre-treated with 0.5%
Polyethylenimine (PEI) for 3 hours at 4 °C. The plates were placed on vacuum manifold (Pall corporation, Port Washington, NY) and washed twice with 200 ml of binding buffer (BB - 50 mM Tris-HCl, pH 7.0 and 150 mM NaCl). The radioligand-receptor binding were performed in 1.5 ml eppendorf tubes containing in each 60 µg of total protein in 60 ml of 0.1% BSA in BB (pH 7.4) buffer at six radioligand concentrations. To determine non-specific binding, cold CP-55940 (2 µM) was added at concentration 200 folds higher than the radioligand. After 1 hour incubation at 30 °C, the samples were added, as triplicates, to selected wells of a PEI-pre-treated-washed, 96-well GF/B filtration plate. The plate with the samples was placed on a vacuum manifold and washed with 0.1% BSA in BB (pH 7.4) buffer (pH 7.4) under 25 mm Hg vacuum. 40 µl of scintillation fluid (Microscint™ 20 from Perkin Elmer) was added to each well and the plates were counted using TopCount NXT™ Microplate Scintillation and Luminescence Counter (Perkin Elmer). The data obtained was processed using Microsoft Excel and Prism 5 (GraphPad, La Jolla, CA). All concentration points were performed in triplicate and data points used for plotting are base-line corrected. $B_{\text{max}}$ and $K_d$ values were calculated by nonlinear regression using Graphpad Prism version 5.03 (one site-binding analysis equation $Y = B_{\text{max}} \times X / (K_d + X)$) on a Windows platform. Single point binding assays, at a radioligand final concentration of 10 µM, were performed for testing ΔhCB1his6 samples and the plates were not pre-treated with PEI. Only ΔhCB174Lhis6 samples were analyzed with 6 concentrations of radioligand using the above stated procedure.
Expression of CB1 receptor in *E. coli*:

**a. Transformation of pET26kzflagΔhCB1_{T4L}his6 plasmid into bacterial cells:**

The pET26kzflagΔhCB1_{T4L}his6 plasmid was transformed into BL21(DE3) competent cells (New England Biolabs) or Rosetta-gami(DE3)pLysS competent cells (Millipore, Billerica, MA) using the manufacturer’s recommended protocol. Briefly, a tube containing 30 µl of BL21(DE3) competent *E. coli* cells or Rosetta-gami(DE3)pLysS competent cells was thawed on ice for 10 min. 1 µl (150 ng) of pET26kzflagΔhCB1_{T4L}his6 plasmid DNA was added to the cell mixture and placed on ice for 30 min. This mixture was heat shock at 42 °C for exactly 30 seconds and placed back on ice for 5 minutes. 400 µl of SOC (at RT) was added and incubated at 37 °C for 60 minutes with vigorous (250 rpm) shaking. The transformed cells were selected on LB agar plates using appropriate antibiotic. Several colonies were selected and glycerol stocks were prepared using standard procedures. The glycerol stocks were stored at -80°C.

**b. Expression of flagΔhCB1_{T4L}his6:**

The previously prepared glycerol stocks of bacterial cells transformed with pET26kzflagΔhCB1_{T4L}his6 plasmid were used to streak a LB agar plates with appropriate antibiotic. Following, overnight incubation at 37 °C, 5 ml LB media was inoculated with one colony from the plate and incubated overnight at 37 °C. This culture was added to 500 ml of LB media containing appropriate antibiotic and allowed to grow overnight at
33 °C. The cells were then induced (when OD ~3) with 0.3 mM IPTG for 4 hours at 25 °C. The cells were harvested by centrifuging at 5000 g for 15 min and stored at -80 °C.

c. Preparation of *E. coli* membranes:

