LIGAND-ASSISTED PROTEIN STRUCTURE CHARACTERIZATION AS A METHOD FOR EXPLORING THE BINDING AND FUNCTIONAL MOTIFS OF LIGANDS ON THE CB2 CANNABINOID RECEPTOR 2

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Dissertation directed by

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Dedication

To my father and mother,
for their support and love.
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Abstract of Dissertation

This dissertation describes an investigation of the G-protein coupled human cannabinoid receptor 2 (GPCR hCB2) binding sites and mechanism of activation utilizing Ligand-Assisted Protein Structure (LAPS), an experimental method developed within the Center for Drug Discovery. Coupled primarily to \( G_{i/o} \) proteins, CB2 mediates numerous physiological responses and are important pharmaceutical targets. The aim is to study the covalent interactions between covalent probes specifically designed to activate/deactivate the receptor and hCB2. We utilize these probes to determine binding motifs at the receptor active site(s) and investigate functional consequences of covalent binding. We found that a distinct binding motif of individual covalent probes can lead to distinct, functional pathways (agonist or antagonist) for CB2.

The isothiocyanato (−NCS) is an electrophilic covalent group that specifically targets cysteine residues. AM841 is a CB2 megagonist that has an NCS group at the terminal carbon (C7′) of the C3-akyl tail end. AM4073 is an analog of AM841 that has the NCS group at the C11 headgroup position. AM4099 has two NCS groups at the C7′ and the C11 positions respectively. The two new analogs were identified to irreversibly bind to C2.59(89), and function as agonists. The helix-2 cysteine is discovered to be a unique binding site for cannabigeric ligand recognition.

A C3′-functionalized adamantyl series of compounds contain an adamantyl group at the 3′-carbon of the phenolic ring and covalent groups associated to the adamantyl unit. AM994 carrying a methylene-NCS chain was identified to react irreversibly with C7.38(284) and C7.42(288) on helix 7 of hCB2 and induce an inverse agonist state on CB2.

The azido (−N3) is a photoactivatable group capable of covalent bond formation at the receptor site upon 245-nm ultra-violet (UV) irradiation. Two aliphatic azides, AM993 and
AM8135 were tested through LAPS but contrary to our expectations, the two compounds do not form covalent attachment with any cysteine in the transmembrane region on the intact hCB2. We suggest future experiments to answer this question.

An array of classical cannabinergic ligands incorporating heteroaroyl groups at the C3 position were employed as probes to the cannabinoid receptors. Compound 41, a classical cannabinergic carrying a benzothiophenone moiety, was determined to have the best affinity and selectivity to mCB2.
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List of Abbreviations

$\Delta^8$-THC  (-)-$\Delta^8$-tetrahydrocannabinol
$\Delta^9$-THC  (-)-$\Delta^9$-tetrahydrocannabinol
2-AG    2-arachidonoyl glycerol
$^3$H    tritium-labeled
AEA      N-arachidonoylethanolamine (anandamide)
ATP    adenosine triphosphate
BEVS    baculovirus expression vector system
B_max    maximal binding capacity
BSA    bovine serum albumin
cAMP    cyclic adenosine monophosphate
CB1    cannabinoid receptor 1
CB2    cannabinoid receptor 2
cDNA    complementary deoxyribonucleic acid
CNS    central nervous system
CP55940 (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol
C-terminus carboxyl-terminus
DC    detergent compatible
DMEM    Dulbecco’s modified eagle’s medium
DMSO    dimethyl sulfoxide
EC    extracellular loop
EC_{50}    half maximal effective concentration
EDTA    ethylenediaminetetraacetic acid
E_{max}    maximum efficacy
FAAH    fatty acid amide hydrolase
FBS    fetal bovine serum
FSK    forskolin
G418    Geneticin
GDP    guanosine 5’-diphosphate
GPCRs    G-protein-coupled receptors
G-protein guanine nucleotide-binding protein
GTP    guanosine triphosphate
hCB1    human cannabinoid receptor 1
hCB2    human cannabinoid receptor 2
HBSS    Hank’s balanced salt solution
HEK293    human embryonic kidney 293
HEPES    4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HHC 9-nor-9β-hydroxyhexahydrocannabinol
IBMX 3-isobutyl-1-methylxanthine
IC₅₀ half maximal inhibitory concentration
IL intracellular loop
K₄ dissociation constant
Kᵢ inhibition constant
LAPS ligand-assisted protein structure
LC liquid chromatography
LC/MS/MS liquid chromatography-tandem mass spectrometry
LogP partition coefficient, distribution coefficient
mCB2 mouse cannabinoid receptor 2
MS mass spectrometry
MS/MS tandem mass spectrometry
MTSEA (2-aminoethyl)methane thiosulfonate hydrobromide
N₃ azido functional group
NMR nuclear magnetic resonance
NCS isothiocyanato functional group
N-terminus amine-terminus
pcDNA plasmid cytomegalovirus promoter deoxyribonucleic acid
PBS phosphate buffered saline
PCR polymerase chain reaction
pH power of hydrogen, -lg[H⁺]
pKa acid dissociation constant
rCB1 rat cannabinoid receptor 1
RPM revolutions per minute
R state inactive state
R* state active state
SAR structure activity relationship
SEM standard error of measurement
SR141716A N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
SR144528 (N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3 carboxamide)
TME Tris/MgCl₂/EDTA
TMH transmembrane helix
TMSBr trimethylsilyl bromide
TR-FRET time-resolved fluorescence resonance energy transfer
UV ultra violet
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CHAPTER 1:

GENERAL INTRODUCTION
The endogenous cannabinoid system, also known as the endocannabinoid system, was named after the cannabis plant that initiated this field of study (Figure 1.1 [1]). The cannabis plant has been used for millennia as a herb for medication and therapy [2]. In modern times, cannabis, commonly known as marijuana, has drawn considerable attention and increasing interest from the medical field. Extracts from the plant have been used for multiple pathologies, such as pain relief and the treatment of cancer [3-9]. The mechanism of action of cannabis was not understood until the early 1990s, when it was discovered that two orphan G-protein coupled receptors (GPCRs) are activated by compounds found in cannabis [10-15]. This discovery initiated understanding of the broader endocannabinoid system that, in addition to the two GPCRs, encompasses a family of endogenous lipids, including anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and related enzymes that biosynthesize and degrade these signaling molecules. Two particularly important enzymes are fatty acid amide hydrolase (FAAH), which hydrolyzes AEA, and monoacylglycerol lipase (MGL), which hydrolyzes 2-AG [16-19].
(-)-\(\Delta^9\)-Tetrahydrocannabinol (\(\Delta^9\)-THC) is the most potent psychoactive component of marijuana, and it affects a number of cell-mediated responses by activating the endocannabinoid GPCRs [20-22]. The two principal GPCRs of the endocannabinoid system cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), are activated by three major groups of compounds: endocannabinoids, phytocannabinoids, and synthetic ligands [16, 23-26]. Cannabinergic ligands utilized in this dissertation are classified in five groups according to their structures: classical cannabinoids such as \(\Delta^9\)-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 different structural
classes have been designed and applied to the exploration of GPCR structure and function, and the discovery of new medications [23-29].

Radiolabeled synthetic classical cannabinoids of high affinity were first developed as tracers to determine the properties of the binding domain in receptors. The compound [³H]-CP55940 is typically utilized in biochemical experiments as this molecule has a high binding affinity to both cannabinoid receptors, and the binding within the transmembrane domains defines the orthostatic binding site [28, 30-32].

![Diagram of cannabinoids](image)

**Figure 1.2** Five major classes of cannabinergic ligands

1.1 G-Protein Coupled Receptors

G-protein coupled receptors are one of the largest families of membrane proteins found in mammals [33]. They mediate cellular responses to hormones and neurotransmitters, and transmit external signals through the cell membrane. They are well known as therapeutic targets, as 30-
50% of medications in the U.S. pharmacopeia are known to bind to and exert their effects via GPCRs. The Nobel Prize of 2012 in the field of Chemistry was awarded to Brian K. Kobilka and Robert J. Lefkowitz for their studies of GPCRs that were “crucial for understanding how G-protein-coupled receptors function”, underscoring their importance to modern medicine [34].

All GPCRs are membrane-bound proteins and they share similar tertiary structures. Seven membrane-spanning alpha helices form a bundle, with each containing 20-24 amino acids. The helices are connected by three intracellular loops (ILs) and three extracellular loops (ELs) [35, 36]. The N-terminus faces the extracellular space, and the C-terminus is located intracellularly, and the coupled guanine nucleotide-binding protein (G protein) is on the cytosolic surface of the receptor. When the receptor is engaged with an external signal, such as the binding of a small molecule, the receptor undergoes a conformational change that causes activation of the G protein, which in turn triggers multiple intracellular signal pathways (Figure 1.3) [37].

Further signal transduction depends on the type of G protein involved. G proteins are made up of three subunits, with $G_\alpha$ tightly associated to the $G_\beta$ and $G_\gamma$ subunits. Activation releases $G_\alpha$, with an exchange from GDP to GTP [38]. There are four classes of $G_\alpha$ referred to as $G_{\alpha i/o}$, $G_{\alpha s}$, $G_{\alpha q}$, and $G_{\alpha 12}$. $G_{\alpha i/o}$ and $G_{\alpha s}$ function through the same effector molecule, cyclic adenosine monophosphate (cAMP), but have opposite outcomes. The activation of $G_{\alpha i/o}$ inhibits adenylyl cyclase from generating the second messenger cAMP, which causes a decrease in the cAMP concentration. The activation of $G_{\alpha s}$ however increases the production of cAMP. The $G_{\alpha q}$ pathway modulates $Ca^{2+}$ ion channels, and $G_{\alpha 12}$ is responsible for cytoskeleton regulation [36, 38].

In the absence of ligand, GPCRs exist in, possibly, multiple conformations that are in equilibrium. In the two-state model, an active state ($R^*$) and an inactive state ($R$) are in
equilibrium [39, 40]. This allows for constitutive activity, which is the capability of a receptor to generate intracellular signaling in the absence of a bound ligand or in the presence of low concentrations of endogenous agonists [41, 42]. Constitutive activity occurs in over 60 wild type GPCRs among three families and between species. However, in many receptors including CB1 and CB2, these levels of constitutive activity are relatively low [41, 43, 44]. Upon binding of an agonist, receptor activation is due to stabilization of the active conformation by the ligand.

Compounds are categorized based upon the biological response one ligand generates when it interacts with a receptor. Agonists are compounds that interact with an active-state GPCR to shift the equilibrium towards R* and provoke its biological response. Full agonists, upon binding, produce the maximal response that the target receptor is capable of. Partial agonists have affinity for the receptor but do not insult in full activation of the receptor thus producing less than the maximal signaling strength the receptor is capable of [45]. Antagonists, also known as neutral antagonists, are compounds that have affinity but no efficacy for the target receptor, and they inhibit the agonist- or inverse agonist-mediated response [46]. Inverse agonists also bind to the receptor, but have an apparent opposite action to that of the agonists. This is due to the ability of inverse agonists to block any constitutive activity to reduce the biological response and stabilize the inactive-state receptors [44]. Neutral agonists, by comparison, do not have the ability to block this constitutive activity.
Figure 1.3 A diagram of the diversity of G-protein-coupled receptor signaling pathways

1.2 Cannabinoid Receptors

Cannabinoid receptors (CBs) currently include two subtypes, CB1 and CB2, and they were cloned shortly after their discoveries in the early 1990s [14, 15]. They have generated increasing interest for the discovery of new medications, because the two receptors are ubiquitous in the tissues of the body to modulate a variety of physical activities, and therefore they are implicated as drug targets for many diseases. CB1 and CB2 are family-A GPCRs that have seven transmembrane helices that are arranged and numbered in a clockwise manner. This transmembrane helix bundle composes the binding pocket, which is the focus in the following chapters of this dissertation. The two subtypes share high homology in their amino acid
sequences: there is 44% similarity for the full length protein, and for the transmembrane region it is 68%

Cannabinoid receptors are primarily associated with G\textsubscript{i/o} proteins, so the signaling transduction follows the inhibitory pattern of the α\textsubscript{i/o} subunit [36]. Attachment of cannabinergic ligands at different binding residues in the transmembrane domain cause different conformational changes of the receptor, which triggers agonist or antagonist functions. Exploring the connection between the binding site and the signaling pathway is one of the aims of this project.

1.2.1 Cannabinoid Receptor 1 (CB1)

Cannabinoid receptor 1, containing 461-472 amino acids, is primarily found in the central nervous system, most specifically in the brain, and with smaller amounts in peripheral tissues [47]. It was first identified in rat brains in 1988, and cloned and recombinantly expressed in 1990 [14, 32]. We detected CB1 binding sites in mouse brains and mouse lymphoma cells by labeling a photoaffinity ligand, and identified one or more binding sites in the CB1-mediated actions of THC [48]. The human CB1 receptor (hCB1) has a length of 472 amino acids and it shares 97% sequence similarity with the rat CB1 receptor (rCB1) [49]. Therefore, in biochemical assays rCB1 can be used as an accurate model of hCB1, and it is commonly employed in our laboratory.

1.2.2 Cannabinoid Receptor 2 (CB2)

Cannabinoid receptor 2 is mainly expressed in the immune system, but is also found in other tissues such as the spleen, and is expressed at a low level in the brain. CB2 was first cloned in 1993 and is made up of 360 amino acids – significantly fewer than CB1 [15]. In addition, there are significant species differences in the human CB2 (hCB2) sequence compared to mouse and
Biochemical assays using hCB2 and mouse CB2 (mCB2) often yield significantly different results [47].

As cannabinoid receptors are large and hydrophobic GPCRs, it is not trivial to determine their three-dimensional (3D) structures with traditional techniques [50]. A combination of mutation, functional and biophysical studies, however, has isolated several structural features that are important for ligand binding and function of CB2. These features also play important roles in binding and signaling of other members in the GPCR families.

There is a CWxP motif on helix 6 that is common in class-A GPCRs, and this motif is located in the middle of the helix and is very flexible, acting like a hinge [51]. For the two cannabinoid receptors, this motif is CWGP on CB1, and CWFP on CB2. The cysteine C6.47 in both hCB1 and hCB2 in the CWxP motif has been implicated in cannabinergic ligand binding and agonistic signal transduction [26, 27]. Another highly conserved motif is DRY, which is located at the end of helix 3 and the beginning of IL 2, and plays an important role in binding and signaling in hCB2. When the DRY sequence is mutated to AAA or ARY, WIN55212-2 has a significantly reduced affinity for hCB2, while mutations of R and Y lead to no major reduction. Modification of Y to A inhibits the agonist response induced by WIN55212-2. These results suggest that the D residue is critical for agonist binding and Y is important for the agonistic transduction [52].

As mentioned, in the absence of the ligand the receptors are in equilibrium of the active state and the inactive state. It has been shown that when a receptor is in the inactive state, a salt bridge is formed between the R residue of DRY on helix 3 and D6.30 on helix 6. This salt bridge drives the two-state equilibrium of the receptor to favor the inactive state [53]. During the activation of the receptor, an agonist binding promotes rotation of the aromatic W residue of the CWxP motif
and modulates the hinge. Subsequently, helix 6 rotates away from helix 3 and the salt bridge is broken and increases the distance between helices 3 and 6. This is referred to as the ‘rotamer toggle switch’ [39, 51, 54]. Therefore, the equilibrium moves towards the active form with an agonist/active signaling transmission.

For CB2 on helix 7, the serine S7.46(292) is more functionally critical than S7.39(285). The S7.46(292)A mutant receptor shows reduced potency based on the inhibition curves of cAMP activity by CP55940 and HU210 (a potent CB agonist), while the decreases of cAMP concentrations were retained on the S7.39(285)A mutant by the two agonists [55]. The double cysteine residues, C7.38(284) and C7.42(288) were identified to be associated with an inverse agonist/antagonist function when a ligand covalently binds to either of them [25].

In order to further investigate the properties of cannabinoid receptors, especially concerning their structures and binding sites, our laboratories in the Center for Drug Discovery (CDD) have developed a set of experimental tools that we refer to as Ligand-Assisted Protein Structure (LAPS). This method assisted us efficiently to obtain structural and functional information of GPCR CB2 receptors and to have a better understanding of protein-ligand complex interactions.

1.3 Ligand-Assisted Protein Structure (LAPS)

The LAPS methodology combines several experimental techniques; including novel covalent compound design, biochemical binding assays, site-directed mutagenesis, biochemical evaluation of function and mass spectrometry [25, 26]. We applied LAPS particularly towards ligand binding sites on the receptor known to be involved in CB2 function. This method complements structural information that may be obtained from traditional techniques such as X-ray crystallography and nuclear magnetic resonance spectroscopy. Although remarkable
progresses have been obtained in protein crystal structures, GPCRs are notoriously difficult to crystalize due to the large amounts of protein required, and the challenges of appropriately solubilizing the transmembrane helix bundle and the often poor thermodynamic stability and high hydrophobicity of the transmembrane bundle [50, 56].

Our method, LAPS focuses on the residues of the transmembrane region of the receptor, instead of the full-length conformation, with the assistance of a mutant series. The biochemical evaluation of function informs on the effect of a cannabinergic ligand on hCB2, and can be associated with the ligand binding information. Based on structural and functional knowledge of the ligand-protein complex we engage in rational drug design of novel cannabinergic compounds in which we incorporate knowledge related to the specific attachment of individual covalent ligands to the CB2 receptor.

Currently used crystal structures of GPCRs are mostly homology models established based on well-determined proteins, such as rhodopsin, in active and inactive forms. Experiments performed in LAPS utilize native cannabinoid receptors and cells that contain active and inactive states. Thus, the receptors challenged by cannabinergic ligands of different classes provide information of ligand binding and their various functions. Such information not only helps confirm the existing homology models, but also assists in the future discovery of new agonists and inverse agonists. LAPS is not only useful in the characterization of the cannabinoid receptors, but can also be applied to enzymes that belong to the endocannabinoid system [57]. In this dissertation the focus is the applications of LAPS upon hCB2 in order to obtain structural and functional information related to receptor activation and deactivation.

1.3.1 Covalently Active Groups
Classical cannabinoids, which have tricyclic backbones, bear four pharmacophores that have been determined to be critical for protein affinity of a ligand: C1-phenolic hydroxyl group, C9-northern head group, C6-southern group, and C3-side chain (Figure 1.4) [58]. The benzopyran ring is not a pharmacophore but its presence is required for ligand activity. Modifications at these sites affect ligand binding. A free or esterified C1-hydroxyl unit retains the activity, and a lengthened and branched C3-side chain enhances the activity [59, 60]. For instance, AM841 has a side chain of 7 carbon atoms and two additional methyl groups linked to C1’, and its affinity on hCB2 is 1.5 nM [26]. AM1336 is a biarylphazazole; its N1-pentyl chain has high flexibility to interact with hCB2 with affinity of 0.54 nM [25, 61]. This dissertation primarily focusses on classical cannabinoids that have modifications on the northern head group and the C3-side chain.

Figure 1.4 Classical cannabinoid and two principal covalent compounds
We have developed a number of ligands with covalently active groups that represent several different structural classes of cannabinergic ligands and are capable of binding to the receptors irreversibly. These covalent ligands are designed using scaffolds that are structurally stable and have a high reversible-binding affinity with the target protein. Thus, the covalently active group forms a link with a specific amino acid residue at or near the receptor’s binding site, and the different placement of the covalent groups around the scaffold targets different residues. These compounds are used as structural probes to covalently and irreversibly modify their target receptors. There are two major types of covalently active groups that we employ; one has an electrophilic warhead and the other reacts by photoactivation.

Electrophilic covalent functional groups are designed to react with a nucleophile at the binding site. The isothiocyanate (-NCS) moiety is a common electrophilic covalent warhead that reacts primarily with the nucleophilic thiol (-SH) groups [62, 63]. When an isothiocyanato probe interacts with the cannabinoid receptor binding site the NCS moiety forms a covalent bond by an addition reaction with the side chain of the cysteine residues that are oriented appropriately in or near the binding pocket. AM841 and AM1336 are two known isothiocyanates that both carry an NCS moiety at their C3-akyl tail ends (Figure 1.4). The two compounds were examined in our previous LAPS studies that found them covalently binding to hCB2 [25, 26].

Photoactivatable affinity probes contain a chemical group that reacts when irradiated with an ultraviolet (UV) light source. Initially these high-affinity compounds interact with the protein non-covalently, and upon UV irradiation they react with a proximal amino acid residue to form a covalent link. Aromatic azides (-N3) have long been used as photoactivatable moieties for labeling proteins because they are highly reactive when they are exposed to a short-wavelength UV light source, and yet are stable in storage [64]. The CDD has developed many unique
aliphatic azido probes which were used for mass spectrometry-based characterization to localize the binding residue involved in the ligand-receptor interaction.

A new class of light-active probes that irreversibly targets cannabinoid receptors is arylphenone analogs, an array of tricyclic cannabinoids incorporating a heteroaryl group at their C3 positions. Unlike azides that are activated by 245-nm UV light, benzophenones react when exposed to a long-wavelength UV light source (365 nm) [65].

1.3.2 Competition and Saturation Binding Assays

For the studies outlined in this dissertation, two types of biochemical binding assays were performed through modified protocols to analyze ligand binding with hCB2. To reveal the structure-activity relationships (SAR), competition binding assays are utilized for measurement of the affinity (inhibition constant, $K_i$), with which a compound competes for the binding sites of the radiolabeled ligand on the receptor. This assay allows us to identify compounds with high binding affinities as candidates for further experiments, among the ligand database. This assay is also used to determine if more than one binding site is present [66].

Saturation binding assays to examine the maximal specific binding sites ($B_{max}$) as well as the dissociation constant ($K_d$) to describe the affinity between a radiolabeled ligand and the receptor. When the receptor is covalently labeled, the saturation assays test the remaining available specific binding sites following the covalent ligand attachment [27, 67].