The membranes of *E. coli* cells were prepared using previously published protocols. (26) Briefly, the cell pellet was washed twice with 0.1 M Tris-HCl, pH 8.0, buffer and resuspended in cold resuspension buffer (0.1 M Tris-HCl buffer containing 20% sucrose. To cell suspension, Halt protease inhibitor cocktail (final concentration of 1x) was added, incubated at 37 °C for 15 min, followed by lysozyme (final concentration of 0.1 mg/ml) treatment and incubation for 15 min at 37 °C with mild agitation. To this mixture, 10 mM EDTA was added and incubated further for 10 min at 37 °C with mild agitation. The mixture was centrifuged at 12000g for 20 min and washed with resuspension buffer. The pellet was then resuspended in ice-cold water and briefly sonicated. To this cell suspension, Halt protease inhibitor cocktail (final concentration of 1x), DNAse I (1000 U), MgCl₂ (final concentration of 1 mM) and Tris-HCl (final concentration of 50 mM) were added, briefly sonicated and incubated on ice for 1 hr. The extract was centrifuged at 100,000g for 1 hr and the membrane pellet was washed with 50 mM Tris-HCl buffer and centrifuged again at 100,000g for 1 hr. The membrane pellet was resuspended in small volume of 50 mM Tris-HCl buffer and stored at -80 °C in aliquots. Membrane protein was quantified with a Bradford dye-binding method (Bio-Rad Laboratories).
d. Saturation binding assay with flagΔhCB1T4L.his6 membrane preparation:

96-well GF/B filtration plates (Perkin Elmer) were pre-treated with 0.5% Polyethylenimine (PEI) for 3 hours at 4 °C. The plates were placed on vacuum manifold (Pall corporation) and washed twice with 200 ml of binding buffer (BB - 25 mm Tris base, 5 mm MgCl₂, 1 mm ethylenediaminetetraacetic acid (EDTA), 0.1% BSA, pH 7.4). Saturation binding assays were performed in a 96-well format. About 25 mg of protein was added to each assay well. The [³H]CP-55,940 was diluted in TME-BSA buffer to yield 11 ligand concentrations ranging from 0.039 to 40 nM. Nonspecific binding was assayed in the presence of 2 mM unlabeled CP-55,940. The assay was performed at 30 °C for 1 h with gentle agitation. The resultant material was transferred to Unifilter GF/B filter plates pre-treated with 0.5% PEI and unbound ligand was removed using a Packard Filtermate-196 Cell Harvester (Perkin Elmer). Filter plates were washed four times with ice-cold wash buffer (50 mM Tris-base, 5 mM MgCl₂ containing 0.5% BSA, pH 7.4). 40 µl of scintillation fluid (Microscint™ 20 from Perkin Elmer) was added to each well and the plates were counted using TopCount NXT™ Microplate Scintillation and Luminescence Counter (Perkin Elmer). The data obtained was processed using Microsoft Excel and Prism 5 (GraphPad, La Jolla, CA). All concentration points were performed in triplicate and data points used for plotting are baseline corrected. $B_{\text{max}}$ and $K_d$ values were calculated by nonlinear regression using Graphpad Prism version 5.03 (one site-binding analysis equation $Y = B_{\text{max}} \times X/(K_d + X)$) on a Windows platform.
e. Anti-FLAG immunoaffinity purification of FlagAhCB1\textsubscript{T4L,his6}:

Cells pellets were suspended in (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, 1% dodecyl-β-d-maltoside, pH 7.4) and lysed on ice by three, 33 s sonication cycles, each cycle consisting of 1 s bursts at 50 W separated by a 5 s interval. The lysate was centrifuged at 16000x g for 15 min (to remove residual precipitants) and the supernatant was collected. An equal volume of (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 7.4) was added to the supernatant. This mixture was then added to pre-equilibrated (in 50% lysis buffer, 50% purification buffer) ANTI-FLAG\textsuperscript{®} M2 affinity gel (Sigma) (200 ml) and incubated for 2 hours at 4 °C on a rotating wheel. The resin was washed twice with (200 ml of 50 mm sodium phosphate, 300 mm NaCl, 10% glycerol, 0.2% dodecyl-β-d-maltoside, pH 7.4) and the protein was eluted 5 times using 100 ml of buffer containing 150 ug/ml of FLAG\textsuperscript{®} peptide (Sigma). SDS-PAGE and western blotting analysis were performed on the aliquot of samples taken during purification according to the procedure previously described.