In both binding assays a well-characterized radioactive compound with high affinity to cannabinoid receptors is used as a standard (Figure 1.5). We utilize the tritium-labeled compound [$^3$H]-CP55940, occasionally WIN55212-2, the CB1-selective SR141617A and the CB2-selective SR144528 [32, 68-70]. The pretreated receptors, isolated from either cultured cells or animal tissues (principally brain), are well mixed with the radioactive ligand. Then the
mixture is incubated and harvested by passing quickly through a filter plate to separate the receptor-bound ligand from the free. The bound radiolabeled ligand remains within the membrane preparation on the plate surface and the radioactivity on the binding site is measured.

![Diagram of biochemical binding assay](Diagram.png)

**Figure 1.5** A work flow of biochemical binding assays. 1) Incubation: 37 °C, 1 hr with agitation; 2) harvest: wash buffer stored at 4 °C; 3) TopCount: scintillation fluid.

1.3.3 Mutant Library from Site-directed Mutagenesis

The essential utility of LAPS is that it can identify residues that are close enough to interact with the covalent tag of the ligand, and in this way shed light on ligand binding at the active site. Our experimental design requires designing mutants that cover several possible binding sites. We considered cysteine as possible binding sites because this amino acid is the strongest nucleophilic binding residue in the proteins and our covalent probes applied in LAPS are mostly electrophiles [71, 72]. Transmembrane cysteine residues are individually modified by site-directed mutagenesis, taking advantage of the selective protein chemistry that can be performed on cysteine residues that disappears when they are mutated to serine [73].

There are five cysteine residues located on transmembrane helices 1, 2, 6 and 7 of hCB2 (Figure 1.6 [26]), and five single cysteine-to-serine mutants were produced. Helix 7 has two
cysteine residues that are in close proximity, being on top of each other and separated by one helical turn. For this reason a double cysteine-to-serine mutant was created. We chose serine as the replacement for cysteine because the two amino acids are isosteric analogs with polar, uncharged side chains. Such a substitution ostensibly should have little influence on the entire conformation of hCB2. Cysteine-to-alanine mutants were generated when the cysteine-to-serine mutation has untoward effects. Alanine is a small, non-polar and non-nucleophilic amino acid, and its replacement of cysteine is expected not to affect protein conformation although reduces the effects of side chains of cysteine and serine (polarity and nucleophilicity).

Each mutant is named based on the amino acids before and after mutation, and the Ballesteros-Weinstein nomenclature is used for residue location on GPCRs that in this system the most conserved residue in each helix is given the number 50 [74]. As an example, for the hCB2 mutant C6.47(257)S: C is cysteine as the wild-type amino acid, 6 signifies helix 6, 47 is the number of the cysteine on helix 6 for which the most conserved residue is a proline (P is numbered 50), 257 is the number in the primary amino acid sequence for the cysteine , and S is serine after mutation.
When a compound is found to covalently bind to wild-type hCB2, the mutant library consisting of single and double cysteine mutants is assayed following the same procedure performed to the wild type. The $B_{\text{max}}$ value differences obtained from saturation assays demonstrate the level of ligand covalent attachment on each mutant receptor, and to identify the residue(s) directly involved in binding. For example, the helix-6 cysteine mutant fails to covalently bind with AM841 while other mutants perform similarly as hCB2 wild type. This suggests that AM841 covalently binds to C6.47(257), the very cysteine located on the conserved CWFP motif [26, 51]. The two helix-7 cysteine residues are sites for AM1336 covalent labeling. Either residue was shown to interact equally since each helix-7 single mutant has diminished levels of covalent labeling while the double mutant has no apparent labeling [25]. This is congruent with the proximal positioning of these cysteine residues.
GPCRs often express poorly in a recombinant system, and it is often easier to use homogenized animal tissues as the source of the receptor for biochemical experiments. In our experiments with cannabinoid receptors, hCB2 and mCB2 are recombinant proteins expressed in stably-transfected mammalian cell lines, whereas rCB1 is obtained from homogenized rat brains.

Human embryonic kidney 293 cells (HEK293) are an excellent cell line for the recombinant expression of mammalian proteins as they are easy to transfect, culture, and maintain, and for those reasons we use this cell line for CB2 expression [75]. In addition, the wild type HEK293 cells have an extremely low specific binding level of the radiolabeled cannabinoid receptor, ligand standard ($[^3]$H]-CP55940) used in the binding assay will not impact the reliability of results from binding assays performed on hCB2 and mCB2, such as $B_{\text{max}}$ values (Figure 1.7).
Other host cells have been used for recombinant membrane protein expression. The baculovirus-infected insect cells and Escherichia coli cells (E.coli) are the most commonly used [76-81]. The baculovirus expression vector system (BEVS) is capable of expressing a relatively high amount of recombinant cannabinoid receptors, and has been used to produce receptor for
mass spectrometric characterization [80]. Receptor produced in *E. coli* yields high specific binding, however the dissociation constants are not similar to those obtained with receptors from mammalian cells [81, 82]. The biochemical binding data in this dissertation were obtained with the receptors produced in HEK293 cells, and the cell-based functional assays were performed at the stably transfected HEK293 cells. The hCB2 mutants were transfected and cultured in HEK293 cells.

Although the mutant library is an extremely helpful tool in elucidating the structure of the receptor labeling, it does have limitations. The level of resolution of the receptor binding site is limited by the location of the wild-type cysteine residues. It is also possible that ligand covalent attachment can occur at residues other than cysteine and this just be considered in interpreting results.

### 1.3.4 Mass Spectrometry-based Characterization

Mass spectrometry (MS) is an increasingly important technique in protein characterization, and it can perform many types of analysis from peptides, post-translational modifications, to intact proteins with high molecular weights [83]. The full-length hCB2 has been cloned in a baculovirous system with two affinity tags to assist in purification: a FLAG (DYKDDDDK) tag on the N-terminus and a *hexa*-histidine (HHHHHH) tag on the C-terminus [80, 84, 85]. The purified protein is then digested with trypsin before being analyzed by tandem mass spectrometry.

Tandem mass spectrometry (MS/MS) can directly and unambiguously identify the binding sites, and does not require making single-point mutations of a particular amino acid to determine the site. This method was used to confirm C6.47(257) as the site of covalent attachment of AM841 on hCB2. The liquid chromatography-tandem mass spectrometry (LC/MS/MS)
experiments were used to identify peak shifts between the control helix 6 and the labeled sample that was exposed to AM841 [80].

Sample preparation is critical for MS. Highly pure protein of sufficient quantity as well as near complete characterization of the enzymatic digestion products of the transmembrane portion of the receptor proteins are required and are major stumbling blocks for obtaining good results with cannabinoid receptors. In this dissertation, biochemical binding assays against the cysteine mutant library were used as the primary tool to identify the binding residues on hCB2 directly involved in the ligand covalent attachment.

1.3.5 Functional Evaluation

CB2 is primarily coupled to $G_{i/o}$ proteins consisting of an $\alpha_{i/o}$ subunit in addition to the $\beta$ and $\gamma$ subunits. Activation of the $\alpha_{i/o}$ subunit on the G protein inhibits adenylyl cyclase and hence limits the generation of cAMP, an important signaling molecule [16]. The evaluation of the functional signal is a cell-based assay measured as the change in the concentration of cAMP [86].

The cAMP accumulation assay is designed as a highly sensitive and robust homogenous time-resolved fluorescence resonance energy transfer (TR-FRET) immunoassay performed via a LANCE Ultra cAMP kit [87]. The experiment requires the intracellular levels of cAMP to be raised via stimulation with forskolin [88]. The cAMP concentrations, which are produced upon modulation of adenylyl cyclase activity, are recorded as TR-FRET signal, as the test compound is added gradually over a concentration range. The presence of free cAMP causes a decrease in TR-FRET signal and the lack of free cAMP produces high signal. This observation is used to classify the test ligands and characterize their functional properties. A reduction in the cAMP concentration implies an agonist, the response level being blocked to have no change suggests a neutral antagonist, and the increase of in the cAMP level signifies an inverse agonist/antagonist.
Two parameters, efficacy ($E_{\text{max}}$, a value indicating the maximum response) and half maximal effective concentration ($EC_{50}$) for a drug, are calculated from the concentration-response curve to describe the performance of a ligand at hCB2 cells. Being an agonist at hCB2, the $EC_{50}$ (0.08 nM) of AM841 is roughly 15-fold more potent than its binding affinity (1.51 nM), making this agonist a megagonist; in addition, the covalent bond formed between the NCS group of AM841 and C6.47(257) improves the potency by 40 times, compared to the non-covalent analogs [26]. The addition of AM1336 increases the cAMP level considerably (over 300%), suggesting it as an inverse agonist/antagonist at hCB2; the helix-7 double mutant nevertheless has diminished cAMP concentration (100-200%) as the receptor fails to covalently attach to the ligand [25].

1.3.6 Molecular Modeling

Molecular modeling was employed as a means to unravel protein structures in the last three decades to assist in understanding proteins’ biological functions and how they perform in health and disease, and to predict new medications [89]. Additionally, some proteins were successfully crystallized and their tertiary structures visualized [90, 91]. However, due to the present level of difficulty to crystalize GPCRs, 3D structural models of the cannabinoid receptors are homology models of a X-ray determined family-A GPCR – rhodopsin – on the basis of their structural similarity [35, 90, 92].

Two receptor conformations (inactive/R and active/R* states) exist in equilibrium in the absence of the ligand. When a functional ligand interacts with hCB2, the agonists bind with higher affinity to the R*-state conformation, while inverse agonists/antagonists switch the equilibrium in favor of the R state to lead to a new conformational equilibrium. In LAPS, we utilize native receptors and cells in the experiments, from which we obtain information useful for
ligand-receptor interaction dynamics. The results can also be used for the future design of agonists and inverse agonists. Identification of binding sites for covalent ligands on hCB2 is achieved either by covalent assays on mutant receptors or mass spectrometry. Functional evaluation results tell which state is used for ligand docking by identifying the ligand agonistic or antagonistic function. Being a very potent agonist, AM841 was docked into the R*-state hCB2 homology model with its C3-alkyl tail fitting in the helix-6 groove and the NCS group forming a covalent bond with C6.47(257); the ligand is also stabilized by hydrogen bonds formed with other residues in addition to the covalent attachment [26].

Our laboratory has synthesized a number of compounds that have high affinity to hCB2, and identified their covalent attachment sites [25, 26, 80, 93, 94]. Results obtained from previous LAPS research offered us knowledge of the inside view of hCB2, and linked ligand attachment with signal pathway efficiently. Those works are employed as an important starting point of this dissertation.

1.4 Outline of this Dissertation

Chapter 1 gives a general introduction on GPCRs, the cannabinoid receptors, cannabinergic ligands, and the methodology of LAPS.

Chapter 2. AM841 with an NCS moiety at C7 of the C3-alkyl chain covalently binds to C6.47(257) on hCB2 as a megagonist. This suggested the generation of two new isothiocyanates; AM4073 is labeled at the C11 position, and AM4099 is labeled at the C11 and the C3-alkyl tail with the NCS groups. Initially AM4073 was determined to bind covalently and exclusively to C2.59(89) on helix 2. We hypothesized that a two-site-attachment would occur to helices 2 and 6 via the double NCS-labeled AM4099. However, AM4099 attaches exclusively to helix 2 in a
mode similar to AM4073. Both are agonists at hCB2. These results demonstrated that C2.59(89) of hCB2 is an important binding and signal-transmitting site.

**Chapter 3** covers 3'-adamantyl compounds carrying either an NCS or an aliphatic N$_3$ moiety. SAR and covalent assays on CB1 and CB2 show that the ligands, which contain an extra methylene (-CH$_2$) group between the adamantane and the covalent group, have higher affinities to rCB1 and hCB2, and also produce greater covalent labeling. We chose AM994 for further study and found it covalently and equivalently interacts with the two helix-7 cysteine residues, and behave as a weak inverse agonist/antagonist at hCB2.

Photoactivatable azides have previously been used as covalent probes. In **Chapter 4**, we tested two high-affinity aliphatic azides, AM993 and AM8135 that covalently label hCB2 upon UV irradiation, through the hCB2 cysteine-mutant library to identify their covalent attachment sites. Our results suggest that none of the transmembrane cysteine residues are involved in the covalent attachment of the two azides on the intact hCB2 receptor. The different experimental conditions may have affected the reactivity of the amino acids targets by the covalent ligands.

In **Chapter 5** we used a group of tricyclic cannabinoids ligands, incorporating a heteroaroyl group at C3, as probes to explore CB1 and CB2. Based on SAR results, when the heteroaromatic group is either 3-benzothiophenyl (41) or 3-indolyl (50), the compounds are mCB2 selective. Compound 41 labels mCB2 through covalent attachment, while 50 does not react. An isomer of 41, compound 40, can also irreversibly bind to mCB2 with high efficiency, although the binding affinity of 40 is much weaker than that of 41 or 50.

Based on past and present LAPS studies, the identified cysteine residues, which are involved in ligand covalent attachment and signal transmission on hCB2, are summarized in **Chapter 6**.
We demonstrate a relationship between the binding residue and the signaling pathway on hCB2, which provides a basic understanding that will help future drug design.
1.5 Reference


CHAPTER 2:

ROLE OF C2.59(89) IN LIGAND RECOGNITION FOR HUMAN CANNABINOID RECEPTOR 2
2.1 Introduction

G-protein coupled receptors (GPCRs) play important roles in the mediation of a large number of physical responses, and are implicated in a variety of disease states, which make GPCRs very promising and popular drug targets [1, 2]. Cannabinoid receptors 1 and 2 (CB1 and CB2) are GPCRs of family A, and activation of a receptor by engagement of an agonist leads to a decrease in the cyclic adenosine monophosphate (cAMP) concentration [3]. As druggable targets, cannabinoid receptors have been studied primarily for their major functions in the central nervous system and the immune system [4-6].

Although there are issues that bring difficulties in utilizing traditional techniques, remarkable progress has been seen in the past years in the structural biology of GPCRs. The family-A receptor rhodopsin was the first GPCR to be crystallized due to the high level of expression in native tissues, while more recently the crystal structures of G-protein-coupled neurotensin and β-adrenergic receptor have been published [7-11]. Homology models of CB1 and CB2 were built based on the rhodopsin model so structural information about CB receptors and ligand binding sites has been derived from these models although they are not precise.

Experimental methods are relatively limited in the structural information they can provide because they do not offer an overall view of protein structures. The features and structural motifs known to govern GPCR functions are discussed in Chapter 1. The common CWxP motif is particularly relevant to this study and is present in human CB2 (hCB2) as CWFP on helix 6 [12].

Our laboratory at the Center for Drug Discovery has put together a set of experimental techniques, named Ligand-Assisted Protein Structure (LAPS), for characterizing protein-ligand interactions. This method comprises several aspects such as molecular biology, functional evaluation and molecular modeling [13-17]. Knowledge obtained from biochemical and
functional assays combined with the modeling information provide us an inside view of cannabinoid receptors, as well as directions for designing new drugs.

We previously reported a classical cannabinergic compound AM841 that contains an isothiocyanato (-NCS) group at the terminal carbon atom of its C3-alkyl side chain (Figure 2.1). This ligand has high binding affinity to CB1 and CB2 and covalently reacts with the cysteine C6.47 on both receptors through its covalently active NCS moiety. It functions as a potent agonist at CB1 and CB2 with its potency significantly higher than its binding affinity (on hCB2, \(EC_{50}=0.08\) nM vs. \(K_i=1.5\) nM), which makes AM841 a megagonist [14, 15]. We showed that the potency of the covalent AM841 is improved by 40-fold when compared to a high-affinity but non-covalent ligand (AM4056) that has the same scaffold and functions as an agonist (\(EC_{50}=3.27\) nM). This suggests that formation of a covalent link between AM841 and hCB2 does not affect the ligands of the same scaffold, with or without a covalent group, to be agonists, but such a link enables the covalent AM841 to be considerably more potent as an agonist [14].
Figure 2.2 is an illustration of AM841-labeled hCB2 with helices 2, 3, 6 and 7 presented [14]. The long alkyl chain of AM841 penetrates the groove formed by helix 6 in the binding pocket and the terminal NCS group reaches C6.47(257) and forms a covalent link. This is the most deeply located transmembrane cysteine residue and is located on the CWFP motif of hCB2 that plays an important role in receptor activation [12, 14]. According to the model, the tricyclic component is positioned at a relatively upper region of the binding pocket, forming hydrogen bonds with helical serine residues to stabilize ligand binding, such as the one between the C11-hydroxy group and S7.39(285). A salt bridge appears between D275 on extracellular loop 3 and K3.28(109) without sterically blocking the binding pocket [14]. This molecular model offers us a hypothesis of how AM841 positions itself when covalently reacting with hCB2, but raises the question as to whether the covalent attachment of AM841 distorts the binding position of the
Because of the unusually potent agonistic nature of AM841, we used it as a template for further modifications and designed two similar new ligands (Figure 2.1). AM4073 is a classical cannabivnergic compound, with the addition of an NCS moiety at C11 on its tricyclic head group. The NCS functional group is used as a chemical probe because it reacts with the nucleophilic side chain (-SH) of cysteine residues, which are strong nucleophilic binding sites in proteins [18]. AM4073 was tested on wild type hCB2 and showed about 60% irreversible labeling. The analog AM4099 has two NCS moieties, one located at the C11 position and the other at the terminal carbon atom of its C3-alkyl chain. It covalently labels wild type hCB2 at a level of 60%.

We identified binding residue(s) for ligand covalent labeling and functional effects on hCB2 by LAPS. AM4073 forms a covalent bond through the C11-head-NCS group with C2.59(89) on helix 2 and functions as an agonist. These results, combined with previous finding for AM841, show that the tricyclic components of AM841 and AM4073 are at the upper region of the binding
pocket of hCB2, close to the extracellular surface, while the C3-alkyl side chains are through the pocket.

When a ligand enters the binding domain of a receptor, it forms a complex with the protein dependent upon the array of non-covalent intermolecular forces determining the ligand-receptor complex. Formation of the covalent attachment may affect the precise location of a covalent ligand, such as AM841 when compared with to the location of its non-covalent counterpart. However we postulate that this effect is not significant.

Our hypothesis was that since AM4099 has two NCS groups, it may form two covalent bonds with cysteine residues on hCB2. However our experimental data show that this agonist, AM4099, irreversibly labels hCB2 only via the NCS moiety at the C11 position at the C2.59(89) residue, in a similar fashion as AM4073. Helix 6 is not involved in the covalent attachment as was initially expected. C2.59(89) of hCB2 was identified as a potent binding site for cannabergic ligands.

2.2 Materials and Methods

2.2.1 Materials

General laboratory chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) at the highest purity/grade unless otherwise noted. AM4073 and AM4099 were synthesized in the Center for Drug Discovery, Northeastern University (Boston, MA) (Figure 2.1). CP55940 and [3H]-CP55940 were kindly supplied by the National Institutes of Health (Bethesda, MD). pcDNA 3.1+ was obtained from Invitrogen (Carlsbad, CA). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). The DC colorimetric protein assay system was from BioRad (Hercules, CA).
2.2.2 Site-Directed Mutagenesis, Cell Transfection and Culture

There are five transmembrane cysteine residues in hCB2, located on helices 1, 2 and 6, and two on helix 7 (Figure 2.3, mutant series established by Dr. Richard W. Mercier). These cysteine residues were modified as either single or double cysteine-to-serine mutants. The full-length cDNA encoding hCB2 was kindly provided by MRC Laboratory of Molecular Biology, Cambridge, UK. The methods utilized to generate hCB2 mutants and to express wild type and mutant receptors in human embryonic kidney 293 (HEK293) cell lines were performed as previously described in the literature and briefly described below [14].

![Figure 2.3 Site-directed mutagenesis of hCB2: cysteine-to-serine mutant library [14]. Transmembrane cysteine residues subjected to mutation are circled in red.](image)

Site-directed mutagenesis of the gene encoding hCB2 in the pcDNA 3.1+ vector was performed following the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) procedure as provided by the manufacturer. Mutagenic oligonucleotide primers were annealed
and extended during 18 cycles of thermocycling with an Eppendorf Mastercycler (Westbury, NY) and Pfu DNA polymerase (Stratagene). After a 30-sec initial melting step at 95 ºC, each cycle consisted of 30 sec at 95 ºC, 60 sec of annealing at 55 ºC, and 5 min of extension at 68 ºC. Primers used to make the following mutants at hCB2, C1.39(40)S, C2.59(89)S, C6.47(257)S, C7.38(284)S, C7.42(288)S and C7.38(284)7.42(288)S, were: Forward 5’-GCT GTT GCT GTG TCG ACT CTG CTG-3’, Reverse 5’-CAG GCC CAG AAG AGT GGA CAA CAC AGC AAC AGC-3’, Forward 5’-AGT GTG GTC TTT GCA TCC AGC TTT GTG AAT TTC C-3’, Reverse 5’-ATT CAC AAA GCT GGA TGG TTC TCC CCA GTG CTG-3’, Reverse 5’-CAG CAC TGG GAA CCA GCT GAT GAG GAG CAC AGC-3’, Forward 5’-GCC TTT GCT TTC TCC TCC ATG CTG TG-3’, Reverse 5’-CA CAG CAT GGA GGA GAA AGC AAA GGC-3’, Forward 5’-GC TCC ATG CTG TCC CTC ATC AAC TCC-3’, Reverse 5’-GGA GTT GAT GAG GGA CAG CAT GGA GC-3’, Forward 5’-GCT TTC TCC ATG CTG TCC CTC ATC AAC TCC-3’, Reverse 5’-GGA GTT GAT GAG GGA CAG CAT GGA GGA GAA AGC-3’. 

DpnI-treated DNA was transformed into One Shot Top10 competent Escherichia coli cells (Invitrogen). Plasmid DNA was isolated by the QIAGEN Mini-Prep Kit (Valencia, CA). Plasmid DNA sequencing by SeqWright DNA Technology Services (Houston, TX) confirmed that only the desired mutations had been performed.