Mass spectrometric analysis of purified CB1 receptor:

a. Reduction, alkylation and in-solution trypsin digestion of purified CB1 receptor:

The samples for MS analysis were prepared using the previously reported standard procedures. Briefly, 35 µl of purified CB1 receptor was reduced using dithiothreitol (DTT, 20 mM) and alkylated using iodoacetamide (IAM, 50 mM) by incubating with each at RT for 1 h. The mixture was then desalted into 25 mM ammonium bicarbonate with 0.05% Cymal-5 using Micro BioSpin 6 columns (Biorad)
using the manufacturer’s recommended protocol. The sample was then subjected to overnight digestion with Tryspin Gold, MS-grade (Promega, Madison, WI) at 37 °C. The digests were analyzed immediately or stored at -80 °C until further processing. Alternatively, tryptic peptide mixture was further concentrated using Zip-tip-based extraction using manufacturer recommended protocol. Briefly, the C4 Zip-tip was wetted with 50% ACN and equilibrated with 0.1% TFA. The peptide mixture was bound to the equilibrated zip-tip and washed with 0.1% TFA and eluted with 95% ACN in 0.1% TFA.

b. MALDI-TOF/TOF analysis of FlaghCB2his6 receptor tryptic peptides:

The samples were analyzed, directly or following Zip-tip-based extraction, using AB SCIEX MALDI TOF/TOF™ 4800 (AB SCIEX) and TripleTOF 5600 (AB SCIEX) instruments in both reflectron and linear modes using standard procedures. All MS spectra were externally calibrated using a mixture of peptide standards [des-Arg1-bradykinin at MH+ 904.4681; angiotensin I at MH+ 1296.6853; Glufibrino peptide at MH+ 1570.6774; ACTH (clip 1-17) at MH+ 2093.0867; ACTH (clip 18-39) at MH+ 2465.1989; and ACTH (clip 7-38) at MH+ 3657.9294]. MS/MS spectra were acquired on selected ions of interest. The instrument was calibrated in the MS/MS mode using five daughter ions (at m/z 175.119, 684.346, 813.389, 1056.475 and 1441.634) generated from the fragmentation of Glu-fibrino peptide (MH+ 1570.6774.) MS/MS spectra were acquired under the following conditions: precursor isolation resolution of 200; collision energy of 2 kV; cell pressure of 2 x 10⁻⁵ torr; air as collision gas. All spectral data points were accumulated following analysis in multiple locations on each sample spot. The
theoretical molecular weights of expected peptides following reduction, alkylation and trypsin digestion were calculated using MS digest (UCSF MS facility, San Francisco, CA). The MS spectra was then be analyzed by comparing the monoisotopic m/z values obtained from MALDI-MS analysis with the theoretical molecular weights using FindPept software (Swiss Institute of Bioinformatics, Geneva, Switzerland).

5.7 Results and discussion:

5.7.1 Cell-free expression of truncated CB1 receptor:

The current techniques of in-vivo expression of GPCR have been extremely time consuming, expensive to produce pure, functional receptors for structural studies such as X-ray crystallography, NMR, etc. Cell-free expression systems have been shown to overcome some of these problems and could prove to be the right tool in our quest to understand these complex proteins in their native state. We had previously expressed a 102 residue N-terminal truncated human cannabinoid 1 receptor in HEK293 cells and found that it does not affect ligand binding (unpublished results). This truncated construct was used as the starting point for the cell-free expression of human CB1 receptor. pET-based plasmids were designed containing a N-terminal 102 amino acid truncated hCB1 receptor gene ending with a hexa-histidine tag (∆hCB1-His6), under the control of the T7 promoter with (pET26kzs∆hCB1his6) or without (pET15kz∆hCB1his6) a N-terminal pelB signal sequence – MKYLLPTAAAGLLLLAAQPAMA (Figure 5.1). The cell-free expression condition optimization experiments were performed using either the pET15kz∆hCB1his6 or pET26kzs∆hCB1his6 constructs.
Figure 5.1 Schematic diagrams of different constructs used for cell-free expression of human cannabinoid receptor.

The commercially available MembraneMax protein expression kit from Invitrogen was used for the cell-free expression of hCB1. The initial trials of hCB1 expression were performed using PCR products of the truncated hCB1 gene, generated using T7 primers from pET15kzΔhCB1his6 or pET26kzsΔhCB1his6 templates. The western blot analysis concluded that the cell-free expression using PCR products of both constructs resulted in no expression of the truncated CB1 receptor (ΔhCB1). In contrast, bacteriorhodopsin, the positive control provided by the manufacturer, was well
expressed. When the plasmid DNA constructs were used directly for cell-free expression with manufacturer’s recommended protocol, no ΔhCB1 expression was observed. But, further removal of protein impurities in the plasmid preparation by phenol extraction; ΔhCB1 receptor was expressed (As seen by two bands at 37kDa and 70 kDa corresponding to the monomer and dimer receptor respectively).

To improve protein yields, ribonuclease inhibitor (RNaseI) was included in the cell-free expression reactions in different amounts (0.65 units, 2 units or 4 units per ul of expression reaction mixture) (Figure 5.2). To optimize the amount of plasmid DNA template necessary for highest yield, different concentrations (5 ng, 10 ng, 20 ng/ul of reaction) were tested (Figure 5.2) To optimize the incubation time, trials using different incubation time points (2 and 4 hours) were also performed and analyzed (Figure 6.). The best expression was observed with a plasmid DNA, and RNase inhibitor concentration of 10 ng/µl and 4 units/µl, respectively and an incubation time of 2 hours with the addition of the feed buffer after the first 30 minutes. Moreover, using higher than 10 ng/µl plasmid DNA concentration and/or extension of the reaction incubation time (to 4 hours) did not increase the yield of the receptor (Figure 5.2). Using optimal condition and both plasmid constructs, a considerable amount of ΔhCB1-His6 receptor was expressed and observed at 37 kDa by the western blot. However, one point saturation binding assays (both filtration and size-exclusion methods) showed that expressed ΔhCB1 receptor did not show any specific binding to [3H] CP55940, a standard cannabinoid receptor ligand with an expected Kd of 1-10 nM. It was therefore concluded that the expressed receptor was
functionally inactive, which may be due to either improper folding or lack of incorporation of the flexible receptors into the nanodiscs.

**Figure 5.2:** Anti-His western blot analysis of RNAsel and DNA concentration optimization of ΔhCB1-His6 cell free expression: **Lane 1** – Standard Protein ladder; **Lane 2, 3, 4** – 0.65, 2 and 4 units of RNAsel per µl of ΔhCB1 expression reaction respectively; **Lane 5, 6, 7** – 5, 10 and 20 ng of plasmid (pET15kzΔhCB1his6) DNA per µl of ΔhCB1 expression reaction respectively; **Lane 8** – Bacteriorhodopsin (Positive control). All lanes were loaded with 5 µl of expression reaction mixture. The band at ~15 kDa is a non-specific band.

Based on study by Yang et al., we hypothesized that the incorporation of the T4Lysozyme into the 3rd intra-cellular loop (ICL3) would stabilize the receptor in its
active form and help in the incorporation of the receptor into pre-formed nanodiscs, thus maintaining the receptor in its active conformation. In order to introduce the T4lysozyme into the intracellular loop 3 region of the receptor, an overlap extension method was used. We designed T4Lysozyme primers with CB1 overhangs and amplified the T4Lysozyme DNA fragment from a plasmid containing the T4Lysozyme. The amplified fragment of the T4Lysozyme, with overhanging CB1 complimentary ends, was purified and then used for the overlap extension procedure (Figure 5.3). To further assist in affinity purification, a N-terminal flag-tag was introduced by restriction digestion in place of the signal sequence and pET26kzFlagΔhCB1T4Lhis6 was generated.

**Figure 5.3: Schematic representation of the overlap extension procedure:** The T4Lysozyme DNA (in black) containing the CB1 complimentary ends (in green and red) was inserted into the pET26kzsΔhCB1his6 plasmid (in blue) and amplified using...
multiple cycles of denaturation, annealing and extension to obtain the pET26kzsΔhCB1T4Lhis6 plasmid construct (in yellow). The original pET26kzsΔhCB1his6 plasmid DNA was digested with DpnI enzyme and newly amplified pET26kzsΔhCB1T4Lhis6 plasmid was transformed into XL-1 blue E. coli cells.