HEK293 cells (ATCC, American Type Culture Collection, Manassas, VA) were transfected with verified mutagenic plasmid DNA using Lipofectamine 2000 (Invitrogen) with an appropriate amount of linearized plasmid DNA harboring the transgene cassette according to the vendor’s manual. Typically 3-5 independent transfections were performed in parallel and duplicated over a 3-day period to maximize cell line integrity. Cultures were selected with 600
μg/mL Geneticin (G418) as an antibiotic over a 10-day period, then passaged to larger adherent cell culture flasks, and were cryopreserved under liquid nitrogen.

HEK293 cells and HEK293-derived cell lines were cultured using Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 4.5 g/L glucose and 2 mM glutamine [14, 19]. Cells were propagated on 245 mm × 245 mm plates and harvested at a high confluence in 1 mM ethylenediaminetetraacetic acid (EDTA) phosphate buffered saline (PBS) buffer by manual scraping.

The cell lines were routinely examined for the presence of the introduced transgene by PCR amplification, using primers corresponding to the parental vector sequence flanking the receptor encoding cDNA (5’ and 3’) matched with internal primers corresponding to the receptor sequence. Through this protocol we can generate only amplified fragments of the transgene cassette, avoiding amplification of the endogenous cannabinoid receptors.

2.2.3 Cell Membrane Preparation and Expression

Confluent cell monolayers were harvested on ice with PBS buffer containing 1 mM EDTA and washed three times with the buffer. Cell pellets were disrupted by cavitation in a pressure cell at 65 Torr for 15 min and on ice. Membrane fractions were isolated by ultracentrifugation (Beckman Coulter Optima L-90K, 44,000 RPM, 45 min, 4 °C), and the pellet was retained [20]. Protein concentration for each preparation was obtained by Bradford protein assay [21].

Saturation binding assays were performed in a 96-well format as described previously [15]. Membrane pellets were resuspended in TME (25 mM Tris-base, 5 mM magnesium chloride, and 1 mM EDTA, pH 7.4) containing 0.1% bovine serum albumin (BSA), and 25 µg of protein was added to each well. [³H]-CP55940 was diluted in TME buffer containing 0.1% BSA to yield a ligand concentration range from an order of magnitude below and above the predicted Kₐ (0.86
nM for hCB2) [22]. Non-specific binding was measured in the presence of 5 μM non-radioactive CP55940. After addition of all the assay components the plate was incubated at 30 ºC for 1 hr with gentle agitation. After incubation samples were transferred to Unifilter GF/B filter plates, and the unbound ligand was removed by a Packard Filtermate-96 Cell Harvester (PerkinElmer Packard, Shelton, CT). Filter plates were rinsed five times with ice-cold wash buffer (50 mM Tris-base, 5 mM magnesium chloride with 0.5% BSA, pH 7.4).

Bound radioactivity was quantitated in a Packard TopCount Scintillation Counter. Non-specific binding was subtracted from the total bound radioactivity to calculate the specific binding of the radiolabeled ligand (measured in pmol/g in saturation curves). Saturation assays were performed independently at least three times, and each individual data point/condition was done in triplicate. Data points were presented as the mean ± SEM. B_max and K_d values were determined by fitting the data to non-linear regression curves using GraphPad Prism (GraphPad Software, San Diego, CA).

Competition binding assays were performed in a 96-well format [23]. Membrane pellets were resuspended in the TME buffer containing 0.1% BSA, and added to each well for a total amount of 0.25 μg protein. [³H]-CP55940 was added to a final concentration of 0.76 nM, with a total volume of 200 μL assay mix per well. Concentrations of the test ligand were determined using IGOR Pro (Lake Oswego, OR) software by inputting relevant data, including an IC_{50} as predicted from prior studies and a log-range of 6. Conditions for the binding incubations and sample filtering were the same as described above. K_i values were determined by fitting the data to non-linear regression curve using GraphPad Prism software and presented as the mean with 95% confidence intervals from at least 3 independent experiments and each sample was made in triplicate.
2.2.4 Covalent Affinity Labeling Assay

Covalent affinity assays were carried out with hCB2-HEK293 membranes prepared as above and the assay procedure was performed as described [13, 14]. \( K_i \) values of AM4073 and AM4099 for hCB2 are 3.30 nM and 12.6 nM, respectively, calculated from the competition binding assays.

Four each sample, 5 mg membrane protein was diluted with TME containing 0.1% BSA to a volume of 5 mL. The test ligand, whose stock concentration was 10 mM in dimethyl sulfoxide (DMSO), was diluted to 10 μM with TME containing 1% BSA. One protein sample was treated with the ligand at a concentration of 10-fold \( K_i \): for example, a volume of 16.5 μL of 10 μM AM4073 was added for a concentration of 33 nM in 5 mL of hCB2. A parallel control devoid of the ligand was diluted in the same way. After being incubated in a 37 °C water bath for 1 hr with gentle agitation, both samples were centrifuged (Beckman Coulter, JA 20, 17,000 RPM, 10 min, 25 °C) and homogenized, twice with TME containing 1% BSA to remove the unbound ligand and once with TME (no BSA) to remove BSA. Saturation binding assays were carried out using \([^3\text{H}]-\text{CP55940}\) as the radiolabeled ligand.

2.2.5 cAMP Assay

Functional evaluation was performed by a homogenous time-resolved fluorescence resonance energy transfer (TR-FRET) cyclic AMP (cAMP) assay, measured as the inhibition of cAMP accumulation stimulated by forskolin in a cell-based bioassay modified from the literature (performed by Dr. Aneetha Halikhedkar) [19, 24].

HEK293 cells stably expressing hCB2 were used for the experiments. The cells were cultured in the culture media introduced above for 24 to 48 hr to 70% confluence and harvested using the non-enzymatic cell dissociation reagent Versene, and followed by centrifugation.
(Fisher Science IEC Marathon 2100R, 1,000 RPM, 5 min, 20 °C). The cells were washed once with Hank’s Balanced Salt Solution (HBSS) and resuspended in the stimulation buffer (1X HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA, pH 7.4). The assays were carried out in 384-well plates with 1,000-1,500 cells/well using PerkinElmer’s LANCE Ultra cAMP kit. The test compounds (5 μL diluted with 4 nM forskolin in the stimulation buffer) were added to the plate followed by 5 μL cell suspension. The plate was incubated for 30 min at room temperature.

Subsequently 5 μL Eu-cAMP tracer solution provided and 5 μL ULight-anti-cAMP solution were added to the plate – both solutions were diluted in the cAMP detection buffer (50 mM HEPES, 10 mM calcium chloride, 0.35% Triton X-100, pH 7.4). The plate was incubated at room temperature for 1 hr. The plates were read on PerkinElmer Envision and the data obtained from 665 nm were analyzed using GraphPad Prism. The maximum efficacy (E_{max}) was calculated from the maximal stimulation or inhibition value that corresponded to the difference between the control (cAMP level measured from forskolin-induced sample) and a plateau value.

2.3 Results

2.3.1 Characterization of hCB2 Expression

The hCB2-transfected HEK293 cell lines, expressing either wild type or cysteine-to-serine mutants, were generated by transfection and selection with G418 the antibiotic. Each cell line was scaled up to large culture plates to obtain a sufficient amount of cells containing the receptors. Each cell line was previously determined as functional lines by the CB agonist CP55940 when the lines were established (performed by Dr. Ying Pei and Dr. Richard W. Mercier) [13, 14]. After membrane preparation, we did saturation assays to characterize cell expression qualities. B_{max} and K_{d} values were obtained from saturation curves (Table 2.1 and
Table 2.1 Expression for hCB2 wild type and cysteine-to-serine mutants
Saturation assays examine the expression for wild type hCB2 and mutant receptors after membrane preparations.

<table>
<thead>
<tr>
<th></th>
<th>$B_{\text{max}}$ (pmol/mg)</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>11.3 (10.9~11.8)</td>
<td>2.04 (1.75~2.33)</td>
</tr>
<tr>
<td>C1.39(40)S</td>
<td>1.46 (1.31~1.62)</td>
<td>7.20 (5.42~8.99)</td>
</tr>
<tr>
<td>C2.59(89)S</td>
<td>11.5 (11.0~12.0)</td>
<td>1.14 (0.95~1.33)</td>
</tr>
<tr>
<td>C6.47(257)S</td>
<td>15.2 (14.6~15.9)</td>
<td>3.80 (3.35~4.25)</td>
</tr>
<tr>
<td>C7.38(284)S</td>
<td>7.41 (7.14~7.69)</td>
<td>1.00 (0.84~1.15)</td>
</tr>
<tr>
<td>C7.42(288)S</td>
<td>1.66 (1.60~1.72)</td>
<td>0.74 (0.60~0.88)</td>
</tr>
<tr>
<td>C7.38(284)7.42(288)S</td>
<td>0.69 (0.66~0.72)</td>
<td>0.50 (0.37~0.62)</td>
</tr>
</tbody>
</table>
Figure 2.4 Saturation assays for wild type hCB2 and cysteine-to-serine mutant membrane
preparations using \([{}^{3}\text{H}]\)-CP55940 as the radiolabeled ligand

2.3.2 Covalent Labeling of AM4073 on hCB2

We performed covalent assays through the cysteine mutant library to examine the ability of AM4073 to interact and covalently bind to hCB2. A neat control and a sample mixed with AM4073 were prepared and assayed in parallel. Figure 2.5 demonstrates saturation curves of the control and AM4073-treated samples for each line.

A comparison of the treated and untreated wild type hCB2 saturation curves clearly demonstrated that the AM4073-treated sample has fewer available specific binding sites for the radiolabeled ligand based on the significant difference in \(B_{\text{max}}\) values (Figure 2.5 (a)). Ostensibly this is due to AM4073 forming a covalent bond between its NCS group and hCB2, irreversibly occupying the specific binding site. The efficiency of covalent labeling was quantified by calculating the ratio of the difference in \(B_{\text{max}}\) between the control and the treated samples; for the wild type hCB2 it is above 60%. Because the NCS functional group primarily reacts with sulfhydryl on the side chain of cysteine, the possible transmembrane-cysteine-residue ligand attachment site(s) were tested by utilizing the same protocol against our cysteine mutant library [18]. For the receptors with cysteine-to-serine mutations on helices 1, 6 and 7, the effect of AM4073 was the same as the wild type receptor, suggesting AM4073 does not react with the cysteine residues in those helices (Figure 2.5 (b), (d), (e), (f) and (g)).

The saturation curves for C2.59(89)S (Figure 2.5 (c)) suggest that AM4073 may bind with a high affinity to hCB2, but not covalently. At high concentrations of \([{}^{3}\text{H}]\)-CP55940 the binding is normal, ostensibly from outcompeting AM4073 at the binding sites. If AM4073 was irreversibly bound to this site by formation of a covalent link even a high concentration of \([{}^{3}\text{H}]\)-CP55940
would not be able to successfully compete with it for the binding site. So C2.59(89) is critical for AM4073 covalent binding to hCB2.

Figure 2.5 Saturation binding curves demonstrating the effect of AM4073 treatment on hCB2 wild type and mutants
Figure 2.6 is a bar graph summarizing the percentage of covalent labeling by AM4073 for the wild type and mutant hCB2 receptors. The mutants from helices 1, 6 and 7, within error, have the same percentage (about 60%) of covalent labeling by AM4073. Yet the mutant C2.59(89)S exhibits no apparent reaction with the ligand, suggesting that the absence of the sulfhydryl at that site prevents formation of the covalent bond, and therefore, C2.59(89) is the binding residue for AM4073 to irreversibly attach to hCB2.

**AM4073 Covalent Labeling on hCB2 WT and Mutants**

![AM4073 Covalent Labeling on hCB2 WT and Mutants](image)

**Figure 2.6** Covalent labeling by AM4073 of hCB2 wild type and mutant receptors. Results are the means of at least 3 independent experiments performed in triplicate.

AM841 with the covalent NCS group at the terminal of C3-alkyl chain reacts with C6.47(257) of the CWFP motif on helix 6 in the middle of the receptor binding pocket. The NCS moiety of AM4073 is at the C11-head position and we found that this head NCS group forms a covalent bound with C2.59(89), which according to modeling is at the upper region of hCB2 and closer to the extracellular surface. These results show that AM841 and AM4073 position
themselves in the binding pocket in a similar pattern that the tricyclic head groups sit at the top part of hCB2 and the alkyl tails stretch to fit in the helix-6 groove through the pocket. The tail-NCS group of AM841 covalently reacts with the deeper located cysteine C6.47(257), and the head-NCS group of AM4073 targets the upper cysteine C2.59(89). Both cysteine residues are located very close to the respective NCS group to then form a covalent bond. The individual locations of the two ligands may differ slightly in the binding pocket but the orientation is similar in each case.

2.3.3 Covalent Labeling of AM4099 on hCB2

As we determined the covalent attachment points on hBC2 for the NCS groups are at different places on the same scaffold, we designed AM4099 that contains covalent groups at both head and tail positions. This analog was determined to have high affinity on hCB2 (Kᵢ=12.6 nM) and irreversibly bind to the receptor by 60% (Figure 2.7 (a)). As mentioned, AM4099 is expected to reversibly interact with the binding domain of hCB2 in a similar pattern. Our hypothesis was that covalent groups at both head and tail positions might allow AM4099 to label the receptor at two sites, C2.59(89) and C6.47(257) by formation of two covalent links.

We performed covalent labeling experiments for AM4099 through the cysteine-mutant library. Figure 2.6 displays the saturation curves of the control and AM4099-treated samples for each receptor, and the results were very similar to those observed for AM4073 treatment of hCB2. For the mutants of helices 1, 6 and 7 (Figure 2.7 (b), (d) and (e)) AM4099 retains the ability to covalently modify the receptor and block the specific binding sites. The saturation curves for mutant C2.59(89)S (Figure 2.6 (c)) suggest that AM4099 binds reversibly to the receptor but does not covalently react with it. At high concentrations of [³H]-CP55940 this ligand can outcompete AM4099 at the binding site.
Figure 2.7 Saturation binding curves demonstrating the effect of AM4099 treatment on hCB2 wild type and mutants

Figure 2.8 is a bar graph displaying the percentage of covalent labeling of AM4099 on hCB2. The mutants from helices 1, 6, and 7, within error, have the same percentage of covalent labeling by AM4099 to the wild type (about 60%). However for the mutant C2.59(89) there is no apparent change in maximal binding after treatment with AM4099, suggesting that the loss of the sulfhydryl side chain at that cysteine site prevents formation of the covalent bond.
These results did not support our hypothesis, according to which AM4099 would simultaneously form two covalent bonds at two cysteine residues (Figure 2.9). Based on the experiments with AM841 and AM4073 discussed in the introduction, one bond should be formed between the NCS group at C7' of the C3-alkyl chain and C6.47(257), and the other is between the C11 head group and C2.59(89). However, our results strongly suggest that only C2.59(89) undergoes irreversible modification by AM4099, as the mutant C6.47(257)S was unaffected by AM4099. These data also imply that the only covalent group of AM4099 interacting with hCB2 is the one at the C11 position.
Figure 2.9 Expected binding position of AM4099 in hCB2. AM4099 was expected to bind to hCB2 with the tricyclic head group close to the extracellular surface and the alkyl side chain penetrating the binding pocket. C2.59(89) is close to the C11-NCS group while C6.47(257) is accessible to the C7'-NCS group. Transmembrane helix (TMH) 3 is omitted from this view for simplicity.

2.3.4 Functional Evaluation of hCB2

We performed forskolin-stimulated cAMP accumulation assays with cells recombinantly expressing hCB2 for AM4073 and AM4099. Figure 2.10 shows that with increasing concentrations of AM4073 or AM4099, there is a concomitant decrease in the cAMP levels, which were initially enhanced by forskolin stimulation. These results suggest that AM4073 and AM4099 are hCB2 agonists, because activation of hCB2 via an agonist pathway is linked to the
inhibition of cAMP concentrations [3, 25]. The two ligands do not eliminate the response of the signal completely, which is similar to the results obtained with AM841 in this assay. However, the EC$_{50}$ values of AM4073 and AM4099 are in the same nanomolar range when compared to their K$_i$ values (Table 2.2). Thus, AM4073 and AM4099 are CB2 agonists and not as potent as the megagonist AM841, whose potency is extremely high (EC$_{50}$=0.08 nM) when compared to its binding affinity (K$_i$=1.5 nM) [14]. The sudden decrease of the cAMP levels at high concentrations of AM4099 also suggests that this ligand may have a slow dissociation rate when interacting with hCB2.
Figure 2.10 cAMP accumulation assays for AM4073 and AM4099 with cells recombinantly expressing hCB2. Forskolin-stimulated cAMP levels in hCB2 cells are inhibited by both (top) AM4073 and (bottom) AM4099, suggesting CB2 agonists.

Table 2.2 Inhibition of forskolin-stimulated cAMP accumulation in hCB2 cells

<table>
<thead>
<tr>
<th>#</th>
<th>EC$_{50}$ (nM)</th>
<th>Efficacy (%)</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM4073</td>
<td>18.67</td>
<td>39.95</td>
<td>3.30</td>
</tr>
<tr>
<td>AM4099</td>
<td>22.88</td>
<td>74.05</td>
<td>18.6</td>
</tr>
</tbody>
</table>
In summary, AM 4073 forms a covalent bond between its electrophilic NCS moiety and the site C2.59(89) on hCB2, and behaves as an agonist. AM4099 featuring two NCS groups, at the terminal carbon atom of the C3-alkyl chain and C11-head position respectively, does not covalently react to form two links as expected. It binds irreversibly and exclusively to hCB2 on C2.59(89) ostensibly through its C11-position NCS moiety, and acts as an agonist.

2.4 Discussion

2.4.1 Binding of AM4073 on hCB1 and hCB2

Both hCB1 and hCB2 have five cysteine residues in the transmembrane bundles, but the locations are not the same on the two receptors. For hCB2, cysteine residues are at C1.39(40), C2.59(89), C6.47(257), C7.38(284) and C7.42(288) (Figure 2.3). Those on hCB1 are C1.55(139), C4.47(238), C6.47(355), C7.38(382) and C7.42(386) (Figure 2.11). Thus, a big difference that is critical in identification of covalent binding sites is that hCB1 does not have a helix-2 cysteine residue but has one on helix 4. The helix-1 and helix-4 cysteine residues of hCB1, whose locations are close to the intracellular side of the receptor, face out toward the lipid bilayers, while the other three residues are close to the extracellular surface [26, 27]. On the active-state hCB1, C6.47(355) and C7.42(386) are oriented toward the binding pocket and are shown to be assessable for cannabinergic ligand binding [15, 28, 29]. C7.38(382) is located at the interface of helices 6 and 7 and is less likely to be a reactive site for covalent reaction with ligands [27].

Previously we found that AM4073 has good binding affinity on hCB1 (K_i=1.67 nM) and it covalently labels the receptor by 50%, and functions as an agonist (EC_50=2.3 nM) [16]. A hCB1-cysteine-mutant library (Figure 2.11) was employed as guides for localization of binding
site, from which we learned that AM4073 binds to hCB1 through a covalent link between its NCS moiety at the C11-head position and C7.42(386), the second cysteine residue on helix 7 that is an accessible binding site in an active bundle.

Figure 2.11 The amino acid sequence of hCB1 and the cysteine mutant library

In the study of AM4073 binding to hCB2, we initially tested the helix-7 mutants, namely, C7.38(284)S, C7.42(288)S and C7.38(284)7.42(288)S. However, neither of the helix-7 cysteine residues was involved in the covalent bond formation since these mutants retained the covalent
labeling levels as hCB2 wild type (60%). After testing the full mutant library we found that only C2.59(89) on helix 2 is the putative covalent binding site on hCB2 for AM4073, a residue located at the same approximate depth within the membrane as C7.38(284) at the upper region of hCB2 binding pocket.

As an agonist on hCB1 and hCB2, AM4073 irreversibly reacts with different cysteine residues on the two receptors, which reflects the receptor structural distinction in their binding pockets. It is important to note that hCB1 has no helix-2 cysteine, unlike hCB2, so AM4073 does not target hCB1 at helix 2. Although C7.42(386) of hCB1 and C2.59(89) of hCB2 are on two helices, both residues are close to the extracellular side of respective receptor. Therefore, AM4073 interacts with either hCB1 or hCB2 with its tricyclic head group oriented at the upper region of the binding pocket.

2.4.2 Calculation of Residual Non-covalently Bound Ligands

We observed a rare but interesting binding effect when identifying the covalent binding sites on hCB2. Our results show that the mutant C2.59(89) has no apparent covalent labeling with AM4073 or AM4099 (Figures 2.6 and 2.8). However, the saturation curves for the ligand-treated samples had a temporary decrease in specific binding at low concentrations of [3H]-CP55940 (Figures 2.5 (c) and 2.7 (c)), while the final B_{max} values of the treated and the control receptors were similar. This observation is not the same with previous non-covalent-binding experiments, in which the respective two saturation curves from control and ligand-treated fully overlap with each other.

A possible explanation for one observation with AM4073 is that in the ligand-treated sample there is residual non-covalent bound ligand which competes with CP55940. With increasing addition of CP55940, it displaces the residual AM4073 and restores the B_{max} value at the level of
the control sample. The concentration of the postulated residual AM4073 in the mutant sample can be calculated by the Langmuir equation (when $[L]_{\text{total}} \gg [R]$):

$$\frac{[RL]}{[R]} = \frac{1}{1 + \frac{K_d}{[L]}}$$

$$\frac{[RL]}{[R]} = \frac{1}{1 + \frac{K_d^A}{[L]}(1 + \frac{[A]}{K_d^A})}$$

in which, $[RL]$ is the concentration of bound receptor, $[R]$ is free protein, $[L]$ is the radioactive CP55940, and $[A]$ is for the residual AM4073 [30]. $K_d^A$ is the affinity constant for CP55940 and $K_d^A$ is the affinity constant for AM4073. $K_d^A$ and $K_d^A$ are calculated based on the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}}$$

in which, $K_i$ and $IC_{50}$ values are obtained from competition assays [31]. The one-site competition assays determined the ligand concentration for 50% inhibition of $[^3H]$-CP55940, so the value of $EC_{50}$ is that of $IC_{50}$.