Figure 5.4: Anti-His western Blot analysis of the cell-free expression purification of ΔhCB1_{T4L}his6 and flagΔhCB1_{T4L}his6 receptor: **Lane 1:** Protein ladder showing the 50 kDa band. **Lane 2:** Cell-free expression reaction mixture of ΔhCB1_{T4L}his6 (5µl). **Lane 3:** Cell-free expression reaction mixture of flagΔhCB1_{T4L}his6 (5µl)

Using the previously optimized expression conditions and other additives, PelBΔhCB1T4Lhis6 and FlagΔhCB1T4Lhis6 were expressed and confirmed using the western blot analysis (Figure 5.4). FlagΔhCB1T4Lhis6 receptor was found to express in
significantly higher yields than PelBΔhCB1T4Lhis6 receptor. In addition to the introduction of Flag-tag, this increase in expression yield can also be explained partially by the minor optimization of T7 promoter distance to start codon and the removal of HA signal sequence.

PEI pre-treated GF/B plates were used in the radioligand binding assays to increase the binding of the soluble, lipophilic receptors, incorporated in nanodiscs, by neutralizing the negative charge of the glass fibers. PEI-pretreatment was also expected to decrease the non-specific binding of the receptors to the filter plates. A 6-point saturation binding experiment, using CP55940, produced curves (Figure 5.5) with a Bmax of 1359 pmol/g and 2965 pmol/g and a Kd of 8.57 nM and 8.38 nM for PelBΔhCB1T4Lhis6 and FlagΔhCB1T4Lhis6 constructs respectively. This confirmed that the expressed both PelBΔhCB1T4Lhis6 and FlagΔhCB1T4Lhis6 receptors were functionally active with respect to ligand binding. Furthermore, the higher expression yield of Flag-tagged CB1 directly corresponds to the ligand-binding competency, indicating that significant amount of the expressed protein is functionally active.

Both PelBΔhCB1T4Lhis6 and FlagΔhCB1T4Lhis6 constructs were purified by affinity chromatography. PelBΔhCB1T4Lhis6 was purified using immobilized metal affinity chromatography with Cobalt-based TALON resin beads. During initial trials, dodecyl maltoside was used to solubilize the receptor and disassemble nanodisc, which resulted in protein recovery of <50%. This poor purification yield was attributed to the
aggregation of the unstable receptors and protein precipitation. Therefore, the use of any detergents was avoided all further purification steps. Using the optimized purification method, it was confirmed by western blot analysis that about 60-70% of total PelBΔhCB1T4Lhis6 protein was recovered. However, coomassie-stained SDS-PAGE analysis of His-tag purification eluate revealed the purity to be less than 50%. This can be attributed to the high non-specific affinity of some of the E. coli proteins present in reaction mixture to Talon resin.

![Saturation Binding curve of Cell-free expressed shFlagCB1-T4L-His6 and B) Flag ΔhCB1T4Lhis6 receptor](image)

**Figure 5.5: Saturation binding assay with cell-free expressed A) PelBΔhCB1T4Lhis6 and B) Flag ΔhCB1T4Lhis6 receptor:** The 6-point binding assay was performed with [3H]-CP55940 radioligand using the 0.5% PEI pre-treated-washed, 96-well GF/B filtration plates. To determine the non-specific binding, cold CP-55940 (2 μM) was used. The plates were counted using TopCount NXT™ Microplate Scintillation and Luminescence Counter after the addition of 40 μl/well of scintillation fluid. The data
obtained was processed using GraphPad Prism 5 as detailed above. (One concentration point was removed from the plot due to a large error).

In contrast, FlagΔhCB1T4Lhis6 was purified using both N-terminal Flag-tag (FLAG M2 immunoaffinity chromatography) and C-terminal His6-tags (IMAC TALON affinity chromatography). The western blot analysis of affinity purification (Figure 5.6) revealed that FlagΔhCB1T4Lhis6 purified well with both his-tag and flag-tag based purification methods. The coomassie-stained SDS-PAGE analysis revealed that the Flag-tag based purification yielded higher purity of hCB1 receptor.

Figure 5.6: A) Anti-his western blot analysis of representative Flag-tag based and his-tag based purification of FlagΔhCB1T4LHis6: Lane 1 – Ladder, Lane 2, 7 – Unbound fractions from flag tag and his-tag based purification respectively Lane 3, 4 and 8, 9 – Wash fractions from flag tag and his tag based purification respectively, Lane 5, 6 and 10, 11 – Eluate 1 and 2 respectively from flag-tag and his-tag based purification
respectively. B) **Coomassie-stained SDS-PAGE analysis of Eluate 1 from Flag-tag based purification of cell-free expressed FlagΔhCB1T4LHis6.**

### 5.6.2 Bacterial expression of truncated CB1 receptor:

As an alternative approach, we explored the feasibility of expressing and purifying CB1 using an *E. coli* expression system. *Escherichia coli* offers important advantages as a potential expression host over cell-free expression, including robustness and scalability of expression and absence of expensive media requirements. The expression of FlagΔhCB1T4Lhis6 receptor was conducted in *E. coli* strains BL21(DE3) and Rosetta-gami(DE3)pLysS cells using the newly designed pET26kzflagΔhCB1T4Lhis6 plasmid under different conditions including incubation time, temperatures, cell densities and IPTG concentrations. Following detergent-solubilization, the soluble versus insoluble (inclusion bodies) receptor expression was evaluated at various time points (3, 6 and 9 hours) using anti-his western blot analysis. When FlagΔhCB1T4Lhis6 receptor was expressed in Rosetta-gami(DE3)pLysS cells at 25°C, almost 90% of the expressed receptor was found in the detergent insoluble fraction, most likely as inclusion bodies. It was observed that the expression of FlagΔhCB1T4Lhis6 receptor in BL21(DE3) cells, with an incubation of 4 hours at 25 °C following induction with 0.4 mM IPTG, facilitated the best receptor expression profile (highest ratio of detergent solubilized receptor to receptor in inclusion bodies) (Figure 5.7).
A saturation binding experiment with the cannabinergic ligand $[^3H]CP$-55,940 and the spheroplast based membrane preparation yielded a saturation curve with a $B_{\text{max}}$ of 813 pmol/g and a $K_D$ of 17.7 nM (Figure 5.8). This result correlates well with ligand binding profile seen with cell-free expressed FlagΔhCB1T4Lhis6. However, it should be noted that the non-specific binding, when determined using 2 µM of unlabeled CP55940, constituted about 55% of total binding. This high non-specific binding has been attributed to the presence of large number non-specific interaction with bacterial protein and was also observed with human CB2 receptor.\textsuperscript{26}

**Figure 5.7: Anti-His western Blot analysis of FlagΔhCB1$_{T4L}$his6 receptor expression in BL21(DE3) cells:** Lane 1: Protein ladder with the molecular weights of each band mentioned on its left. Lane 2-4: Total (T), detergent-solubilized fraction (S) and
detergent-insoluble fraction (P) of BL21(DE3) cells expressing FlagΔhCB1T4Lhis6 receptor right before induction with IPTG. **Lane 5-7:** Total (T), detergent-solubilized fraction (S) and detergent-insoluble fraction (P) of BL21(DE3) cells expressing FlagΔhCB1T4Lhis6 receptor 3 hours after induction with IPTG. **Lane 8-10:** Total (T), detergent-solubilized fraction (S) and detergent-insoluble fraction (P) of BL21(DE3) cells expressing FlagΔhCB1T4Lhis6 receptor 6 hours after induction with IPTG. **Lane 11-13:** Total (T), detergent-solubilized fraction (S) and detergent-insoluble fraction (P) of BL21(DE3) cells expressing FlagΔhCB1T4Lhis6 receptor 9 hours after induction with IPTG at 25°C.

**Figure 5.8: Saturation binding assay with FlagΔhCB1T4Lhis6:** The 6-point binding assay was performed with [³H]-CP55940 radioligand using the 0.5% PEI pre-treated-washed, 96-well GF/B filtration plates. To determine the non-specific binding, cold CP-55940 (2 µM) was used. The plates were counted using TopCount NXT™ Microplate
Scintillation and Luminescence Counter after the addition of 40 µl/well of scintillation fluid. The data obtained was processed using GraphPad Prism 5 as detailed above.