Based on the equations we ran simulations for the case of AM4073 binding to C2.59(89)S. When the residual ligand on the mutant is at a concentration of 4-fold $K_i$ (13 nM), the simulated saturation curves of the control and AM4073-treated receptors are the same to those from the experiment (Figure 2.12 top). We did calculations for AM4099 on C2.59(89)S and found that the residual concentration of AM4099 is 2.6-fold $K_i$ (33 nM), as this concentration generates stimulated curves matching the experimental curves (Figure 2.12 bottom).

One explanation of this observation is that the ligands have slow dissociation rates, and a fraction of the ligand is present in the preparation after the washes. This may be because the
wash buffers we used that contain BSA are unable to remove AM4073 or AM4099 efficiently through washing.

![Graphs showing Covalent Assay of AM-4073 and AM-4099](image)

**Figure 2.12** Experimental and simulated saturation curves for (top) AM4073 and (bottom) AM4099 on C2.59(89)S. When the residual AM4073 is 4-fold $K_i$, the simulated curves are the same to the experimental curves. When the residual AM4099 is 2.6-fold $K_i$ the simulated curves match the experimental curves.

### 2.4.3 Significance of C2.59(89) and Novel Drug Design

This research project was undertaken not only to identify the covalent binding residue(s) of two novel ligands, but also to identify a site of ligand attachment on hCB2. Song and Reggio demonstrated that C2.59(89) is accessible within the ligand binding crevice for MTS derivatives [32]. Nevertheless, this cysteine on helix 2 of hCB2 has never previously been considered part of binding site for cannabinergic ligands. Our results obtained by using LAPS demonstrate that C2.59(89) is part of the binding pocket for cannabinergic ligands, and suggest that ligands that covalently react with this cysteine are capable of activating hCB2, functioning as agonists.
As a structural analog of AM841 and AM4073, AM4099 was designed to have its covalent groups at two positions, which are a composite of the positions used in the two reference compounds. Based on previous experimental results, we hypothesized that AM4099 would be an agonist and would form two covalent bonds with hCB2. The two binding sites ostensibly would be C6.47(257) as with AM841, and C2.59(89) as with AM4073. Our results however determined that AM4099 binds to C2.59(89) exclusively on hCB2 through the covalent labeling of its C11-NCS moiety.

Although AM4099 is not a bivalent-reactive covalent ligand, understanding its molecular mechanism has yielded valuable information for future cannabinoid-receptor ligand/drug design. After formation of the covalent link between C2.59(89) and the C11-NCS group, both hCB2 and AM4099 adjust conformations and orientations, which may make the helix-6 cysteine unreachable to the tail-position covalent moiety for bond formation. Further modifications to the AM841 scaffold, such as a two-NCS-containing ligand with a longer C3-alkyl chain (8 or 9 carbons), may successfully target two residues on hCB2 simultaneously and be very potent irreversible agonists.
2.5 References

CHAPTER 3:

C3’-FUNCTIONALIZED ADAMANTYL CANNABINERGIC LIGANDS
FOR CB1 AND CB2 CANNABINOID RECEPTORS
3.1 Introduction

Cannabinoid receptors 1 and 2 (CB1 and CB2) are membrane proteins of the family A  
G-protein coupled receptors (GPCRs) [1-4]. When a ligand binds to the receptor, a  
conformational change occurs to activate the linked $G_{i/o}$ protein at the cytosolic exposed surfaces  
of the receptor and initiate intracellular signaling, the most significant of which is a negative influence on the second messenger cyclic adenosine monophosphate (cAMP) [5-7].

Cannabinoid receptor 2 (CB2) is mostly found in the peripheral tissues of the immune system, with the greatest density in the spleen [2]. As a potent drug target and because of its functions in modulating disease onset, much research has focused on CB2 during the decades [8-10]. Traditional techniques, such as X-ray crystallography, are not amenable in determination of the structure of CB2, because of the difficulty of producing protein crystals of high purity for detection [11]. However, previous results from experimental work on human CB2 (hCB2) have isolated several structural features that are important for ligand attachment and function, as discussed in Chapter 1.

Our laboratory at the Center for Drug Discovery has developed an experimental method, Ligand-Assisted Protein Structure (LAPS), to explore the ligand covalent binding sites and subsequent functional effects on hCB2 [12-15]. Compounds of high affinity to hCB2, which carry covalently active groups and have a high level of covalent labeling, were selected by competition binding assays and covalent labeling assays [4]. We assayed those compounds against a mutant library to identify covalent attachment site(s) on hCB2. Cyclic AMP accumulation experiments measured how the ligand functionally affects the cAMP concentration at the cells [16, 17].

Previous LAPS studies determined that AM841 forms a covalent bond with the helix-6
cysteine C6.47(257), which is part of the CWFP motif in the middle of helix 6 on hCB2 [13, 15]. This covalent attachment stabilizes an AM841 binding motif that interestingly, leads to a hyper-activation of hCB2. For this reason we refer to AM841 as a megagonist [13]. AM1336 is a hCB2-selective compound, and it covalently labels C7.38(284) and C7.42(288) on helix 7 equally. Formation of the covalent bond in the ligand-receptor complex enhances the inverse agonistic/antagonistic response of AM1336 on hCB2 [12].

In this work, newly designed C3’-adamantyl cannabinergic ligands with different side chains attached to the adamantane unit were assessed for their structure-activity relationships (SARs) (Figure 3.1). AM991, AM992, AM993 and AM994, whose side chains contain covalently active groups (isothiocyanato-/NCS or azido/-N3), are potent to both CB1 and CB2 and have efficient levels of covalent attachment. AM993 (R=CH2N3) and AM994 (R=CH2NCS) give higher binding affinities and covalent attachment levels compared to AM991 (R=N3) and AM992 (R=NCS). The presence of a methylene (-CH2) group separating the reactive moiety from the adamantyl ring improves the hydrophobicity and flexibility of the ligand and allows a better fit in the hydrophobic receptor binding pocket. We further determined that AM994 is a weak inverse agonist/antagonist on hCB2 and its NCS group reacts with C7.38(284) and C7.42(288) in helix 7 equally.
3.2 Materials and Methods

3.2.1 Materials

General laboratory chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) at the highest purity/grade unless otherwise noted. The series of C3’-adamantyl analogs were kindly provided by Dr. Marcus Tius, University of Hawaii (Honolulu, HI). CP55940 and $[^3]H$-CP55940 were kindly supplied by the National Institutes of Health (Bethesda, MD). pcDNA 3.1$^+$ was obtained from Invitrogen (Carlsbad, CA). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). The DC colorimetric protein assay system was from BioRad (Hercules, CA). The ultraviolet (UV) light for irradiation was from Spectrolinker XL-1000 UV Crosslinker (Spectronics Corporation, Westbury, NY).
3.2.2 Site-Directed Mutagenesis, Cell Transfection and Cell Culture

Full-length wild type mouse CB2 (mCB2) and hCB2 cDNA (MRC Laboratory of Molecular Biology, Cambridge, UK) were transfected into human embryonic kidney 293 (HEK293) cells and maintained via the protocol described in Chapter 2, Materials and Methods section.

We generated the hCB2 cysteine-to-serine mutant library by site-directed mutagenesis (Stratagene, La Jolla, CA) and transfected the mutants in HEK293 cells (Figure 3.2, mutant series established by Dr. Richard W. Mercier) as described in Chapter 2, Materials and Methods section [13].

![Figure 3.2 Site-directed mutagenesis of hCB2: cysteine-to-serine mutant library [13]. Transmembrane cysteine residues subjected to mutation are circled in red.](image)

3.2.3 Cell Membrane Preparation and Expression

Wild type rat CB1 (rCB1) was prepared from purchased frozen unstripped rat brains (Pel-Freez Biologicals, Rogers, AR). Rat brains had cerebella removed to reduce non-specific
binding of the receptor, and then were cut into small pieces [18]. The plasma membrane containing rCB1 was homogenized in homogenization buffer composed of 0.32 M sucrose in TME (25 mM Tris-Base, 5 mM magnesium chloride, 1 mM EDTA, pH 7.4). After removing cell debris by centrifugation (Beckman Coulter Avanti J-E, JA20, 5,700 RPM, 10 min, 4 °C), the supernatant was loaded onto a discontinuous sucrose gradient (1.2 M and 0.8 M in TME), and the membranes were collected at the sucrose buffer interface. Sedimented by ultracentrifuge (Beckman Coulter Optima L-90K, 44,000 RPM, 45 min, 4 °C), the rCB1 membrane pellet was retained and resuspended in TME [4, 19].

mCB2 and hCB2 membrane preparations were obtained from transfected HEK293 cell lines [20]. Experimental details of membrane preparations were described in Chapter 2, Materials and Methods section. Saturation binding assays were performed to examine the expression quality [12, 20].

3.2.4 Affinity and Covalent Attachment Assays

Wild type rCB1, mCB2 and hCB2 were used in competition assays for SAR studies and covalent labeling experiments. Competition assays (two-point and eight-point) were performed successively using \[^{3}\text{H}\]-CP55940 to indicate ligand binding affinity (K_i) [20]. A two-point competition assay used the compound at a final concentration of 30 nM and 300 nM to estimate binding affinity. For the compounds whose K_i values are estimated to be below 1,000 nM, the accurate K_i values were obtained from an eight-point competition binding experiment run in triplicate.

We did covalent assays on wild type membrane preparations for covalent-group-containing ligands with K_i below 100 nM (later on hCB2 mutant series for AM994). Samples treated with the azido (-N_3) adamantyl cannabinergic ligands were activated by exposure under
short-wavelength (245 nm) UV light for 1 min, with the control [21]. The isothiocyanato (-NCS) compounds required an incubation period of 1 hr at 37 °C with gentle agitation. Experimental details were described in Chapter 2, Materials and Methods section.

3.2.5 cAMP Assay

Functional evaluation was performed by a homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) cyclic AMP (cAMP) assay, measured as the inhibition of cAMP accumulation stimulated by forskolin in a cell-based bioassay (performed by Dr. Aneetha Halikhedkar) [22, 23]. Experimental details were described in Chapter 2, Materials and Methods section.

3.3 Results

3.3.1 Structure Activity Relationships

When a ligand enters the binding domain of a receptor, it forms a complex with the protein dependent upon the array of non-covalent intermolecular forces that drive the reversible affinity, regardless of the presence of a covalently active group in the ligand. The results of competition binding assays for the C3'-adamantyl series of compounds are shown in Table 3.1. AM977, AM995 and AM10503 were not selected for additional experiments due to their poor binding affinity to the receptors. The other six compounds show high reversible binding affinities for CB1 and CB2, below 100 nM with several in the low nanomolar range.

AM976 (R=CN) and AM978 (R=CH₂CN) have low Ki values, especially for mCB2 and hCB2, in the low nanomolar range (below 10 nM). AM978, with an extra carbon, has higher rCB1 affinity than AM976. The cyanide (-CN) functional groups are not covalently active in our study, because nucleophilic addition to the CN group requires a high activation energy. Therefore,
AM976 and AM978 were not tested for covalent attachment.

AM991, AM992, AM993 and AM994 are associated with covalent groups in their side chains. The covalent moieties of AM991 (R=N$_3$) and AM992 (R=NCS) are directly attached to the adamantane unit directly, while AM993 (R=CH$_2$N$_3$) and AM994 (R=CH$_2$NCS) have the covalent group and the adamantane moiety joined by a CH$_2$ spacer group (Figure 3.1).

Affinities of AM993 and AM994 for rCB1 and hCB2 are generally higher. For rCB1 their $K_i$ values are about 5-10-fold less when compared to AM991 and AM992. These results suggest that the incorporation of a CH$_2$ spacer group in the ligand plays a role in the enhancement of ligand affinity to the receptor. The spacer group also improves the ligand’s hydrophobicity in the receptor, such as that the partition coefficient (LogP) value of AM994 (4.95) is higher than that of AM992 (4.27). Binding affinities for mCB2 for the four ligands are similar, regardless of the functional groups or the presence of the CH$_2$ unit. However, comparing the $K_i$ values between mCB2 and hCB2 shows that the four analogs, especially AM993 and AM994, are slightly more selective to hCB2. This related two- or three-fold species difference of hCB2 versus mCB2 is consistent across the series of compounds.
Table 3.1 Binding affinity ($K_i$) for C3'-adamantyl cannabinergic ligands

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>rCB1</th>
<th>mCB2</th>
<th>hCB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM976</td>
<td>CN</td>
<td>45.8</td>
<td>5.23</td>
<td>4.24</td>
</tr>
<tr>
<td>AM977</td>
<td>COOH</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>AM978</td>
<td>CH₂CN</td>
<td>2.6</td>
<td>4.37</td>
<td>4.58</td>
</tr>
<tr>
<td>AM995</td>
<td>CONH₂</td>
<td>297.6</td>
<td>946.9</td>
<td>337.3</td>
</tr>
<tr>
<td>AM10503</td>
<td>CH₂NH₂</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>AM994</td>
<td>CH₂NCS</td>
<td>3.0</td>
<td>34.6</td>
<td>10.3</td>
</tr>
<tr>
<td>AM993</td>
<td>CH₂N₃</td>
<td>4.4</td>
<td>26.4</td>
<td>9.62</td>
</tr>
<tr>
<td>AM992</td>
<td>NCS</td>
<td>35.4</td>
<td>31.7</td>
<td>13.1</td>
</tr>
<tr>
<td>AM991</td>
<td>N₃</td>
<td>18.6</td>
<td>38.4</td>
<td>24.8</td>
</tr>
</tbody>
</table>

3.3.2 Covalent Attachment for Potent Ligands

The high-affinity azides (AM991 and AM993) and the isothiocyanates (AM992 and AM994) were selected for covalent attachment. Samples mixed with different covalent probes were treated differently according to their covalent functional groups. The membranes prepared for the azido compounds were exposed to the 245-nm UV light for 1 min as the N₃ group is photoactivatable [21]. The samples prepared for the isothiocyanates were incubated at 37 °C for 1 hr with gentle agitation, as NCS is an electrophile and it takes time for the addition reaction to occur. Control receptor preparations devoid of ligands were subject to similar treatment and run in parallel.
Saturation assays were performed on the covalent-ligand treated samples to obtain the respective $B_{\text{max}}$ values on wild type receptors. The covalent labeling level was calculated as the ratio of the difference in $B_{\text{max}}$ between the control and the ligand-treated sample (Table 3.2).

AM991, AM992, AM993 and AM994 label rCB1, mCB2 and hCB2 covalently, with rCB1 showing the highest level of covalent attachment at 60% of binding sites. AM993 and AM994 covalently bind to CB1 and CB2 at a level of 60-80%. AM991 and AM992 have lower covalent binding levels (20-30%) to hCB2 compared to AM993 and AM994.

As discussed in SAR studies, the presence of the CH$_2$ group improves the binding affinity of AM993 (R=CH$_2$N$_3$) and AM994 (R=CH$_2$NCS) for CB1 and CB2 by improving ligand flexibility and hydrophobicity. In the ligand-receptor complex, the ligand may form a covalent bond with a particular amino acid in close proximity to the covalent group. In covalent attachment assay, AM993 and AM994 label the receptors at higher levels than AM991 (R=N$_3$) and AM992 (R=NCS), especially on CB2. This observation demonstrates that the extra carbon atom assists ligand covalent labeling on the receptors.

As an electrophile, the NCS group primarily targets the thiol (-SH) group of cysteine residues on the receptor [24]. Therefore, the high-affinity AM994 was chosen for further mutational study to identify the covalent attachment site(s) on hCB2. Although AM993 is also of high affinity and it covalently labels the receptor efficiently, the reaction mechanism between the aliphatic azide and hCB2 remains unclear as the mutant library utilized at this stage in LAPS was unable to identify accurate binding sites for covalent aliphatic azides on hCB2. Thus, AM993 was singled out for additional experiments, study described in Chapter 4.
Saturation assays for covalent attachment on wild type receptors using $[^3]$H-CP55940. Isothiocyanate-treated samples were incubated at 37°C for 1 hr, and azide-treated samples were activated by 245-nm UV light for 1 min.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Covalent labeling (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rCB1</td>
</tr>
<tr>
<td>AM994</td>
<td>63</td>
</tr>
<tr>
<td>AM993</td>
<td>67</td>
</tr>
<tr>
<td>AM992</td>
<td>54</td>
</tr>
<tr>
<td>AM991</td>
<td>61</td>
</tr>
</tbody>
</table>

### 3.3.3 Characterization of hCB2 Mutant Expression

The hCB2-transfected HEK293 cell lines, expressing either wild type or cysteine-to-serine mutants, were generated [13]. Cell lines were determined to be functional (by Dr. Ying Pei and Dr. Richard W. Mercier) [12]. Expression quality was shown in **Chapter 2, Results** section.

### 3.3.4 Covalent Labeling of AM994 on hCB2

The hCB2 mutants were tested independently via the same assay protocol. Figure 3.3 shows saturation curves of each mutant line, and the degree of covalent attachment is measured based on the amount of specific binding relative to specific binding in the absence of AM994. For wild type hCB2, the $B_{\text{max}}$ value decreases considerable when the receptor is incubated with AM994. This shows that AM994 has irreversibly occupied the binding site and lowers the apparent $B_{\text{max}}$ due to the formation of a covalent bond with hCB2 and the irreversible nature of the binding. The cysteine-mutants of helices 1, 2 and 6 had similar results to the wild type, a 70% reduction in $B_{\text{max}}$, implying that no individual cysteine on these helices is involved in the covalent attachment.
However, the cysteine mutations on helix 7 had a robust effect on covalent binding compared to the wild type receptor. This is shown in Figure 3.4 that shows labeling percentages averaged from three independent experiments for each receptor. The single mutants on helix 7, C7.38(284)S and C7.42(288)S have reduced levels of covalent attachment, approximately 30-40%, half as much as the wild type (70%). The double cysteine mutant C7.38(284)7.42(288)S has no apparent covalent labeling. Therefore, AM994 is irreversibly interacting with either of the two cysteine residues on helix 7 of hCB2 at an equal probability of interaction with each site, as they are located close to each other. When one cysteine is modified, the other residue retains the ability to form a covalent bond with the NCS group of AM994, although the level of covalent attachment is at approximately 40%. When both cysteine residues are replaced by serine, the covalent group fails to covalently bind, so the nature of ligand binding remains reversible. This reversibility is further suggested by the fact that the ligand is easily removed by simple washes.

The structure of AM994 provides an insight into understanding these results. Although the adamantane group is a bulky and rigid unit, the CH₂NCS side chain is somewhat flexible. The flexibility enables the ligand to orient itself to reach either the upper cysteine C7.38(284) or the lower C7.42(288).
Figure 3.3 Saturation binding curves demonstrating the effect of AM994 treatment on hCB2 wild type and mutants.
AM994 Covalent Labeling on hCB2 WT and Mutants

![Covalent Labeling Graph]

Figure 3.4 Covalent labeling by AM994 of hCB2 wild type and mutant receptors. Results are the means of at least 3 independent experiments performed in triplicate.

3.3.5 Functional Evaluation of AM994 at hCB2

Cyclic AMP levels in hCB2 cells pre-treated with forskolin were measured after the addition of AM994. Cannabinoid receptors are G<sub>i/o</sub>-protein coupled and agonists are expected to therefore decrease the cAMP concentration stimulated by forskolin [7, 25]. The cAMP levels were not affected at low concentrations of AM994 but were elevated at high ligand concentrations (Figure 3.5). The function curve gives merely a 40% change in efficacy with an EC<sub>50</sub> value of 2.4×10<sup>5</sup> nM. The slight enhancement of cAMP at very high ligand concentrations suggests that AM994 is a weak inverse agonist/antagonist at hCB2. This EC<sub>50</sub> value is significantly higher than the K<sub>i</sub> value (10.3 nM), which also suggests that AM994 function is very weak.
Figure 3.5 cAMP accumulation assay for AM994 with cells recombinantly expressing hCB2. Forskolin-stimulated cAMP levels in hCB2 cells are slightly enhanced by high concentrations of AM994, suggesting that this ligand is a weak CB2 inverse agonist/antagonist.

In summary, we studied a series of the C3'-adamantyl cannabinergic ligands to determine their structure-activity relationships. Four high-affinity covalent ligands, AM991, AM992, AM993 and AM994, were tested for covalent attachment levels on rCB1, mCB2 and hCB2. AM993 (R=CH₂N₃) and AM994 (R=CH₂NCS) have higher affinity and more efficient covalent labeling levels compared to AM991(R=N₃) and AM992 (R=NCS), because the addition of a CH₂ spacer group improves the ligand flexibility and hydrophobicity to allow a better fit in the hydrophobic binding pocket of the receptor. We did further LAPS study on AM994 by covalent binding assays against the hCB2 cysteine-to-serine mutant library and functional evaluation of cAMP, and determined that AM994 covalently reacts with C7.38(284) and C7.42(288) on helix 7 of hCB2 and functions as a weak CB2 inverse agonist/antagonist.

3.4 Discussion

3.4.1 The Presence of CH₂ Improves Affinity and Selectivity
Although the four adamantyl ligands (AM991, AM992, AM993 and AM994) have high binding affinity for CB1 and CB2, the SAR data show that the incorporation of a methylene (-CH₂) group connecting the covalent moiety (-N₃ or -NCS) and the adamantane results in higher affinity for rCB1 and hCB2. The CH₂ tether unit enables AM993 and AM994 to be slightly more selective to hCB2 with an approximate two-fold difference in their Kᵢ values. These data suggest that the C3'-adamantyl ligands have selectivity between receptor subtypes and species. In addition, the extra carbon atom results in a greater efficiency of covalent attachment on hCB2 as AM993 and AM994 have much higher levels of irreversible labeling (more than two folds) than their respective structural analogs, AM991 and AM992.

In the case of AM994 on hCB2, our mutational study shows that the NCS moiety of AM994 reacts equally and irreversibly with C7.38(284) and C7.42(288) on helix 7. The increased flexibility of the CH₂NCS side chain allows contact of the covalent group to either cysteine. The presence of the CH₂ group also improves the ligand’s reversible binding affinity and selectivity to hCB2. This is also a likely result of the increased flexibility of the CH₂NCS group that permits a more favorable interaction for AM994 in the binding region [26]. The hydrophobic CH₂ may also enhance stability in the hydrophobic transmembrane domain environment.

The comparison between AM976 (R=CN) and AM978 (R=CH₂CN) also shows the improvement of binding affinity to rCB1 with the addition of CH₂.

3.4.2 Ligand Binding Sites and Signal Transmission

We previously reported a hCB2-covalently-selective compound AM1336 and its covalent binding sites and function on hCB2 with the application of LAPS [12]. AM1336 is a biarylpyrazole compound (SR144528 analog) associated with a N1-pentyl chain to which an NCS group is linked at the terminal carbon atom. As shown in Figure 3.1, this compound has a
bulky adamantane group, so it enters hCB2 without penetrating deeply into the binding pocket (Figure 3.6, produced by Dr. Anna L. Bowman) [12]. Staying near the top region of the hCB2 binding pocket, AM1336 has its flexible alkyl chain orient so that the covalent group at the chain terminal is close to either cysteine on helix 7 and thus, it forms a covalent bond with C7.38(284) or C7.42(288). By contrast, AM841 penetrates further into the helix-6 groove with its flexible side chain permitting the covalent NCS group on the 7-carbon chain to position itself close to and form a covalent bond with C6.47(257) [13].

![Figure 3.6 AM1336 covalent docking models on hCB2 at (left) C7.38(284) and (right) C7.42(288)](image)

We examined another high-affinity isothiocyanato probe, AM994. It was selected from a list of C3′-adamantyl cannabinergic compounds to further test its covalent attachment site(s) on hCB2 because it irreversibly labels hCB2 at a 70% level. Like AM1336, AM994 has a bulky adamantane moiety in the structure and it interacts with the upper region of hCB2 due to the size.
Although the adamantane unit lacks mobility, the side chain (R=CH₂NCS) linked to it is flexible because of the presence of an extra carbon atom. So the covalent NCS group is able to react with either C7.38(284) or C7.42(288) to attach to hCB2 irreversibly.

We evaluated the functions of AM994 and AM1336 on hCB2 cells, which share the same covalent attachment sites. Interestingly, both compounds function as inverse agonists/antagonists. AM994 increases cAMP levels by merely 40% when the ligand in hCB2 cells is at high concentrations, and its EC₅₀ is in a micromolar range, suggesting that AM994 is a weak CB2 inverse agonist/antagonist.

AM1336, whose EC₅₀ is 20 nM, significantly increases the cAMP concentrations at hCB2 cells by 330%; mutating either one or two helix-7 cysteine residues reduces the cAMP level to 130-270%, suggesting that formation of a covalent bond in the ligand-protein complex enhances the efficacy [12]. Structural analogs of AM1336, whose N1-alkyl chains are one-carbon shorter or longer, selectively and covalently label C7.42(284) and function as inverse agonists/antagonists on hCB2 [12, 27].

The similarity we observed in the results of AM994 and AM1336 projects suggests that there is a link between the binding sites on helix 7 and functional pathways on hCB2. Ligands that covalently bind to C7.38(284) and/or C7.42(288) of hCB2 are associated with an inverse agonist/antagonist function. When a ligand irreversibly attaches to hCB2 at the helix-7 cysteine, a particular conformational change occurs to activate the coupled G-protein in an inverse agonistic/antagonistic pathway.

More sample cases on hCB2 that indicate links between binding sites and functional pathways are presented in Chapter 6 of this dissertation with more descriptions.
3.5 References


CHAPTER 4:

ALIPHATIC AZIDES ON HUMAN CANNABINOID RECEPTOR 2
4.1 Introduction

G-protein coupled cannabinoid receptors 1 and 2 (GPCR CB1 and CB2) are widely utilized as drug targets to modulate physical responses [1-4]. When the receptor is bound by an agonist, the activation of the coupled $G_i/o$ protein initiates downstream signaling by inhibiting adenylyl cyclase activity [5-9].

In order to explore structural information for cannabinoid receptors, our laboratory in the Center for Drug Discovery (CDD) has developed the Ligand-Assisted Protein Structure (LAPS) method that involves covalent compound design, site-directed mutagenesis, biochemical binding assays and functional evaluations [10]. We utilize a library of high-affinity ligands to first synthesize cannabinergic compounds that carry covalently active groups (such as isothiocyanato/−NCS and azido/−N$_3$) and retain high-affinity reversible binding. The covalent group on the ligand, under appropriate conditions, forms a covalent bond to residues with appropriately nucleophilic side chains that are in close-enough proximity.

The CDD applied LAPS in previous studies and obtained useful data to refine our models of cannabinergic ligands binding to CB1 and CB2 [10-13]. Utilizing the NCS moiety, which is known to primarily react with the thiol (−SH) unit, as the covalent functional group, we isolated the cysteine residues of hCB2 on helix 6 (C6.47(257)) as a critical residue for ligand covalent attachment and an agonist function, and helix 7 (C7.38(284) and C7.42(288)) for covalent labeling and an inverse agonist/antagonist signaling transmission [10, 12].

To further explore the nature of covalent binding we designed aliphatic azido-containing compounds and examined their mechanisms of binding and their functional effects on hCB2. Light-sensitive aromatic azides have been introduced in photo-affinity reagents since 1969, and became widely used because of chemical stability in physiological conditions in the dark, and
their ability to be activated upon the short-wavelength ultraviolet (UV) illumination [14-16]. Upon UV irradiation, the azido group forms a nitrene accompanied by the elimination of nitrogen gas (Scheme 4.1). The reactive nitrene is able to react with nearby nucleophiles in the binding region of a receptor [17, 18]. Azides have been applied as valuable intermediates during organic synthesis and functional groups in pharmaceuticals [16, 19-21].

![Scheme 4.1 Possible mechanism of nitrene formation. An azido ligand is irradiated by a short-wavelength UV light source to generate a nitrene intermediate.](image)

We previously performed mass spectrometry (MS)-based characterization of aliphatic azido ligands binding to helical peptides that mimic hCB2 helix 6 to identify the covalent attachment site and refine our detection methods. We found that when activated, the aliphatic azido probe selectively and covalently reacted with the cysteine residue. Among 20 amino acids, cysteine is the most nucleophilic amino acid, followed by histidine and lysine [22-24]. Serine may serve as a nucleophile at the active site of enzymes, such as fatty acid amide hydrolase and monoacylglycerol lipase [25-28]. The MS study confirmed the expectation that the cysteine residue on the peptide is the covalent attachment site of that azido ligand (unpublished results). Based on the reaction scheme and these MS results, we performed covalent binding experiments for two aliphatic azido cannabinergic ligands with full-length intact hCB2 (Figure 4.1).

In Chapter 3, we discuss the high-affinity ligand AM993, which carries a methylene-azido (−CH$_2$N$_3$) side chain linked to the adamantyl unit at the C3’ position, and show that it covalently labels hCB2 to 60% of the available sites. We determined that its analog AM994 (−CH$_2$NCS)
reacts with both cysteine residues on helix 7 of hCB2, and functions as an inverse agonist/antagonist. Another azide AM8135 was designed based on a reported megagonist AM841 (−NCS) that binds to the helix-6 cysteine on hCB2 irreversibly [10].

In this LAPS work we first determined that AM993 and AM8135 have high reversible binding affinities to hCB2, and they covalently label the receptor by 60% and 85%, respectively. We found that each azide functions in a similar fashion with its isothiocyanate counterpart on hCB2. AM993 is an inverse agonist/antagonist on CB2, and AM8135 is a CB2 agonist. We performed experiments against a cysteine-to-serine mutant library to identify which cysteine residues on hCB2 are critical for the two azides covalent labeling. Unexpectedly, the cysteine residues in the transmembrane-helix bundle of hCB2 are not involved in the covalent attachment. To foreclose the possibility that the azido group may react with serine, we additionally replaced cysteine to alanine on helices 6 and 7. The new mutants do not affect the covalent labeling on hCB2 for either aliphatic azide.

These observations did not match the hypothesis that the two azides would irreversibly react with cysteine on hCB2 upon UV irradiation, and this may be due to several reasons. The most critical is that the previous MS study used a synthesized peptide to mimic the structure of helix 6 of hCB2, while the present test was performed on the intact hCB2 in the tertiary conformation. It is likely that the binding motif of the ligand, the flexibility of the functional groups and the conformational restrictions imposed on the receptor by occupation of the binding site all play a role on the final covalent reaction that takes place.
4.2 Materials and Methods

4.2.1 Materials

General laboratory chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) at the highest purity/grade unless otherwise noted. AM993 was kindly provided by Dr. Marcus Tius, University of Hawaii (Honolulu, HI). AM8135 was synthesized in the CDD, Northeastern University (Boston, MA). CP55940 and [³H]-CP55940 were kindly supplied by the
National Institute of Health (Bethesda, MD). pcDNA 3.1+ was obtained from Invitrogen (Carlsbad, CA). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). The DC colorimetric protein assay system was from BioRad (Hercules, CA). The UV light source was Spectrolinker XL-1000 UV Crosslinker (Spectronics Corporation, Westbury, NY).

4.2.2 Site-Directed Mutagenesis, Cell Transfection and Culture

Full-length wide type hCB2 and mutant receptors were stably transfected and maintained in human embryonic kidney 293 (HEK293). The hCB2 cysteine-to-serine/alanine mutant library was generated through site-directed mutagenesis (Stratagene, La Jolla, CA) (Figure 4.2, generated by Dr. Richard W. Mercier) [10, 29]. Two additional modifications were established and primers used to generate C6.47(257)A and C7.38(284)7.42(288)A were: Forward 5’-GCT GTG CTC CTC ATC **GCC** TGG TTC CCA GTG CTG-3’, Reverse 5’-CAG CAC TGG GAA CCA **GGA** GAT GAG GAG CAC AGC-3’, Forward 5’-GCT TTC **TCC** TCC ATG CTG **TCC** CTC ATC AAC TCC-3’, Reverse 5’-GGA GTT GAT GAG **GGA** CAG CAT GGA **GGA** GAA AGC-3’. Techniques and materials applied were described in the literature and Chapter 2, Materials and Methods section [10, 30].
4.2.3 Cell Membrane Preparation and Expression

Transfected HEK293 cells expressing wild type hCB2 and mutant receptors were previously determined as functional line [10, 12]. Membrane preparations were obtained as previously described [30-32]. Saturation binding assays were performed to examine the expression quality [1, 33]. Experimental details of membrane preparations and saturation assays were described in Chapter 2, Materials and Methods section.

4.2.4 Covalent Affinity Labeling Assay

We performed assays on wild type and mutated hCB2 receptors [10, 12]. Reversible binding affinity values of AM993 and AM8135 on hCB2 were obtained from competition assays. For covalent assays samples were exposed to 245-nm UV for 1 min because the photoactivatable azide has the highest absorption in this UV range [9, 17]. Experimental details were described in
Chapter 3, Materials and Methods section.

4.2.5 cAMP Assay

Functional evaluation was performed by a time-resolved fluorescence resonance energy transfer (TR-FRET) cyclic adenosine monophosphate (cAMP) assay, measured as the inhibition of cAMP stimulated by forskolin in a cell-based bioassay (performed by Dr. Aneetha Halikhedkar) [30, 34]. Experimental details were described in Chapter 2, Materials and Methods section.

4.3 Results

4.3.1 Covalent Attachment on Wild Type hCB2

The binding affinity (K_i) of AM993 is 9.5 nM, and K_i of AM8135 is 1.5 nM, obtained from competition assays. Both ligands have suitably high reversible binding (low nanomolar range) to justify testing irreversible labeling levels on the receptor in covalent assays. For each azide, the B_{max} value for the azide-treated curve decreases significantly compared to the neat control (Figures 4.3). The decrease signifies that a number of specific binding sites are occupied by the ligand during the photoactivation, and thus, these sites are no longer available to bind with [³H]-CP55940. The efficiency of covalent labeling was quantified by calculating the ratio of the difference in B_{max} between the control and the treated sample. AM993 covalently labels wild type hCB2 at a level of 60%, and AM8135 occupies the binding sites of 85%.

We performed control experiments using analogs of the same compounds that lack the covalent N_3 groups. AM10503 (–CH_2NH_2), the analog for AM993, showed a significantly low affinity for reversible binding on hCB2 (K_i > 1,000 nM, see Chapter 3). The analog of AM8135 is AM4056 that has no apparent covalent binding on hCB2 [10]. These results confirmed that the
irreversible bonds formed by the aliphatic azides (AM993 and AM8135) and hCB2 are due to the addition of the azido functional group.

![Covalent Assay of AM-993 (hCB2)](image)

![Covalent Assay of AM-8135 (hCB2)](image)

**Figure 4.3** Saturation binding curves demonstrating the effect of azide treatment on wild type hCB2. Pre-treatment with AM993 (top) eliminates the specific binding sites by 60%, and (bottom) AM8135 by 85% upon UV irradiation.

### 4.3.2 Functional Evaluation at hCB2

The intracellular cAMP levels in cells pre-treated with forskolin were recorded after the addition of AM993 and AM8135 (done by Dr. Aneetha Halikhedkar). Functional assays were
performed without UV irradiation and Table 4.1 lists the parameters obtained from functional curves for the two ligands.

The addition of AM993 does not affect the cAMP levels at low ligand concentrations. However, at micromolar concentrations the cAMP levels increase (Figure 4.4 top). This observation indicates that AM993 is a weak inverse agonist/antagonist on hCB2, because the deactivation of hCB2 has an enhancement effect in generating the second messenger cyclic AMP [6, 35]. The EC$_{50}$ value of AM993 is three orders of magnitude higher than its K$_i$, which also suggests that this ligand is very weak in its inverse agonist/antagonist function. The function of AM993 is similar to that of AM994 on hCB2.

AM8135 inhibits the concentration of cAMP with increasing ligand concentrations, as indicated by a gradual decrease in the curve (Figure 4.4 bottom). The inhibition by the presence of ligand suggests AM8135 as an agonist, because activation of hCB2 via an agonist pathway is related to the inhibition of cAMP concentration [6, 35]. The EC$_{50}$ value of AM8135 is lower but still in the same concentration range to its K$_i$, so AM8135 is not like AM841 to be a megagonist. However the cAMP assay was performed without UV irradiation so no covalent attachment is formed between the ligand and hCB2.
Figure 4.4 cAMP accumulation assays for AM993 and AM8135 with cells recombinantly expressing hCB2. Forskolin-stimulated cAMP levels in hCB2 cells are (top) enhanced by high concentrations of AM993, suggesting an inverse agonist/antagonist, and (bottom) decreased by AM8135, suggesting an agonist.
Table 4.1 Forskolin-stimulated cAMP accumulation at hCB2 cells

Forskolin-stimulated cAMP assays using stably transfected hCB2 cells. Kᵢ values are listed to compare with the EC₅₀ values for each ligand. Efficacy is measured as a decrease (-) or increase (+) in cAMP concentrations.

<table>
<thead>
<tr>
<th></th>
<th>EC₅₀ (nM)</th>
<th>Efficacy (%)</th>
<th>Kᵢ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM993</td>
<td>6685</td>
<td>+219.8</td>
<td>9.62</td>
</tr>
<tr>
<td>AM8135</td>
<td>0.367</td>
<td>-67.85</td>
<td>1.46</td>
</tr>
</tbody>
</table>

4.3.3 Characterization of Mutant Expression

The HEK293 cell lines that expressed either wild type hCB2 or cysteine-to-serine/alanine mutants were established and tested to be functional (by Dr. Ying Pei and Dr. Richard W. Mercier) [10, 12]. Expression quality of cysteine-to-serine lines was shown in Chapter 2, Results section, and that of cysteine-to-alanine lines was shown in Table 4.2 and Figure 4.5.

Table 4.2 Expression for hCB2 wild type and cysteine-to-alanine mutants
Saturation assays examine the expression for wild type hCB2 and mutants after membrane preparations.

<table>
<thead>
<tr>
<th></th>
<th>Bₘₐₓ (pmol/mg)</th>
<th>Kₐ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>11.3 (10.9~11.8)</td>
<td>2.04 (1.75~2.33)</td>
</tr>
<tr>
<td>C6.47(257)A</td>
<td>3.10 (2.90~3.31)</td>
<td>0.82 (0.57~1.06)</td>
</tr>
<tr>
<td>C7.38(284)7.42(288)A</td>
<td>6.36 (6.01~6.70)</td>
<td>1.01 (0.79~1.24)</td>
</tr>
</tbody>
</table>
4.3.4 Covalent Labeling of AM993 on hCB2

We performed covalent experiments against the mutant series for AM993 to identify its covalent attachment site(s) on hCB2 [12]. With experimental errors in the permissible range, the repeated trials of individual mutant receptors are shown as a bar graph (Figure 4.6), which summarizes the percentage of covalent labeling of AM993 on wild type and mutant receptors.
obtained from saturation curves (not shown here). Contrary to expectations, mutation of cysteine residues did not affect the covalent attachment of AM993 on the receptor. All cysteine mutants show covalent labeling levels of approximately 60%, which is similar to that of wild type hCB2. None of the cysteine-to-serine mutations interferes with the covalent binding.

Although serine is much less nucleophilic than cysteine, it represents the catalytically nucleophiles in activity studies of enzymes [25-28]. For example, S241 in the serine-serine-lysine triad of fatty acid amide hydrolase (FAAH) is an essential residue for enzymatic activity because the mutant S241A completely inhibits the activity of FAAH [25, 36]. Most FAAH inhibitors have been discovered by work on this enzyme by covalent modification on S241 at the active site [37].

Thus there is a possibility that a covalent attachment occurs between the azido group and the serine residues that were mutated in place of cysteine, because side chains of cysteine (−SH) and serine (−OH) share similarities such as nucleophilicity and polarity. Therefore, we modified the most likely binding residues to alanine on helix 7 of hCB2 because alanine has a non-polar and non-nucleophilic side chain (−CH₃). Alanine is of a small size, so the replacement does not bring significant interference to the protein conformation, and we expected that AM993 would no longer covalently modify the receptor of a double alanine mutation.

Unexpectedly, the double mutation, C7.38(284)7.42(288)A tested with the same protocol, showed the same covalent labeling level (60%, Figure 4.6) by the treatment of AM993 under UV irradiation as the wild type. This observation suggested that neither of the two helix-7 cysteine residues was involved in the covalent attachment of AM993 on hCB2. So, none of the cysteine residues in the transmembrane-helix bundle of hCB2 are involved in the covalent bond formation with the azido functional group of AM993.
AM993 Covalent Labeling on hCB2 WT and Mutants

Figure 4.6 Covalent labeling by AM993 of hCB2 wild type and mutant receptors. Results are the means of at least 3 independent experiments performed in triplicate.

4.3.5 Covalent Labeling of AM8135 on hCB2

We further examined whether AM8135 can covalently bind to C6.47(257) of hCB2 in a similar manner as the isothiocyanate counterpart AM841. The results from repeated experiments (Figure 4.7) clearly show that each cysteine-to-serine mutant retains a similar covalent labeling level as the wild type of approximately 80%. Thus, no cysteine-to-serine mutation influences the ability of hCB2 to form a covalent bond with AM8135.

Alanine was introduced to replace the cysteine C6.47(457) based on the same concerns mentioned above. We tested the mutant C6.47(257)A that has little influence to the receptor conformation to foreclose the possibility that the azide of AM8135 reacted with C6.47(257)S covalently. We found that it had no effect to ligand covalent attachment, either (Figure 4.7). Therefore, the covalent attachment of AM8135 on hCB2 does not occur at C6.47(257), or other
cysteine residues in the membrane-spanning domain, but at a different amino acid.

Figure 4.7 Covalent labeling by AM8135 of hCB2 wild type and mutant receptors. Results are the means of at least 3 independent experiments performed in triplicate.

In summary, the two high-affinity aliphatic azides, AM993 and AM8135 covalently bind to hCB2. As with its respective isothiocyanate analog, AM993 functions as a weak inverse agonist/antagonist, and AM8135 is an agonist on hCB2. However, our results from the mutant covalent experiments do not agree with the initial hypothesis. The azido groups on the ligands do not react with cysteine residue(s) in the transmembrane domain by formation of covalent bonds.

4.4 Discussions

4.4.1 Aliphatic Azides are Covalent Cannabinergic Ligands

Traditionally, the photoactivatable azides are used as covalent probes, such as the aryl azides used as crosslinking reagents and the aliphatic azides that are accessible by nucleophiles [15, 17].
The covalent link formed between an aliphatic azido cannabinergic ligand and cysteine – a nucleophile – was confirmed by mass spectrometry in previous LAPS experiments (unpublished results).

Aliphatic azido cannabinergic ligands were described in **Chapter 3**, such as AM991 (−N₃) and AM993 (−CH₂N₃), whose side chains are linked to the adamantane units. The two ligands have high binding affinities (Kᵢ < 25 nM) on hCB2 and label the receptor irreversibly. Compared to AM991, AM993 has a two-fold higher level of covalent labeling because of the presence of an extra carbon in the side chain that allows AM993 to improve hydrophobicity and flexibility to fit in the hydrophobic binding domain of hCB2 [38].

In this study we found that AM8135, an analog of the megagonist AM841 with an azido group at the terminal carbon atom of the C3-side chain, is a high-affinity ligand and covalently binds to hCB2 by 85%. The irreversible attachment of AM8135 is due to the azido moiety because we proved that the analog lacking the N₃ group had no apparent covalent labeling on hCB2 [10]. Covalent ligand binding assays have identified more aliphatic azido cannabinergic ligands that react with hCB2 covalently; however, many of these do not show a sufficiently high level of labeling (> 50%) for further assays on the mutants. These results confirmed that the azido functional group is a covalently active group, and the aliphatic azido cannabinergic compounds which were developed in the CDD can react with the cannabinoid receptors irreversibly.

**4.4.2 Two Azides Do Not React With Cysteine**

Results of functional evaluations of AM993 and AM8135 at hCB2 were consistent with the data from the isothiocyanato counterparts. AM994 (−NCS) and AM993 (−N₃) are weak CB2 inverse agonists/antagonists, and AM841 (−NCS) and AM8135 (−N₃) are CB2 agonists [10].
similarity in functional effects of each pair of analogs suggests the same binding site(s) on hCB2 to activate the $G_{i/o}$ protein in the same pattern. Therefore, we hypothesized that each pair of analogs should react with the same cysteine residue(s), and tested the cysteine mutant series. Unexpectedly, neither of the azido compounds, AM993 or AM8135, reacts with the cysteine residues in the transmembrane region of hCB2.

Mostly the mutant receptors in the LAPS study are modifications of the transmembrane cysteine to serine. The two amino acids are conservative, isosteric analogs, and have polar side chains, and the replacement does not bring significant interference to the protein conformation and function, or cell phenotype [10, 12]. The cysteine residues in the membrane-spanning helices are not engaged in disulfide bond formation; furthermore, the cannabinoid receptors commonly lack the disulfide linkage between extracellular loops 2 and 3, which is conserved in most family-A GPCRs [39, 40]. So the cysteine modification does not cause changes in protein secondary and tertiary conformations.

Although we rarely mutate non-helical cysteine residues scattered on the loops and the N- and C-terminals, it does not affect the reliability of the present mutant series. AM993 and AM8135 can compete with CP55940, and thus, they occupy the orthosteric binding site, and upon UV irradiation, must covalently occupy this binding site in the transmembrane domain of hCB2. In addition, by interacting with hCB2, either ligand can activate the $G_{i/o}$ protein and initiate a particular signaling pathway to affect the cAMP concentrations. This observation further supports our hypothesis that the ligands are in the transmembrane binding pocket where they induce functional and conformational changes.

We considered the possibility that the azido groups react with serine because it also has a weak nucleophilic character. To foreclose the possibility we replaced the cysteine residues on
helices 6 and 7 of hCB2 to alanine. However, the ligands retained the same level of covalent labeling on the cysteine-to-alanine mutants. So, AM993 and AM8135 did not react with serine. Thus, the transmembrane cysteine residues of hCB2 are not the covalent binding sites for AM993 or AM8135.

4.4.3 Result Analysis and Possible Directions

The early MS-based characterization demonstrated that cysteine is the covalent binding site for the alkyl azido cannabinergic compounds, but our LAPS study found that the covalent links between the two azido ligands and hCB2 do not occur at cysteine. These contradicting results may be due to several reasons, of which the most critical one is the target protein preparations that were applied in two studies.

In the MS study, the azide was tested on a model peptide corresponding to helix 6 of hCB2, on which the cysteine residue was easily exposed to the surface and thus, formed a covalent bond with the ligand upon UV irradiation. In our experiments, we performed binding assays using the full-length, intact hCB2 in the tertiary conformation. The cysteine-mutant receptors were intact proteins as well. As the receptor is well structured, the cysteine residues are less exposed compared to those in the helical peptide, but still exposed enough to the receptor binding pocket. However, the different covalent groups (−NCS and −N₃) may affect the reversible binding motifs of the ligands in the pocket of hCB2, and thus, cause the different patterns of covalent attachment with the receptor.

A comparison of the two covalently active groups leads to the following considerations. When an isothiocyanato (−N=C=S) group reacts with the cysteine thiol (Cys−SH), the addition reaction occurs at the nitrogen-carbon double bond (π bond). The thiol group undergoes a nucleophilic addition to remove the π bond by creation of two σ bonds. The final product has a
structure of $\text{NH} - \text{C(=S)} - \text{S} - \text{Cys}$ (Scheme 4.2). Thus, the reaction takes place on the carbon atom, the second atom in the NCS moiety.

Thus, the reaction takes place on the carbon atom, the second atom in the NCS moiety.

![Scheme 4.2](image)

**Scheme 4.2** Addition reaction of an isothiocyanate and cysteine. An addition reaction occurs by incubation and forms a covalent bond.

Although the azido group has three nitrogen atoms, upon UV illumination, nitrogen gas is eliminated (Scheme 4.1). The intermediate nitrene undergoes a rearrangement to form an imine (Scheme 4.3), so the subsequent reaction occurs between the amino acid and the double bond of the imine. When reacting with hCB2 the activated azido group is relatively shorter than the isothiocyanato group. The length difference is about 1.29 Å based on the distance of the nitrogen-carbon double bond. This may explain why AM993 and AM8135 do not bind to the receptor in the same pattern of their isothiocyanato counterparts. The ligand cannot reach any cysteine residue in the proximal area of the azido group upon UV activation.

![Scheme 4.3](image)

**Scheme 4.3** The nitrene forms an imine through rearrangement

Although we did not identify the exact covalent binding sites for AM993 or AM8135 on hCB2, our results still provide us some hints for future research, which may include extending the mutant library to other target amino acids. Apart from cysteine sulfhydryls, histidine imidazoles and lysine amines also serve as nucleophilic binding sites in proteins, although
histidine and lysine are much weaker than cysteine in nucleophilicity [22-24]. Histidine is relatively stronger than lysine because at physiological pH, the higher pK$_a$ values render the side chain and the lysyl amino group significantly less nucleophilic.

There are two histidine residues in the transmembrane domain of hCB2. The one on helix 5 is too deeply located, very close to the intracellular side, so it is unlikely to be involved in the ligand binding (not shown). The other one is at the beginning of helix 6, H6.57 (Figure 4.8). Although the side chain faces the lipid bilayer according to the model, this histidine is a potential candidate that should be experimentally tested. Only one lysine residue is located in the transmembrane bundle and it is K3.28 on helix 3 (Figure 4.8). Its side chain points towards the binding pocket of hCB2, which makes this residue a likely binding site. H6.57 and K3.28 are primary sites to test in future experiments.

![Figure 4.8 Histidine and lysine located in the transmembrane domain](image)

Leucine is another candidate for mutation. Although it is not a nucleophile it is known to react with the azido group via Staudinger reactions [41]. In addition, some leucine residues located on helices 6 and 7 of hCB2 are very close to the cysteine residues (Figure 4.9), such as L6.52, L7.41 and L7.43. These sites should be considered because they may have a chance to
form covalent bonds with the two azides due to their locations.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has shown its advantage in a previous LAPS study [42]. It should be a useful and direct way to identify azide covalent attachment sites on a full-length intact protein, such as hCB2. However, we met practical issues during protein purification and digestion. Future studies will require improvements in methods for cell expression and protein purification as well as sample preparation in order to develop more efficient identification of covalently labeled residues with the GPCR using LC/MS/MS.
4.5 References


36. Patricelli, M.P., M.A. Lovato, and B.F. Cravatt, Chemical and mutagenic investigations of fatty acid amide hydrolase: evidence for a family of serine hydrolases with distinct


CHAPTER 5:

PHOTOLABELING CANNABINOID RECEPTOR 2

WITH C3-HETEROARYL CANNABINERGIC COMPOUNDS
5.1 Introduction

G-protein coupled cannabinoid receptors 1 and 2 (GPCR CB1 and CB2) are potential drug targets for the treatment of a variety of maladies, such as pain relief and inflammation [1, 2]. The best known classical cannabinoid is a phytocannabinoid, Δ⁹-tetrahydrocannabinol (Δ⁹-THC) present in a wide concentration range in different cannabis [3]. The known phytocannabinoids exhibit moderate receptor binding affinity and signaling profiles in vitro; Δ⁹-THC binds with nearly equal affinity to CB1 (Kᵢ= 10 nM) and CB2 (Kᵢ=24 nM), and it functions as partial agonists [4-6].

In order to discover higher-affinity and high-selectivity cannabinergic compounds, novel cannabinergic analogs were designed based on structural information related to the interaction of compounds with their respective receptors. Significant structure-activity-relationship (SAR) studies have been performed to correlate structure with pharmacological effects of the cannabinergic compounds. It has been found that a classical cannabinoid contains a tricyclic backbone and four key pharmacophores, as discussed in Chapter 1, such as Δ⁹-THC and Δ⁸-tetrahydrocannabinol (Δ⁸-THC) (Figure 5.1) [7-9].

Figure 5.1 Classical cannabinoids
The ring that joins the C-9 northern group can bear a double bond (Δ⁹-THC and Δ⁸-THC), or be alicyclic (hydroxyhexahydrocannabinol/HHC, Figure 5.2) [9, 10]. The side chain at C3 is very critical for high-affinity binding to cannabinoid receptors [9]. Earlier work found that 3-naphthoyl and 3-naphthylmethyl tricyclic cannabinergic ligands have modest affinity for CB1 [11]. In addition, the Δ⁸-THC analog and a bicyclic analog, that bear a benzoyl unit at the C3 position, have some CB2 selectivities [12, 13].

Based on these data we designed and synthesized a series of tricyclic cannabinergic ligands that share the backbone with HHC but have modifications on the C3 side chain. In this series, analogs carry a heteroaromatic group with a carbonyl spacer at the C3 position, so they incorporate the northern β-C9 hydroxyl pharmacophore (Figure 5.2, highlighted in red) and an arylphenone group (highlighted in blue) – a photoactivatable component capable of covalent labeling on GPCRs [14-16].

We applied our Ligand-Assisted Protein Structure (LAPS) method, along with traditional medicinal chemistry, to the newly-designed C3-heteroaroyl ligands. First we obtained their SAR on the cannabinoid receptors by competition assays and chose some high-affinity compounds for further experiments as photoactivatable probes. Then we tested those compounds with different heteroaromatic groups for their potential to label the receptors covalently and to be used in LAPS. Our work described the structural features for this class of photo-affinity labels, and selected that the 3-benzothiophenyl analog became the highest selectivity to mCB2 and its benzothiophenyl group was shown to covalently label mCB2. In addition, we optimized the experimental conditions in the assay protocol of arylphenone-containing photosensitive compounds.
Figure 5.2 Chemical structures of principal cannbinergic ligands and arylphenone analogs

5.2 Materials and Methods

5.2.1 Materials
General laboratory chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) at the highest purity/grade unless otherwise noted. Arylphenone ligands were kindly provided by Dr. Marcus Tius, University of Hawaii (Honolulu, HI). CP55940 and $[^3H]$-CP55940 were kindly supplied by the National Institute of Health (Bethesda, MD). The DC colorimetric protein assay system was from BioRad (Hercules, CA). The ultraviolet light for irradiation was from a Longwave Ultraviolet Lamp Model B-100A (Black-Ray, Upland, CA).

5.2.2 Cell Culture and Membrane Preparations

Human embryonic kidney 293 (HEK293) cells that stably express mouse and human CB2 receptors (mCB2 and hCB2) were cultured as described in Chapter 2, Materials and Methods section [17, 18]. Rat CB1 (rCB1) membrane preparations were extracted from purchased unstripped rat brains (Pel-Freez Biologicals, Rogers, AR) as described in Chapter 3, Materials and Methods section.

5.2.3 Competition Binding Assays

We performed two types of competition binding assays (two-point and eight-point) for the arylphenone analogs using $[^3H]$-CP55940 as a radiolabeled ligand, as described in Chapter 3, Materials and Methods section [19].

5.2.4 Covalent Affinity Photolabeling Assay

We did covalent affinity assays with CB2 membrane preparations for ligands with $K_i$ values below 100 nM, as described in Chapter 2, Material and Method section [20, 21]. Membranes were incubated in silanized glass tubes for 30 min at a 37 °C water bath with gentle agitation. Subsequently, the samples were spread on ice-cold siliconized Petri dishes and then irradiated
with a Black-Ray long-wave ultraviolet (UV) lamp of 365 nm for 1 hr [14, 17, 22, 23].

5.3 Results

5.3.1 Structure-Activity Relationships

We did competition assays on the series of C3-heteroaroyl compounds to determine their binding affinity on rCB1, mCB2 and hCB2 (Table 5.1). Previous studies demonstrated that substituting aroyl groups for the C3-alkyl chain of the classical cannabinoid confers CB2 selectivity [11, 12]. All compounds, except compounds 40, 48 and 49, have significantly reduced affinity to rCB1 ($K_i > 1,000$ nM). Compound 40 bearing 2-benzothiopheno has no selectivity since the binding to rCB1, mCB2 and hCB2 are close. Compound 48 containing a meta-fluorine substituent has improved binding on rCB1 but not significant. Compound 49 with a meta-trifluoromethyl group has the highest affinity to rCB1 among the analogs but still CB2 selective. These results agreed with the previous work that these C3-heteroaroyl compounds are CB2 selective.

The compounds that contain a 1’-five-membered heteroaromatic ring (34, 35, 38, 39, 42 and 43) also exhibit greatly diminished affinity for CB2 ($K_i > 1,000$ nM). This is presumably caused by the lack of sufficient (hydrophobic) interaction of this ring with the hydrophobic pocket of the receptor, which typically is a determining factor in the affinity, potency and selectivity of classical cannabinergic ligands [7]. Compounds 44-47 incorporate a nitrogen atom into various positions of the aromatic ring, and they all exhibit further reduction in the affinity of CB2.

The compounds that showed an improved affinity and selectivity for CB2 have fused
bicyclic 3-heteroaroyl substitutions, such as the compounds containing 3-benzofuran (37), 3-benzothiophene (41) and 3-indole (50). The analog with 2-benzothiopheno (40) has improved potency but no selectivity, and the one with 3-(N-methylindole) (51) has low affinity for CB1 and CB2. These binding data suggest that steric factors may play a key role in the effectiveness of the 3-aroyl pharmacophore in interacting with each receptor.

**Table 5.1** Affinity ($K_i$) of C3-heteroaroyl cannabinergic ligands on CB1 and CB2 $K_i$ values were obtained from one eight-point experiment run in triplicate when a previous two-point assay showed $K_i$ values below 1,000 nM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (nM)</th>
<th>rCB1</th>
<th>mCB2</th>
<th>hCB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td></td>
<td>968</td>
<td>247</td>
<td>587</td>
</tr>
<tr>
<td>34</td>
<td></td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td>N.A.</td>
</tr>
<tr>
<td>35</td>
<td></td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td>N.A.</td>
</tr>
<tr>
<td>36</td>
<td></td>
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### 5.3.2 Photolabeling on hCB2

Replacement of the fluorine group (48) with a *meta*-trifluoromethyl group (49) yielded a compound with the highest affinity for hCB2 among the analogs. Therefore, we performed a covalent photolabeling assay on hCB2 (Figure 5.3). The percentage of covalent labeling was calculated using the ratio of the difference in $B_{\text{max}}$ of the two samples to the $B_{\text{max}}$ value of the control sample. Although compound 49 has the best affinity for hCB2, its labeling efficiency is relatively low, at only 30%. We did not do covalent experiments on other ligands that have testable $K_i$ values on hCB2 because the binding affinity is too poor to reach a concentration of 10-fold $K_i$. 
Figure 5.3 Effect of treatment of hCB2 with compound 49. Irradiation of membrane preparation containing hCB2 by a 365-nm UV lamp with compound 49 eliminates the specific binding sites on hCB2 by 30%.

5.3.3 Photolabeling on mCB2

According to SAR, the 3-benzothiophene analog (41) has the highest affinity for mCB2, and there is a significant difference between compound 41 and its 2-regioisomer 40 in affinity and selectivity vs. CB1. Thus, we performed covalent assays for both compounds 40 and 41, and the two benzothiophenone ligands exhibit the highest ability to photolabel mCB2 irreversibly (77% and 67% respectively, Figure 5.4).
Figure 5.4 Effect of treatment of mCB2 with compounds 40 and 41. Irradiation of membrane preparations containing mCB2 by a 365-nm UV lamp with (top) compound 40 eliminates the specific binding sites on mCB2 by 77%, and (bottom) compound 41 by 67%.

The 3-indolyl analog (50), which has good affinity and selectivity for mCB2, was assayed. There was no change in B_max for the sample after ligand treatment compared to the control curve (Figure 5.5). This suggests that this functional group is not covalently active on the receptor.
**Figure 5.5** Effect of treatment of mCB2 with compound 50. Irradiation of membrane preparation containing mCB2 by a 365-nm UV lamp with 50 has no influence to mCB2.

### 5.4 Discussion

#### 5.4.1 Covalent Photolabeling Protocol Development

Compounds that incorporate the arylphenone functional groups are photoactivatable. They require long-wavelength UV (365 nm) irradiation to become activated, not the typical UV light of 254 nm used for the photoactivatable azides [14]. For these analogs we found that the ideal reaction conditions were to place a 365-nm UV lamp over the well-homogenized and mixed samples plated on the silanized Petri dishes, which was to increase the surfaces exposed to the UV light.

When attempting covalent labeling of compound 40 on mCB2, we followed procedures established in the literatures (365-nm UV illumination for 15 min and on ice) [22, 23]. However, initial saturation assay suggested that compound 40 was not covalently bound to mCB2 (data not shown). Other reports suggested that the arylphenone photoactivation was time-dependent [24].
Therefore, we lengthened the incubation time from 15 min to 1 hr.

It was necessary to perform the photoactivation on ice because the proteins and compounds absorb high-intensity UV light, which generates excessive heat. We determined that upon illumination the temperatures of the samples increased by 5 °C after 15 min with no ice, measured by a thermometer placed next to the dishes. In another experiment the samples were photo-irradiated for 1 hr and on ice, and the saturation curves demonstrated that compound 40 irreversibly binds to mCB2 by over 70% (Figure 5.4).

Compounds may not fully covalently react with the receptor during a shorter period of UV-activation time, and proteins will be destroyed at an increasing temperature without ice. Therefore, we incubated the samples under a 365-nm UV lamp for 1 hr and on ice. The same procedure was applied to activate other arylphenone ligands on mCB2 (Figures 5.3-5.5).

5.4.2 Identifying Potent Arylphenone Compounds

According to the SAR results, it is clear that compounds 41, 49 and 50 are promising for further development because they have binding affinities to CB2 below 100 nM. Compound 40, a regioisomer of 41, also has potential for improvement, although its $K_i$ values are above 100 nM. Compound 49 has the best affinity for hCB2, yet this ligand does not covalently label well (30%, Figure 5.3). Similarly, compound 50 has a reasonable high affinity for mCB2, but it also does not covalently attach to the receptor (Figure 5.5), suggesting that a low $K_i$ is not indicative of the success of covalent attachment.

Interestingly, the isomers, compounds 40 and 41 have a significant difference in binding affinity for mCB2, with 41 roughly five-fold higher in affinity and selectivity. However, the two
isomers have similar covalent reactivity with mCB2, giving high levels of covalent attachment (77% by 40 and 67% by 41, Figure 5.4).

To better interpret these unexpected results we explored the rotational space available to compounds 40 and 41, as well as another previously reported CB2-selective compound, the 3-vinyladamantyl analog AM755 (structures in Figure 5.1) [25]. Shown in Figure 5.6 is a comparison of the steric similarity between the respective pharmacophores for the two analogs with greater CB2 affinity and selectivity (41 and AM755), to the conformational spaces of the non-selective compound 40 (produced by Dr. Anna L. Bowman) [26]. The conformational spaces of compound 41 and AM755 are more similar to each other than to the conformational space of compound 40. This underscores the steric factors associated with mCB2 binding, suggesting a pharmacophore model for the design of higher-affinity CB2-selective compounds.

The limited binding data does not represent a full exploration of the arylphenone pharmacophore for the CB2 receptors. This will be attempted in future work when a larger database becomes available.
Figure 5.6 Comparison of rotational spaces on mCB2 for 40, 41 and AM755. Accessible conformers within 6 Kcal/mol of the global energy minimum for 40 (magenta), 41 (orange) and AM755 (blue). Analogs are shown superimposed at their aromatic rings in stick representation.
This project explored the interaction of a novel series of cannabinergic analogs bearing 3-heteroaroyl functional groups with CB1 and CB2, and their potential for use in our LAPS studies. According to the SAR results, compounds with an arylphenone component instead of the typical C3-side chain are CB2 selective. We regard compound 41 as the lead compound because it has the best affinity and selectivity for mCB2 and a high level of covalent attachment, suggesting that the 3-benzothiopheno group has potential as a covalent moiety to be incorporated in cannabinergic photolabeling probes. We also developed the covalent labeling protocol for ligands that require long-wavelength photoactivation, and established experimental conditions for a robust and accurate assay technique.

Supplemental information: Principle C3-heteroaroyl cannabinergic ligands were numbered in the CDD database as AM967 (41), AM983 (49), AM989 (40), and AM973 (50).
5.5 References


17. Pei, Y., et al., Ligand-binding architecture of human CB2 cannabinoid receptor: evidence


CHAPTER 6:

CONCLUSIONS
6.1 Conclusions

As a potential therapeutic target, the G-protein coupled human cannabinoid receptor 2 (GPCR hCB2) has drawn increasing attention [1-5]. This dissertation centers on an investigation of hCB2 binding sites and the mechanism of its activation utilizing Ligand-Assisted Protein Structure (LAPS). This experimental methodology, formulated within the Center for Drug Discovery (CDD), combines novel drug design, molecular biology, proteomic characterization and molecular modeling to reveal details concerning the active site of target proteins and mechanisms that govern protein function upon occupation of that binding site by a ligand [6, 7].

Part of this approach relies heavily on the use of covalent ligands that have been specifically designed to identify different aspects of ligand affinity and/or function. Cannabinergic ligands with a covalently active group must enter the binding pocket of hCB2 and therefore, must have a reasonable high reversible binding affinity to the receptor. When the covalent group attached to the ligand is in close-enough proximity to a particular amino acid with an appropriately reactive side chain, it reacts with that residue to form a covalent link with the receptor, which results in irreversible ligand labeling. The occupation of the cannabinergic ligand within the binding site then initiates the corresponding functional activity such as the activation of the associated Gi/o proteins [2, 5, 8]. The fact that the ligand is permanently tethered to that site allows a more detailed analysis of the binding location, and furthermore, has led to interesting functional consequences that may be analyzed and interpreted with respect to the binding data.

Based on LAPS studies to date, we have found that cannabinergic ligands that are covalently engaged at different residues of hCB2 function differently (synergistically or antagonistically). We identified four transmembrane cysteine residues, out of five transmembrane cysteine sites, on hCB2 that are involved in ligand covalent attachment and signal transmission [6, 7, 9, 10]. Those
cysteine-containing binding sites are located on three helices (2, 6, and 7), and form covalent bonds with high-affinity cannabinergic ligands with different covalently active groups belonging to different structural classes. We now have data concerning all the identified cysteine residues on hCB2 that are involved in covalent ligand attachment, as well as the corresponding functions attributed to different cannabinergic compounds, examined to date.

Table 6.1 highlights compounds that have high reversible binding affinity on hCB2 and irreversibly react with the transmembrane cysteine residues through their respective covalent groups. We postulated that by adopting distinct motifs within the transmembrane binding site of hCB2, the ligands cause different conformational changes of the receptor and correspondingly activate an agonist or inverse agonist/antagonist function. Several compounds listed in the table, namely AM841, AM1336 and AM9017 have been previously reported whereas the other ligands are presented for the first time in this dissertation [6, 7, 11].

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<td>inverse agonist/antagonist</td>
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<td>AM994</td>
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*: Additional LAPS experiments required
The different cysteine residues on hCB2 are highlighted in different colors in Figure 6.1 (generated by Dr. Anna L. Bowman). The helix-1 cysteine of hCB2 has been shown to be unreactive with cannabinergic ligands, and has no known function. According to molecular modeling studies, this cysteine residue faces the binding pocket but is sterically blocked by the helix-7 methionine residue M7.40(286) [12]. Therefore, C1.39(40) is unlikely to be involved in ligand attachment or signal transmission, and it was not further studied or discussed in this dissertation.

The cysteine C2.59(89) is believed to be located on the interface between helices 2 and 3 according to the modeling, and is not blocked by other amino acids [12]. This cysteine was not as well studied as the four other native cysteine residues located in the transmembrane region. However, we demonstrated that the cysteine C2.59(89) is a ligand binding site that is accessible to covalent cannabinergic ligands. AM4073 and AM4099, two classical cannabinergic compounds that have the same chemotype as AM841, were found to covalently bind to hCB2 by forming covalent bonds with C2.59(89) exclusively and both compounds initiate agonist functions. This is the first report identifying the helix-2 cysteine C2.59(89) as a potent binding site for covalent cannabinergic compounds, and also as part of an agonist binding pocket of hCB2.

Cysteine C6.47(257) is located in the CWFP motif on helix 6 of hCB2, and is identified in the models as the most deeply located transmembrane cysteine [13]. The CWFP motif forms a hinge in the middle of helix 6 because of a kink induced by the proline residue. C6.47(257) is an important residue for agonists interacting with hCB2 as it is postulated to undergo a conformational change as part of the rotamer toggle switch upon hCB2 binding of an agonist [13-15]. We previously identified C6.47(257) as a covalent attachment site for AM841, and that
the covalent modification of this residue triggers an agonist pathway [7, 11]. The isothiocyanato (-NCS) functional group at the terminal carbon atom on the C3-alkyl chain of AM841 reacts with this cysteine on hCB2, and this ligand functions as a very potent agonist (megagonist) with high potency [7]. As an anandamide analog, AM9017 irreversibly targets C6.47(257) on hCB2 [11]. Anandamide was previously believed to be a ligand only for CB1, however, our results suggest that anandamide-like compounds can also be high-affinity ligands of hCB2.

C7.38(284) and C7.42(288) on helix 7 are suggested to be in the upper region of hCB2. C7.38(284) is at the same approximate depth within the membrane as C2.59(89), and extracellular to C7.42(288) by one helical turn [7, 12]. We previously determined that these two residues are the covalent attachment sites for AM1336, whose inverse agonist/antagonist efficacy is significantly enhanced by formation of a covalent link [6]. We recently identified AM994 that covalently reacts with the two helix-7 cysteine residues and functions as a weak inverse agonist/antagonist for hCB2. The bulky adamantane units present in the structures most likely cause the ligands to only partially insert into the receptor because of their steric bulk. The N1-pentyl chain of AM1336 and the extra methylene spacer group of AM994 improve the ligand’s flexibility and hydrophobicity, which should increase their affinity for the hydrophobic binding pocket of hCB2, and also enable the covalent NCS groups to approach C7.38(284) and C7.42(288) [6, 16]. The structural similarities may allow the ligands to have similar binding positions in the binding pocket of hCB2 and thus, to confer proximity of the covalent group to the helix-7 cysteine residues. Their similar binding positions thus trigger similar functions, so the ligands that react irreversibly with C7.38(284)/7.42(288) are associated with an inverse agonist/antagonist function on hCB2.
Figure 6.1 Molecular modeling of cysteine-involved covalent binding sites on hCB2. The identified sites on hCB2 are in ribbon representation: C2.59(89) on helix 2 (turquoise), C6.47(257) on helix 6 (orange), and C7.38(284) and C7.42(288) on helix 7 (magenta).

Determining the connection between binding sites and signaling pathways offers important information for the discovery of new medications. AM4099 was designed with two covalent NCS groups, at its head and tail positions, for two-site covalent binding on hCB2, based on the knowledge of AM4073 and AM841. With two NCS groups, AM4099 was expected to covalently label both helix-2 and helix-6 cysteine residues. However, our results demonstrated that AM4099 reacts with C2.59(89) exclusively in the same pattern of AM4073 labeling hCB2. This suggests that AM4099 primarily reacts with helix 2 with its head NCS moiety, and the chain-terminal NCS group fails to attain proximity to C6.47(257). Although this attempt failed in regard to our expectation, we obtained important information on improving the ligand structure design by lengthening the C3-alkyl chain by one or two carbon atoms.

The LAPS experiments did not identify the covalent binding site(s) for AM993 or AM8135 on hCB2, but our results did suggest that the binding of the aliphatic azide on the full-length intact receptor is different from that of the helical peptide mimic of the transmembrane helix. According to our experiments on the cysteine mutants, the covalent labeling sites on hCB2 for the two aliphatic azides are very unlikely to be the transmembrane cysteine residues.

We introduced a new class of side-chain analogs, specifically heteroaroyl groups on the C3 position of classical cannabinergic compounds, and examined their structural-activity
relationships on CB1 and CB2 [17]. The lead compound 41 (3-benzo thiophenyl) has the highest affinity and selectivity to mCB2, and it covalently labels 67% of the receptor population. Our work demonstrated that some C3-heteroaroyl cannabinoids have good binding affinities (K_i below 100 nM) and are capable of covalent attachment to CB2; therefore, those heteroaroyl groups can be utilized in future drug design.

6.2 Future directions

The LAPS method is a universal and useful biochemical experimental approach, and it can be used to characterize ligand-receptor complex motifs with other members of the GPCR family. At the CDD, the endocannabinoid enzymes are also being studied with the LAPS methodology in order to obtain structural information [18, 19]. In our LAPS experiments on hCB2, however, we encountered some limitations in the two approaches used to determine the covalent binding sites (specific amino acid residues).

The first limitation was the hCB2 mutant library employed in our experiments. The mutant receptors, generated by site-directed mutagenesis and stably transfected into human embryonic kidney (HEK293) cells, are mostly cysteine-to-serine mutations, with some cysteine-to-alanine mutations. This library was extremely valuable in identifying the covalent binding sites for isothiocyanato cannabinergic compounds, because of the selectivity of the NCS functional group for the sulfhydryl side chain of cysteine [20, 21]. However, the azido compounds were not nearly as selective and therefore, complicated interpretation of the experimental data. When new covalent groups are introduced into the cannabinergic ligands as structure/function probes, it is critical that they are highly selective for particular amino acids in order for the LAPS methodology to be useful. A larger hCB2 mutant library would be most helpful in future
experiments. This will require development of an efficient transient transfection procedure for the receptor, because of the very time-consuming nature of creating stable transfected and functional cell lines, or an *in vitro* cell-free expression system.

Alternative amino acids should be studied in lieu of cysteine as discussed in **Chapter 4**. Examples of such amino acids that are of particular interest include L6.52, L7.41 and L7.43 (Figure 6.2). Leucine residues are known to react with azido functional groups in Staudinger reactions [22]. These particular leucine residues are close to the cysteine residues discussed above and our models place them in or near the proposed binding sites of our compounds. Lysine and histidine are good candidates for new mutations as nucleophilic binding sites, although their nucleophilicity is weaker than cysteine [23-25]. H6.57 and K3.28 are located in the transmembrane bundle of hCB2 and should be considered for testing (Figure 6.3).
Figure 6.2 Leucine residues close to cysteine residues on (top) helix 6 and (bottom) helix 7

Figure 6.3 Histidine and lysine located in the transmembrane domain
Another limitation of using the LAPS approach with the cannabinoid receptors is the difficulty of studying them using mass spectrometry (MS). As discussed in Chapter 1, MS is a powerful tool that can precisely determine the site of covalent modification via tandem MS/MS experiments [26, 27]. However, sample preparation is critical in order to obtain high quality MS data. Because of problems in expression and purification of hCB2 it is at present difficult to prepare protein samples of enough high-quality for MS characterization. In addition, tryptic digestion of the highly hydrophobic transmembrane helices is often only partial with many missed cleavages, and greatly complicates data interpretation. Therefore, future development of better recombinant protein expression of hCB2 and more efficient protein purification methods will greatly aid the MS characterization of these physiologically important and druggable GPCRs.
6.3 Reference


APPENDICES

APPENDIX I:

SUMMARY OF CANNABINERGIC LIGAND BINDING AND FUNCTION
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Functional cAMP assays were performed by Dr. Annetha Halikhedkar
Positive (+) efficacy %: inverse agonists/antagonists
Negative (-) efficacy %: agonists

### Compounds with binding tested

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*: Compounds with functions tested, results listed in Appendix I.
APPENDIX III:

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APPENDIX IV:

PUBLICATIONS
Abstract. G protein-coupled receptors (GPCRs) play critical physiological and therapeutic roles. The human cannabinoid 1 GPCR (hCB1) is a prime pharmacotherapeutic target for addiction and cardiometabolic disease. Our prior biophysical studies on the functional significance of hCB1 transmembrane helix 7 (TMH7) and its cytoplasmic extension, helix 8 (H8), [hCB1(TM7/H8)] demonstrated that the helices are oriented virtually perpendicular to each other in membrane-mimetic environments. We identified several hCB1(TM7/H8) structure-function determinants, including multiple electrostatic amino-acid interactions and a proline kink involving the highly conserved NPXXY motif. In phospholipid bicelles, TMH7 structure, orientation, and topology relative to H8 are dynamically modulated by the surrounding membrane phospholipid bilayer. These data provide a contextual basis for the present solid-state NMR study to investigate whether intermolecular interactions between hCB1(TM7/H8) and its phospholipid environment may affect membrane-bilayer structure. For this purpose, we measured $^1$H–$^{13}$C heteronuclear dipolar couplings for the choline, glycerol, and acyl-chain regions of dimyristoylphosphocholine in a magnetically aligned hCB1(TM7/H8) bicelle sample. The results identify discrete regional interactions between hCB1(TM7/H8) and membrane lipid molecules that increase phospholipid motion and decrease phospholipid order, indicating that the peptide’s partial traversal of the bilayer alters membrane structure. These data offer new insight into hCB1(TM7/H8) properties and support the concept that the membrane bilayer itself may serve as a mechanochemical mediator of hCB1/GPCR signal transduction. Since interaction with its membrane environment has been implicated in hCB1 function and its modulation by small-molecule therapeutics, our work should help inform hCB1 pharmacology and the design of hCB1-targeted drugs.

KEY WORDS: conformational switch; integral-membrane protein topology; molecular hinge; NMR; signal transduction.
brane helix 7 (TMH7) is contiguous with a small cytoplasmic helical extension, helix 8 (H8), and contains a highly conserved NPXXY motif (2,13,14). The C-terminal domain of traditional class-A GPCRs holds particular functional significance. The NPXXY motif is a candidate structural feature of GPCR activation and conformational switching among multiple GPCR activity states, and H8 is a component of the intracellular GPCR binding site to G proteins (14–17).

A prominent pharmacotherapeutic target for substance abuse, drug addiction, weight-control, and cardiometabolic disease/metabolic syndrome (18–20), the human cannabinoid receptor 1 (hCB1) is a class-A, rhodopsin-like GPCR that can be activated by intrinsic lipid mediators (endocannabinoids) and synthetic cannabimimetic agents (21). Indirect evidence from site-directed mutation studies and computational homology modeling suggests that TMH7 and H8 are critical to ligand recognition and signal transmission by hCB1 (22–26). Consequently, the importance of the hCB1 C-terminal domain to the design and targeting of drugs that modulate hCB1 activity for therapeutic benefit is well appreciated (19,20,23,26). In marked contrast to the detailed knowledge of the structural features of rhodopsin photoactivation (13–15), the interactions and dynamics between the hCB1 C-terminal region and its plasma-membrane environment across the hCB1 activity spectrum are not well characterized. The relatively limited homology between rhodopsin and hCB1—particularly with respect to their C-terminal domain—precludes direct extrapolation of the rhodopsin structure to hCB1 (25,26). Our and others’ investigations on hCB1 higher-order structure indicate that H8 acquires a helical conformation in membrane-mimetic environments with a juxtamembrane orientation parallel to the membrane surface and perpendicular to the TMH7 intramembrane bundle (4,27–30). Multiple electrostatic amino-acid interactions underpin TMH7/H8 conformation (4), and interactions between these two hCB1 α-helical segments have been implicated in ligand-induced hCB1 conformational transitioning (3).

We have recently provided NMR and site-directed spin labeling/electron paramagnetic resonance spectroscopy (SDSL/EPR) evidence of hCB1 TMH7 conformational plasticity such that its intramembrane orientation and its topology relative to H8 are influenced by membrane-bilayer thickness (3,27). These results have led us to postulate that the dynamic interaction of the hCB1 C-terminal domain with its membrane phospholipid environment could be an important modulator of hCB1 activation and determinant of hCB1 ligand pharmacology. Our postulate gains strength from accumulating evidence that GPCR/hCB1 function (ligand-binding competency, signal transmission) is influenced decisively by lipid microdomains in the plasma membrane (31–33). From the perspective of hCB1-related medicines, inhibition of cancer-cell proliferation by the first-in-class hCB1 inverse-agonist drug, rimonabant, requires hCB1 interaction with lipid rafts/caveolae (34), suggesting that lipid microdomains serve as hCB1 regulatory signaling elements potentially amenable to therapeutic exploitation (35).

The present report extends our prior investigations on the structural biology of the hCB1 C-terminal domain (3,4,27). We first recall selected results from our solution and solid-state NMR analyses of a chemically synthesized peptide corresponding to an extensive hCB1 TMH7 segment and its entire contiguous H8 domain (i.e., fourth cytoplasmic loop). This peptide is designated as hCB1(TM7/H8). The experiments allowed us to solve the NMR solution structure of hCB1(TM7/H8) in a membrane-mimetic environment, determine the precise orientation of TMH7 and H8 in model-membrane phospholipid bicelles, and detail the influence of bilayer phospholipid thickness on TMH7 structure. The results form the contextual foundation for the current study, which utilizes this hCB1(TM7/H8) peptide as reconstituted and aligned in a defined dimyristoyl-sn-glycero-3-phosphocholine (DMPC) model-membrane system. Aligned bicelle samples afford high spectral resolution and are well-accepted for analyzing the structure of membrane proteins and investigating membrane properties using heteronuclear dipolar couplings (36–39). With this experimental system, we now extend our previous findings by identifying and characterizing directly specific intermolecular interactions between hCB1(TM7/H8) and its membrane phospholipid environment. For this purpose, we have applied a highly sensitive, solid-state NMR approach with proton-decoupled local field (PDLF) sequence to measure potential 2D 1H–3C-dipolar interactions between the lipid molecules in the DMPC bilayer and hCB1(TM7/H8) reconstituted therein (40). We demonstrate that specific regional interactions between hCB1(TM7/H8) and membrane phospholipid molecules in its immediate environment reduce the lipid order parameter, suggesting that the hCB1 C-terminal domain, by partly traversing the membrane bilayer, may induce localized motion and conformational disorder/deformation in the membrane phospholipids. Since membrane environment can influence hCB1 activity and its response to small-molecule drugs (31,32,34), our findings implicate discrete intermolecular crosstalk between the C-terminal domain of hCB1 and plasma-membrane phospholipids in the regulation of hCB1 signaling.

**MATERIALS AND METHODS**

**Peptide Synthesis and Purification**

The 40-mer \[^{[77]} TVFAFCSMlAlLNSTVPNPIYAL RSKDLRHAfRSMfPSAE^{[41]}_

Sample Preparation and NMR Experiments

**NMR Sample Preparation.** Sample preparation for the solid-state NMR experiments (below) was conducted as previously detailed (3,27), yielding the aligned bicelle system composed of hCB1(TM7/H8) reconstituted in a DMPC bilayer.

**Solid-State NMR.** Solid-state NMR experiments were conducted at 37°C on a 700-MHz Bruker AVANCE II NMR spectrometer using a 5-mm double resonance probe under static sample conditions. Solid-state \(^{13}\)C NMR-oriented spectra were collected utilizing a standard cross-polarization
pulse sequence (1,5,36,41,42). A ramped cross-polarization sequence with a contact time of 5 ms was used to record the 1D $^{13}$C experiments. 2D $^{13}$C spectra were obtained at 37°C using 128 $\tau_f$ experiments, 128 scans, a 5-s recycling delay, and 20 kHz $^1$H decoupling (36).

Values from the $^1$H–$^{13}$C dipolar coupling spectrum were converted into an order parameter ($S_{CH}$) profile for the DMPC phospholipid molecule by using the relationships:

$$D_{CH} = D_O S_{CH} \left( 3 \cos^2 \theta - 1 \right) / 2$$

(1)

and

$$\Delta \nu = 2 \kappa \left[ D_{CH} + J_{CH} / 2 \right]$$

(2)

where $D_O = 21.5$ kHz is the dipolar coupling constant for rigid C–H bond; the angle $\theta = 90$ defines the orientation of the bicelle normal relative to the magnetic field; $\Delta \nu$ is the experimental splitting; $J_{CH}$ is the scalar coupling constant; and $\kappa$ = scaling factor of the homonuclear decoupling constant, which has a value of 0.42 (36).

**RESULTS**

**Precedent for the Current Work: Key Structural Features of hCB1(TM7/H8) in Membrane-Mimetic Environments**

Except for H8, very little direct structural information is available for the C-terminal region of GPCRs, including hCB1 (2, 14, 30). We previously solved the NMR solution structure of a synthetic peptide 40-mer, hCB1(TM7/H8), representing the hCB1 C-terminal domain (4) and reported the first application of solid-state NMR, along with SDSL/EPR, to study the peptide’s higher-order structure in defined phospholipid bilayers (3, 27). Since certain results from those studies provide a contextual rationale for the new solid-state NMR experiments to follow, select findings are briefly recalled here.

As illustrated by the ribbon depiction in Fig. 1a and elaborated upon in different, previously published schema (4), the structural signature of hCB1(TM7/H8) in membrane-mimetic 30% aqueous trifluoroethanol solution evidences four structurally distinct regions: the rigid TMH7 α-helix; a loop-like region interconnecting TMH7 and H8 and containing the highly conserved NPXXY motif with a proline kink; a short H8 α-helix, and an unstructured C terminus end. Multiple short-distance electrostatic interactions appear to be critical determinants of this hCB1(TM7/H8) solution structure: cation-phenolic and cation-π interactions promote formation of an interhelical microdomain that may act as a hinge during ligand-induced hCB1 conformational transitioning, as depicted in Fig. 1b and visualized differently in other schematics published elsewhere (4). Our prior solid-state NMR study of hCB1(TM7/H8) reconstituted in defined phospholipid model membranes indicates that the two hCB1 TM7 and H8 α-helical segments are oriented virtually orthogonally to each other, as they are in solution: TMH7 is disposed within the membrane bilayer, and H8 is parallel to the phospholipid membrane surface (3, 4, 27). Our molecular dynamics simulations (27) and SDSL/EPR results (3) further demonstrate that TMH7’s intramembrane conformation and relative orientation with respect to H8 are dynamically modulated by its membrane environment. A final characteristic of hCB1(TM7/H8) germaine to the present work, as first defined by us (4, 27) and substantiated by others (30), is the amphipathicity of H8: the relative disposition therein of a hydrophilic cationic cluster contralateral to a hydrophobic “face” of nonpolar residues is likely important for optimal H8 orientation/interaction with both the plasma membrane and the G-protein subunits to which hCB1 couples for information transmission and intracellular signal propagation.

**Solid-State NMR Using Proton-Decoupled Local Field Techniques**

Select data summarized above from our previous studies on the structural biology of hCB1(TM7/H8) in membrane-
mimetic environments (3,4,27) raise the following question: Does hCB1(TM7/H8) interact dynamically with membrane phospholipid in such a way as to affect bilayer molecular topology and act thereby as a determinant of membrane structure? This question prompted us to conduct additional, high-resolution solid-state NMR studies with the hCB1(TM7/H8)-DMPC bicelle system used previously and focus specifically on the phospholipid bilayer component therein. If indeed membrane phospholipid environment plays a role in defining hCB1(TM7/H8) structure and orientation, as background data summarized above indicate, then hCB1(TM7/H8) might invite structural responses by the membrane phospholipid bilayer, either along the phospholipid acyl chains and/or in the head groups. To probe for such intermolecular interactions between hCB1(TM7/H8) and DMPC membranes in magnetically aligned bicelles, we conducted 2D PDLF NMR experiments on this model system. In this approach, correlation is made between the 13C chemical shift spectrum (horizontal dimension) of the DMPC bicelle and the 1H–13C dipolar couplings (vertical dimension) of the bicelle (Fig. 2). Figure 2a shows the 1D 13C chemical shift spectrum of the DMPC bicelles, and Fig. 2b displays the 2D PDLF spectrum of the bicelles. The 2D spectrum evidences highly resolved doublets in the indirect frequency dimension due to the combined dipolar and scalar interactions between directly bonded 1H–13C spin pairs on DMPC. Most of the overlapping peaks in the chemical shift dimensions are also resolved. Notably, in the PDLF spectrum, all the resonances are resolved, which demonstrates the enhanced power of this 2D technique over 1D solid-state NMR experiments.

We next converted the dipolar coupling value for each carbon along the DMPC lipid (Fig. 3a) into an order parameter (S_CCH) to generate the profile shown in Fig. 3b. As compared to a DMPC bicelle itself, bicelles into which hCB1(TM7/H8) had been reconstituted evidenced a decrease of S_CCH (i.e., increased phospholipid-bilayer disorder) at the choline methylene (α- and β-sites) groups as well as at the glycerol protons and the DMPC acyl-chain region, but not at the terminal trimethylammonium (γ-site; Fig. 3b). The overall change in the S_CCH in the glycerol head-group region induced by hCB1(TM7/H8) is indicative of increased motion (43,44). No change was observed at the terminal alkyl group of the fatty-acyl chains, suggesting that the peptide had no significant effect on the more fluid, core region of the membrane bilayer.

DISCUSSION

Definition of the structural features associated with GPCR activation and pharmacological modulation by “drug-gable” small-molecule ligands is a major focus of translational biomedicine (2,14). However, the heptahelical, integral-membrane character of traditional, therapeutically interesting GPCRs such as hCB1 constitutes a formidable impediment to their purification as intact, functional holoreceptors for structure determination by traditional techniques such as X-ray crystallography. As an alternative, a segmental approach has been adopted by us and others whereby peptides representing the hCB1 C-terminal region have been studied by biophysical methods such as NMR and SDS/EPR for direct experimental analysis of their structural features and dynamics, since the C-terminal component of class-A GPCRs is considered to have great functional relevance (3,4,27–30). With respect to the hCB1 C-terminal region, particular attention has been focused on TM7 and its short cytoplasmic extension (H8), for these helices appear to play functional roles in selective ligand recognition and intracellular signal transmission, respectively (24,45).

Most of the work related to the structural biology of the C-terminal domain of hCB1 has focused on peptides representing hCB1 TM7 and/or H8 either in solution or, more commonly, as reconstituted into membrane mimetics. These studies have identified key structural attributes of the helices within the hCB1 C-terminal domain. For example, NMR studies from this laboratory on a synthetic peptide corresponding to an extended segment of hCB1 TM7 and its entire contiguous H8 domain, hCB1(TM7/H8), demonstrated that hCB1 TM7 is oriented virtually perpendicular to H8 whether hCB1(TM7/H8) is in membrane-mimetic solution or reconstituted into a phospholipid bilayer, the amphipathic H8 assuming an α-helical structure and juxtaplomer position quasi-parallel to the membrane surface, and TM7 spanning the bilayer (3,27). The observed structures and dispositions of hCB1 TM7 and H8 are consonant with predictions made from rhodopsin-based hCB1 homology models and are apparently
shared by other class-A GPCRs as well (2,7,14,22–26). Likewise, our experimentally defined structure for hCB1 H8 is reminiscent of that described for a synthetic peptide representing CB1 H8 in phospholipid micelles (28,29). For that peptide and a similar rat CB1 H8 peptide, an absence of secondary structure in an aqueous environment and a high degree of \( \alpha \)-helicity in dodecylphosphocholine and sodium dodecyl sulfate micelles have been observed (28,29,46). Collectively, these data help validate our segmental experimental approach to hCB1 structure and our use of NMR to analyze hCB1(TMH7/H8) reconstituted in membrane-mimetic environments.

We then sought to extend these structural descriptions of hCB1 TMH7 and H8 and probed for interactions both within hCB1(TMH7/H8) and between this peptide and its phospholipid bilayer environment that could impact hCB1(TMH7/H8) structure. Application of biophysical techniques including NMR and SDSL/EPR allowed us to demonstrate multiple close-range electrostatic interactions between amino acids as candidate structural determinants of hCB1(TMH7/H8) and implicate a flexible TMH7 region containing the conserved NPXXY domain with its proline kink as a molecular hinge that may help link TMH7 and H8 functionally (4). The flexible hCB1 TMH7 domain appears more extensive than the canonical, proline-kinked region in other class-A GPCRs (7,13,47). Nonetheless, TMH7 in both bovine rhodopsin and the human \( \beta_2 \) adrenergic receptor is bent due to a proline-based kink and likewise evidences a noncanonical distortion within the NPXXY motif (2,4,7,14). By inducing local helix distortions that modulate this region’s structure, the flexible NPXXY motif may play a crucial role in hCB1 signal transduction for effective coordination of ligand-binding and signal-transmission events between TMH7 and H8 (3,4,27,48,49). We also showed that TMH7 orientation can be altered by the thickness of the hydrophobic membrane bilayer (27), suggesting that hCB1 function may be influenced by its phospholipid bilayer environment.

We have now examined the structural biology of hCB1 (TMH7/H8) from the standpoint of its lipid environment, specifically, with respect to the peptide’s potential influence on membrane phospholipid bilayer motion and order. Two-dimensional \(^1\)H–\(^13\)C heteronuclear dipolar couplings for the DMPC choline, glycerol, and acyl-chain regions were measured in a magnetically aligned hCB1(TMH7/H8) bicelle system as compared to a bicelle lacking the peptide. The overall decrease in \( S_{CH} \) observed even at 0.5 mol% peptide indicates that hCB1(TMH7/H8) induces disorder in the membrane-lipid acyl chains and conformational changes at the phospholipids. Early 1D NMR experiments suggested that hCB1(TMH7/H8) alters the dynamic properties of the lipid component of multilamellar vesicles (43). The current 2D experiments allowed us to demonstrate directly that membrane phospholipid conformation and bilayer order are influenced by hCB1(TMH7/H8) and define discrete interaction loci responsible for the peptide’s impact on membrane structure. In particular, the change in \( S_{CH} \) we
observed when hCB1(TM7/H8) was incorporated into DMPC bicelles likely reflects specific intermembrane interactions between the phospholipid glycerol moiety and the peptide due to phospholipid head-group reorientation.

Data supporting our conclusion that hCB1(TM7/H8) can induce dynamic changes in membrane phospholipid structure have literature precedents which further suggest that such structural changes have functional significance. The interaction of cytotoxic peptides with plasma-membrane phospholipids elicits counter-directional changes at the α and β phospholipid sites and leads to the formation of pores or voltage-gated ion channels that adversely alter cell physiology (5). Stimulation of endothelial cells with fluid shear stress, hypotonic stress, or a fluidizing agent increases cellular bradykinin B2 GPCR activity (50). New data presented herein unequivocally demonstrate the occurrence of dynamic intermolecular interactions between a functionally important hCB1 signaling domain and its membrane phospholipid environment to affect the structure of the membrane bilayer at discrete loci. Collectively, these data suggest that the phospholipid bilayer itself may play a significant (mechanochemical) role in mediating GPCR/hCB1 transmission. Alterations in membrane phospholipid conformation may represent a physiological component of GPCR signal transduction. The potential for mutual structural accommodation between GPCRs (including hCB1) and their biomembrane environment to affect GPCR transmission holds significant implications for GPCR-targeted drug discovery.

Note added in proof: After submission of this work, the crystal structure of an antagonist-ligated human dopamine D3 receptor was reported (Chien EYT, Liu W, Zhao Q, Katritch V, Han GW, Hanson MA, et al. Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. Science. 2010;330:1091–1095). That publication extends the findings in references [7–14] regarding the current repertoire of GPCRs whose high-resolution X-ray structures have been solved.

ACKNOWLEDGMENTS

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C3-Heteroaroyl cannabinoids as photolabeling ligands for the CB2 cannabinoid receptor

Darryl D. Dixon, Marcus A. Tius, Ganesh A. Thakur, Han Zhou, Anna L. Bowman, Vidyanand G. Shukla, Yan Peng, Alexandros Makriyannis

A series of tricyclic cannabinoids incorporating a heteroaroyl group at C3 were prepared as probes to explore the binding site(s) of the CB1 and CB2 receptors. This relatively unexplored structural motif is shown to be CB2 selective with \( K_i \) values at low nanomolar concentrations when the heteroaromatic group is 3-benzothiophenyl (41) or 3-indolyl (50). When photoactivated, the lead compound 41 was shown to successfully label the CB2 receptor through covalent attachment at the active site while 50 failed to label. The benzothiophenone moiety may be a photoactivatable moiety suitable for selective labeling.

The known phytocannabinoids have long been known to exhibit only moderate receptor binding affinities and signaling profiles in vitro, yet they exhibit substantial potency in vivo. The best known of these classical cannabinoids, \( \Delta^8 \)-tetrahydrocannabinol (\( \Delta^8 \)-THC) binds with nearly equal affinity to the two known G-protein coupled cannabinoid receptors, CB1 and CB2. Substantial available SAR data for classical cannabinoids have shown that the northern \( \beta \)-C9 hydroxyl, C1 phenolic hydroxyl and the C3 side chain are key pharmacophores in determining receptor affinity and pharmacological potency for both CB1 and CB2. The design of novel CB1/CB2 analogues possessing higher affinities and selectivities can be based on structural information related to the interaction of cannabinergic ligands with their respective receptors. In the absence of either X-ray crystallographic or NMR data, information on the structural features of the ligand-cannabinoid receptor binding motifs can be gained through the use of carefully designed high-affinity electrophilic or photoactivatable probes. Such compounds interact with the receptor at or near the binding site and attach covalently to one or more amino acid residues. Identification of the attachment site(s) can subsequently be accomplished using targeted mutations within the receptor or by using LC/MS/MS to characterize the ligand-receptor complex. This approach that was developed in our laboratory combines the use of receptor mutants and mass spectrometry and was designated as Ligand-Assisted Protein Structure (LAPS). The present work describes our efforts to develop a new class of photoaffinity labels thus extending current work in our laboratory aimed at characterizing ligand–cannabinoid receptor binding motifs.

Earlier work from our laboratories had shown that 3-naphthoyl and 3-naphthylmethyl tricyclic cannabinoids have moderate affinities for the CB1 receptor. More recently Moore and coworkers showed that the tricyclic \( \Delta^8 \)-THC analogue 1 bearing a benzoyl unit at C3 is CB2 selective while we have shown that the bicyclic analogues such as 2 are also selective for CB2. We have now designed and synthesized a series of tricyclic cannabinoids bearing a heteroaromatic group with a carbonyl spacer at C3. Design of our novel compounds incorporates the northern \( \beta \)-hydroxyl pharmacophore as well as an arylphenone component, a photoactivatable group capable of transforming the ligand into a GPCR covalent label. Earlier work from the laboratories of Martin and coworkers and from our laboratory has shown that the northern \( \beta \)-hydroxyl enhances affinity for both receptors while imparting the molecule with enhanced polar properties and water solubility.

**Figure 1.** Aroyl cannabinoids.
Our SAR approach involves the attachment of different aryl groups to the 3-keto group of the tricyclic cannabinoid moiety.

**Chemistry.** Utilizing a strategy that has been developed in our group,\cite{1,18} we prepared bicyclic intermediate 7 via the acid catalyzed condensation between persilylated phloroglucinol 6 and a mixture of diacetates 4 and 5 (Scheme 1) following a general approach that was applied to the synthesis of nabilone by the Eli Lilly group.\cite{18} It should be noted that persilylating phloroglucinol was essential to improve solubility in the reaction medium so as to ensure a high yield of 7. Ketone 7 was subsequently treated with TMSOTf to promote the rearrangement-cyclization to yield tricyclic compound 8. Selective conversion of the C3 phenolic hydroxyl group to the corresponding triflate led to 9 in 57% overall yield from 7. Reduction of ketone 9 with NaBH4 led to a 95/5 mixture of C9 diastereoisomers in 97% yield. Simultaneous protection of the phenolic and aliphatic hydroxy groups in 10 as methoxymethyl ether groups (MOM) led to 11 in 93% yield.

As in earlier work,\cite{18} we wanted to prepare all compounds from 11, a common advanced intermediate, utilizing a cross coupling procedure. The carboxylate Stille coupling was an attractive option for the installation of the heteroaryl unit due to the variety of commercially available heteroaryl stannanes and their relative ease of preparation from their corresponding aryl bromide or iodide. Treatment of triflate 11 with a slight excess of heteroaryl stannane, LiCl, 4 Å molecular sieves (MS), 2,6-di-tert-butyl-4-methylphenol (BHT) and catalytic 1,1-dibutyl-4-tert-butylylamine (DIBAL) took place in 96% yield.\cite{18} Reduction of ketone 9 with NaBH4 led to a 95/5 mixture of C9 diastereoisomers in 97% yield. Simultaneous protection of the phenolic and aliphatic hydroxy groups in 10 as methoxymethyl ether groups (MOM) led to 11 in 93% yield.

In cases in which the stannane was either not commercially available or was unreactive, a slightly modified synthetic procedure was used in order to prepare the heteroaryl cannabinoids. We have shown in earlier work that triflate 11 can be converted to nitrile 27 (Scheme 3) in 96% yield.\cite{18} Reduction of 26 to aldehyde 28 with diisobutylaluminum hydride (DIBAL) took place in 96% yield. Nucleophilic addition of the aryllithium reagents derived from 3-bromofluorobenzene and 3-bromobenzotrifluoride to 28 followed by oxidation of the respective products with active manganese dioxide led to phenones 30 and 31 in 74% and 81% yield, respectively. Direct addition of various aryllithium or arylmagnesium compounds to nitrile 27 failed to produce the desired phenones, necessitating the two-step procedure. Exposure of 28 to indole in methanolic KOH furnished 33 in 97% yield.

Removal of the methoxymethyl ether protecting groups from 12 to 26 and 30 to 33 with TMSBr led to 3 and 34–51 in moderate to good yields (Scheme 4). The low yield for deprotection of the furyl and thiophenyl compounds can be attributed to the high nucleophilicity of the electron rich aromatic ring. Reaction with the methoxymethyl bromide that is generated during deprotection may be responsible for the appearance of byproducts. Since poor yields were also observed in these cases in the presence of poly(4-vinylpyridine), it is unlikely that the poor yields of protected products can be attributed to the presence of strong acid.

Scheme 1. Reagents and conditions: (a) pTsOH, CHCl3/acetone (4/1), 0 °C, 1 h; rt, 1 h; (b) CH3Cl, cat. DMAP, pyr, Ac2O, 0 °C to rt, 12 h; (c) KOH, MeOH, 0 °C, 1.5 h; 68% from 4 to 5; (d) TMSOTf, MeNO2, 0 °C to rt, 2.5 h; (e) Ph3P,Ni,C(O,N),CH2Cl2, 0 °C to rt; 57% from 7; (f) NaNH2, MeOH, rt, 1 h; p/x ca. 95/5, 97%; (g) MeOCl, CH2Cl2, iPr2NEt, CH3COCH3, 0 °C to rt, 2.5 h, 93%.

Scheme 2. Reagents and conditions: (a) DMF, CO, LiCl, BHT, 4 Å MS, 110 °C, ArSnBu3, PdCl2(dpdf) CH2Cl2, 24 h.

Scheme 3. Reagents and conditions: (a) CH3I, Dibal, –78 °C; 96%; (b) ArBr, n-BuLi, THF, –78 °C; (c) MnO2, CH2Cl2; 30, Ar = 3-fluorophenyl, 74%; 31, Ar = 3-(trifluoromethyl)phenyl, 81%; 32, 52% (2 steps); (d) KOH, MeOH, indole; (e) DMF, NaH, Mel, 97%.

Scheme 4. Reagents and conditions: (a) TMSBr, CH3Cl2, –40 °C, 1.5 h; 0 °C, 1 h.
Other common conditions to remove methoxymethyl ethers such as methanolic HCl or ZnBr2/HCl have also failed to improve the yields.

### Structure–Activity Relationships

Earlier work from our laboratory12 as well as from the Moore and co-workers13 explored the role of aroyl groups as substitutions at the C-3 position in the classical cannabinoid in lieu of the traditionally used alkyl side chain. It was shown13 that introduction of a 3-benzoyl substituent in a Δ9-THC tricyclic structure results in a compound (1) with high affinity for CB2. We have now extended the limited available SAR in this class of cannabinoids with a series of analogues carrying the cannabinoid receptor-favorable 9β-OH group10,15 as well as different heteroaryl groups at the 3-position. These structural modifications were aimed at identifying novel ligands and photoaffinity probes for the CB2 cannabinoid receptor with improved overall profiles. Our work has led to the discovery of a novel effective covalent probe for this receptor. The SAR of all novel arylphenone analogues was evaluated by measuring their respective affinities for the rat CB1 (rCB1), mouse CB2 (mCB2) and human CB2 (hCB2) receptors (Table 1). All synthesized novel analogues exhibited reduced affinities for both CB1 and CB2 receptors when compared with the earlier synthesized benzophenone analogue 1.13

All novel 9β-OH analogues were shown to have reduced binding affinities for both receptors when compared to the Δ9-THC analogue 1. The reason for this observation is unclear. Arguably, the additional 9β-OH group of analogue 3 may be orienting the planar benzophenone side chain differently in the receptor hydrophobic pocket to cause an overall unfavorable interaction. Our SAR data show that all analogues containing 1′-five-membered heteroaromatic ring (34, 35, 38, 39, 42, and 43) exhibited significantly diminished affinities for both CB1 and CB2 receptors. This is presumably due to lack of sufficient (hydrophobic) interaction of this ring with the hydrophobic pocket of the receptor which, in general, is a determining factor for the affinity, potency and selectivity of classical cannabinoids.10 To further probe the interaction of this 3-benzophenone group, we incorporated a nitrogen atom into various positions of the aromatic ring (44-47). All of these compounds exhibited further reduction in affinity for both CB receptors. Incorporation of a meta fluorine substituent in the aromatic ring (48) improved binding at CB1 with a slight loss in binding affinity at mCB2. Replacement of the fluorine group with a lipophilic trifluoromethyl group (49) gave encouraging results. This compound exhibited significantly improved affinity for both CB receptors (Kᵢ = 61.7 nM at rCB1, 45.8 nM at mCB2 and 37.3 nM at hCB2).

Our most promising results with regard to CB2 affinities and selectivities were observed with analogues carrying fused bicyclic 3-heteroaroyl substitutions. The best were those carrying 3-benzothiophene (40; Kᵢ mCB2 169.2 nM rCB1/mCB2 = 17-fold), 3-benzothiopheno (41; Kᵢ mCB2 34.2 nM rCB1/mCB2 = 37-fold), and 3-indole (50; Kᵢ mCB2 60.4 nM rCB1/mCB2 = 17-fold) substituents while those with 2-benzothiopheno (40) or 3-(N-methylindole; (51) had somewhat reduced affinities or CB2 selectivities. The binding data of the analogues included in this study point to steric factors playing a key role in determining the effectiveness of the 3-aroyl pharmacophore’s ability to interact with each of the two receptors. An interesting observation in our SAR is the significant difference in mCB2 affinities between the lead 3-benzothiophene analogue 41 and its 2-regiosomer 40. To better interpret these interesting results we have explored the rotational space available by these two substituents. We also included in our comparison results for the earlier published 3-vinyladamantyl analogue AM755 that also exhibited CB2 selectivity.22 A comparison of the computational data (Fig. 2) points out the steric similarities between the respective pharmacophores for the two analogues with favorable CB2 affinities and selectivities (41, AM755) and the distinct differences when their conformational spaces are compared with

### Table 1

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<tr>
<th>Compound</th>
<th>rCB1 (nM)</th>
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* Binding affinities for CB1 and CB2 were determined using rat brain (CB1) or membranes from HEK293 cells expressing mouse or human CB2 and [³²P]CP-55,940 as the radioligand following previously described procedures.20 Kᵢ values for these compounds were obtained from one experiment (8 point) run in triplicate when experiments using the two point data (in triplicate) showed Kᵢ values below 1000 nM.
the analogue (40) with lower affinity and selectivity for mCB2. The limited binding data included here did not allow us to carry out a full exploration of the arylphenone pharmacophore for the CB2 receptor. This will be attempted in future work when a larger database becomes available. However, our computational exercise underscores the steric factors associated with mCB2 binding and provide a basis for the design of higher affinity analogues.

**Photolabeling of mCB2.** To explore the value of this class of cannabinoid analogues as photolabeling reagents for the CB2 receptors we tested some of our compounds for their abilities to interact covalently with the mCB2 receptor. The experiment was carried out using membrane preparations obtained from a HEK293 cell line expressing mCB2 receptor. The procedure method development in our laboratory with cannabinergic ligands carrying different photoactivatable groups.22 While employing conditions reported earlier for labeling the NK-1 receptor with ligands incorporating a benzophenone moiety,23 the heteroaryl benzophenones tested, the two benzothieno analogues (40 and 41) exhibited the highest ability to photolabel the mCB2 receptor (77% and 67% respectively; Fig. 3). The meta-trifluorinated analogue 42 also labeled the receptor, however, less effectively.25 Conversely, the 3-indolyl analogue (50) failed to label mCB2. These very successful experiments confirm the value of the 3-arylphenone moieties as useful photolabels for the CB2 receptors.

**Conclusions.** In this SAR study we explored the value of cannabinoid analogues carrying 3-arylphenone moieties in lieu of the 3-arylalkyl chains found in the phytocannabinoid structures as potential photoaffinity ligands for the mCB2 receptor. The lead compound 41 provided evidence that the 3-benzothiophene analogue had the highest affinity and selectivity for mCB2. Our results underscore the important steric requirements of the arylphenone pharmacophoric moiety for the CB2 receptor and provide the basis for the design of later generation analogues with improved affinity profiles. Importantly, we demonstrated that 41 is capable of photolabelling the mCB2 receptor in excellent yields. This compound will be used in future studies to obtain information on the binding motifs of the arylphenone cannabinoid analogues for the CB2 receptor. Additionally, our results suggest that the 3-benzothiophene group is an excellent moiety to be incorporated in cannabinergic photolabeling probes.

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**Supplementary data.**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.06.013.