Figure 5.9: Anti-His western Blot (lanes 1-7) and coomassie-stained SDS-PAGE (lanes 8-10) analysis of FlagΔhCB1T4L.his6 receptor affinity purification: Lane 1 and 8: Protein ladder with the molecular weights of each band mentioned under lane labeled kDa. Lane 2: The flowthrough fraction of solubilized flagΔhCB1T4L.his6-BL21 cells after binding to FLAG M2 affinity resin. Lane 3: FLAG-resin wash using wash buffer. Lane 4 and 10: flagΔhCB1T4L.his6 receptor eluate 1 from flag-tag based immunoaffinity purification. Lane 5: flagΔhCB1T4L.his6 eluate 2 from FLAG resin. Lane 6: FLAG resin treated with SDS buffer following receptor elution. Lane 7 and 9: flagΔhCB1T4L.his6
receptor eluate 1 from His-tag based IMAC purification. The red box denotes the bands corresponding to flagΔhCB1_{T4L}his6 receptor.

BL21(DE3) cells, containing recombinant FlagΔhCB1_{T4L}his6 receptor, were resuspended in solubilization buffer and subjected to mild sonication. The solubilized proteins were separated from insoluble inclusion bodies and cell debris by high-speed centrifugation. The efficiency of solubilization was determined by the western blot analysis and a recovery of up to 60%–70% of the recombinant receptor was observed. The purification of FlagΔhCB1_{T4L}his6 receptor was performed using either immobilized metal affinity chromatography on a Talon cobalt resin or immunoaffinity chromatography on an ANTI-FLAG M2 affinity gel, using the previously mentioned procedures. The eluates from the two purification methods were analyzed using anti-His western blotting and coomassie staining of SDS-PAGE gel (Figure 5.9). It was observed that IMAC purification resulted in good yields of the recombinant receptor but was extremely impure whereas FLAG-tag based immunoaffinity purification resulted in marginally higher purity (~50%). However, due to the overall poor protein yields and purity, FlagΔhCB1_{T4L}his6 from bacterial expression was not chosen for further mass spectrometric characterization studies.

5.6.3 Mass spectrometric characterization of cell-free expressed CB1:

Owing to the lack of a crystal structure of the cannabinoid 1 receptor, characterization of the ligand binding domains remains a major goal in our structure-
based drug discovery model. Currently, the characterization of receptor structure is limited to cumbersome mutagenesis studies and unreliable computational modeling studies based on known GPCRs structures with relatively low sequence homology. Previously, through our “ligand assisted protein structure (LAPS)” approach, we were able to develop a number of affinity-labeled cannabinergic ligands to obtain structural information about the receptor by identifying key ligand-associated amino acid residues. The final step in this LAPS approach requires the full proteomic characterization of functional, purified CB1 receptor before and after ligand treatment to identify ligand-modified amino acid residues. Using a baculovirus expression system in \textit{sf21} cells, CB1 receptor was well expressed, purified and using a “Bottom-up” MS-based proteomics method, receptor sequence coverage of >94\% was attained. Unfortunately, using known affinity probes in this model, we have been unable to identify the amino acid residues critical in ligand binding.

Here, we used both the IMAC-purified PelBΔhCB1\textsubscript{T4L}his6 and immunoaffinity-purified FlagΔhCB1\textsubscript{T4L}His6 receptor from cell-free expression system to ascertain the use of this system as a potential strategy for the LAPS studies. Samples of purified CB1 were prepared for proteomic mass spectrometric analysis as described in the methods section. MALDI TOF/TOF analysis of tryptic peptides from both PelBΔhCB1\textsubscript{T4L}his6 and FlagΔhCB1\textsubscript{T4L}His6 was performed in reflectron and linear modes for best sequence coverage (Figure 5.10). The peptides from PelBΔhCB1\textsubscript{T4L}his6 and FlagΔhCB1\textsubscript{T4L}His6 identified using MS analysis are listed in Table 5.1 and Table 5.2 respectively. We
observed a sequence coverage of ~80% with MS analysis of PelBΔhCB1_{T4L}his6. Similarly, we observed a sequence coverage of ~50% with FlagBΔhCB1_{T4L}his6.
Figure 5.10: MALDI-TOF MS spectra of trypsin digest of purified A) PelBΔhCB1T4Lhis6 and B) FlagΔhCB1T4Lhis6: Scan area 600–4000 Da obtained in Reflectron mode.

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<th>Observed Mass (Da)</th>
<th>Predicted Mass (Da)</th>
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<th>Peptide Sequence</th>
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<th>Mod.</th>
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<td>47.8</td>
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<td>1354.768</td>
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<td>199-209 (ICL3) (T4L)</td>
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<tr>
<td>943.608</td>
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<td>-53.9</td>
<td>(R)/IDEGLRLK/(I)</td>
<td>210-217 (T4L)</td>
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<td>664.474</td>
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<td>(R)/LKIYK/(D)</td>
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Table 5.1: Peptides identified in tryptic digest of PelBΔhCB1T4Lhis6 receptor using 4800 MALDI-TOF MS instrument in linear and Reflectron mode: The modification column (Mod.) depicts the following modifications at specific residue (underlined) in each peptide sequence – CAM – Cysteine is alkylated by iodoacetamide and MO - Methionine is oxidized. An empty cell indicates that no modifications were seen in that peptide. Column E indicates the error (in ppm) in mass between observed and predicted, Column C indicates the number of missed cleavages in the peptide. The Pos. column indicates the position of the peptide sequence in the PelBΔhCB1T4Lhis6 sequence. (TMH – Transmembrane helix; ICL – Intracellular loop; T4L – T4 Lysozyme; C – C-terminal).
<table>
<thead>
<tr>
<th>Observed Mass (Da)</th>
<th>Predicted Mass (Da)</th>
<th>E (Da)</th>
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<th>Pos.</th>
<th>Mod.</th>
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</tbody>
</table>
Table 5.2: Peptides identified in tryptic digest of FlagΔhCB1_{T4L}his6 receptor using 5800 MALDI-TOF MS instrument in linear and Reflectron mode: The modification column (Mod.) depicts the following modifications at specific residue (underlined) in each peptide sequence – CAM – Cysteine is alkylated by iodoacetamide and MO - Methionine is oxidized. An empty cell indicates that no modifications were seen in that peptide. Column E indicates the error (in ppm) in mass between observed and predicted, Column C indicates the number of missed cleavages in the peptide. The Pos. column indicates the position of the peptide sequence in the FlagΔhCB1_{T4L}his6 sequence. (TMH – Transmembrane helix; ICL – Intracellular loop; T4L – T4 Lysozyme; C – C-terminal).

Most of the tryptic peptides identified belong to the hydrophilic, extracellular and intracellular regions of human CB1 and T4Lysozyme. The amphipathic transmembrane helices are extremely hydrophobic and therefore expected to have poor recovery. However, between both PelBΔhCB1_{T4L}his6 and FlagΔhCB1_{T4L}his6, TMH 2, 3, 4, 6 and 7 we identified. The low intensity of these peaks confirms the losses during sample preparation and/or mass spectrometric analysis. Future studies might require the use of an optimized LC-MS/MS analytical method for tryptic-digest sample analysis for complete coverage of human CB1 receptor.
In conclusion, we were able to demonstrate the feasibility of expression, purification and characterization of functional CB1 receptor using a cell-free expression system in conjunction with nanodiscs. CB1 is involved in numerous physiological processes and therefore provides a wide scope of potential therapeutic opportunities. Therefore, several drug discovery initiatives are underway to develop CB1-selective agents to treat indications such as overweight/obesity, cardio-metabolic disorders, substance abuse, neuropathic pain, and multiple sclerosis. However, due the lack of direct structural evidence, rational drug design endeavors have been hindered. Heterologous expression and isolation of purified, functional and stable CB1 has been the bottleneck for structural studies. This newly developed platform of efficient expression and purification of a structurally stable engineered receptor might now enable us to study the structural characteristics of ligand binding using a number of biophysical methods such as mass spectrometry, NMR and even X-ray crystallization.

5.7 References:


