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In-Vitro Assessment of CB1/CB2 Receptor Binding and Function

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IN-VITRO ASSESSMENT OF CB1/CB2 RECEPTOR BINDING AND FUNCTION

By

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ABSTRACT
JOEY DAVIS: IN-VITRO ASSESSMENT OF CB1/CB2 RECEPTOR BINDING AND FUNCTION
(Under the direction of Dr. Nicole Ashpole)

Cannabinoid receptors CB1 and CB2 are G protein-coupled receptors that have a variety of physiological effects on the human body. Many natural product agonists and antagonists are presumed to interact with the cannabinoid receptors and are therefore heavily studied in drug discovery. Assays are used to study the binding of compounds to these receptors. However, a limited number of these assays are efficient in determining the functional activity of ligands associated with CB1 and CB2. Of those, many use radioactivity which is both expensive and potentially dangerous to the researcher. In this study, we first validated the assays already found to be effective in evaluating the function of ligands in the laboratory and then compared the results of the CB1 and CB2 assays. We found that although the cAMP-Glo and GTPγS assays were effective methods to measure functional efficacy for CB2, they were ineffective for CB1. Using compiled research methods from a number of academic journals, the establishment of a proper protocol for the functional characterization of ligands to CB1 was attempted but unsuccessful. To determine the possible reason for failure, a western blot was run to test the expression of G proteins in the inactive CB1 membrane in comparison to the G proteins in the active CB2 membrane. It was found that the G proteins in the active CB2 membrane were more heavily expressed than the G proteins in the inactive CB1

membrane. Together, these data concluded that the possible reason for our inability to establish an effective method to measure functional efficacy for CB1 is a lack of active G proteins in the membrane.

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I. Background

Cannabinoid receptors are located throughout the body and are known to have a variety of physiological effects including anti-nociceptive, antispasmodic, sedative/anxiolytic, anti-epileptic, neuroprotection, antiemetic, appetite regulation, anti-oxidant, anti-inflammatory and bronchoregulation. Two classic cannabinoid receptors are CB1 and CB2. CB1 is primarily located in the nerve cells in the brain and has a role in mood, appetite, emesis control, memory, spatial coordination, muscle control and analgesia. CB2, on the other hand, is primarily located in white blood cells in the tonsils and spleen, but it is also located in the peripheral nervous system. Its main roles are immune-modulatory, anti-inflammatory, pain and bone loss (Pertwee 2016).

Both CB1 and CB2 are known to be G protein-coupled receptors (GPCRs). These receptors are located in the cell membrane and indirectly transmit signals from extracellular substances to an intracellular molecule via a G protein. The G protein can either be inhibitory, $G_{i/o}$, or stimulatory, G_s, inhibiting or promoting the activation of adenylate cyclase, respectively. CB1 and CB2 are predominantly thought to be $G_{i/o}$-coupled receptors. Adenylate cyclase is the transmembrane protein responsible for the conversion of ATP to cAMP, which can activate a protein kinase and lead to a cellular response.

Both CB1 and CB2 are known to have multiple selective agonists and antagonists, which can be endogenously produced, produced in cannabis (phytocannabinoids), or synthetically developed. Some of these agonists and antagonists are the same for both CB1 and CB2, and some are specific to one or the other. For example, CP-55,940 is a known synthetic agonist for both CB1 and CB2, but AM251 is an antagonist for the CB1 receptor alone (Alexander et al.,

2013). These known agonists and antagonists for the CB1 and CB2 receptors can be used to test for other agonists and antagonists in *in vitro* comparison studies known largely as assays.

Cannabinoid receptor screening assays are utilized to test the binding of a variety of compounds including extracts, isolated compounds and synthesized compounds. The compounds tested in the Neuropharmacology Core Facility at the University of Mississippi are largely natural products. A natural product is a chemical compound or substance produced by a plant, animal, or microorganism that can be harvested and isolated for human use. Natural products have been important for medical and health purposes throughout human evolution, and their ability to bind to distinct inhibitors and diverse proteins implicated in human disease promotes their continued study (Ji et al., 2009).

Due to their large number and diverse effects, including the synergistic effects of their possible combinations, it is difficult to associate a single natural product or group of natural products with the treatment of a specific disease. Screening assays can test a large amount of natural products at once; they are a crucial tool in identifying natural products useful in drug discovery in a timely manner. However, there are limited *in vitro* test systems available for the functional characterization of cannabinoid receptor ligands. The establishment of sensitive functional cannabinoid receptor assays are integral to the proper assessment of natural products associated with these receptors (Cheng et al., 2007).

In a typical radioactive binding assay for a cannabinoid receptor, a receptor and radiolabeled ligand are incubated with or without a competitor in order to induce binding. Then, the mixture is pulled through filtration paper, excess ligand is washed away, and the radioactivity is quantified as a means to quantify CB1 or CB2-bound ligands. Agonists and antagonists are

used as controls to compare the affinities of the test compounds, and their competition is quantified as a Ki, the concentration of drug required to produce half maximum inhibition.

The GTPγS assay has been utilized as a method to evaluate the function of GPCRs. Upon its activation, the α subunit of a G-protein releases bound GDP binds a molecule of GTP and dissociates from the G-protein βγ subunit complex. This crucial step in the function of GPCRs plays a key role in determining overall agonist efficacy (Breivogel et al., 1998). The establishment of an efficient GTPγS assay for specific GPCRs can be difficult due to the varying levels of GDP, Mg^{2+} and Na^{+} required for observing stimulation of GTPγS binding. Moreover, depending on the composition of the host cell membrane, there may be more than one G protein responding to the receptor which can lead to flattening of the overall stimulation curve (Strange 2010).

The GTPγS assay makes use of the [^{35}S]GTPγS radioisotope to quantify binding. It is, however, preferred to use non-radioactive materials in clinical laboratories because they are expensive and dangerous to the researcher. Assays like the cyclic AMP assay have been used for the functional evaluation of other GPCR associated ligands without the use of radioactivity. cAMP is a downstream mediator of G protein signaling, thus assays of cAMP activity serve as readouts for the activation of GPCRs. For example, an efficient cyclic AMP assay was established for the functional evaluation of β-Adrenergic receptor ligands, which are associated with receptors that are G_s-coupled to adenylyl cyclase (Sandeep et al., 1998). Cannabinoid receptors, on the other hand, are known to be G_i-coupled to adenylyl cyclase and therefore inhibit the conversion of ATP to cAMP inside the cell. Agonist ligands for G_i-coupled receptors have proven to be difficult to determine because inducers of cAMP such as forskolin must also

be used in order to activate the cAMP so that the test compound can inhibit it. Therefore, these assays are sources of variations and errors (Gilissen et al., 2015). Nonetheless, many labs are turning to cAMP assays for assessing Gi-coupled GPCRs.

The first goal of this study was to validate the binding assays in the lab to allow for testing of novel natural product-based cannabinoid modulators. These results were compared between CB1 and CB2. Then our goal was to validate functional assays for CB2 expressing cells and to establish functional assays for CB1 expressing cells. The cAMP-Glo functional assay was attempted for both cell membranes first, and the GTPγS functional assay was used as an alternative method due to the fact it requires radioactivity.

II. Methods

CB1/CB2 Membrane Isolation

CHO cells expressing human CB1 or CB2 were purchased from Perkin Elmer and passaged in house. Cells were maintained in F12 media supplemented with 10% FBS, penicillin streptomycin and G418, which selected for CB1 or CB2 expressing cells. Once the cell dishes reached 90-100% confluency, they were scraped for membrane isolation. Before the membrane was scraped, the centrifuge was precooled to 4°C and the PBS, Tris-HCl and cell scrapers were placed on ice. One plate at a time, media was removed and the plates were gently washed with 10 mL of PBS in order to remove excess media and neutralize the pH of the cells.

The PBS was then removed, and 6 mL of chilled Tris-HCl was added which disengages the membrane from the plate by binding divalent cations such as calcium and magnesium, therefore destabilizing the membrane. A cell scraper was used to scrape the cells from the plate

surface, and the Tris-CB1 solution was collected using a pipet and placed into a 50 mL conical tube. 4 mL chilled Tris-HCl was added, scraped and collected to ensure all cells were removed from the plate. The previous steps were performed for 20 plates.

The 200 mL of Tris-CB1 solution was evenly divided into six 50 mL tubes, and then all six tubes were placed into a centrifuge for 10 minutes at 4°C and 3100 rpm. Once the centrifugation was complete, the supernatant from each tube was decanted and discarded. Each pellet was then resuspended in 5 mL of Tris-HCl, and the six pellets were combined into one 50 mL tube. Sonication was performed until the solution was well-mixed, and the tube was placed back into the centrifuge for 10 minutes at 4°C and 1000 x g. For this centrifugation, a tube containing 30 mL of water was needed to balance the centrifuge.

Once the second centrifugation was complete, the supernatant was decanted into a 50 mL centrifuge tube and saved for later, and the pellet was resuspended in 10 mL of fresh Tris-HCl. Then a third and fourth sonication and centrifugation was performed under the same conditions as the second. Once the fourth centrifugation was complete, the pellet was resuspended in 10 mL of the combined supernatant solution rather than Tris-HCl, and then place into a high speed centrifuge tube for a final centrifugation for 40 minutes at 4°C and 13650 rpm.

After the final centrifugation, the supernatant was removed and discarded, and the pellet was resuspended in 10 mL of fresh Tris-HCl. The final membrane solution was sonicated until it was well-mixed. Then it was aliquoted into 2 mL cryotubes (1 mL each), labeled appropriately, and placed into -80°C freezer.

BCA Protein Assay

In order to perform competitive CB1 receptor binding screening, the protein concentration in the CB1 membrane must be known. To find the concentration, a BCA protein assay was performed. First, diluted BSA standards were created. Nine microcentrifuge tubes were used, labeled A-I. In vial A, 300 µL of BSA was added; B, 375 µL of BSA was diluted in 125 µL distilled water; and C, 325 µL of BSA was diluted in 325 µL distilled water. Vial B and C tubes were vortexed to ensure homogeneity and used to create the 6 other dilutions.

In vial D, 175 µL of vial B dilution was diluted in 175 µL distilled water; E, 325 µL of vial C dilution was diluted in 325 µL distilled water; F, 325 µL of vial E dilution was diluted in 325 µL distilled water; G, 325 of µL vial F dilution was diluted in 325 µL distilled water; H, 100 µL of vial G was diluted in 400 µL distilled water; and I, 400 µL of distilled water was added. Each vial was vortexed after it was diluted to ensure homogeneity before using it as a diluent or pipetting its contents onto a plate.

The CB1 membrane solution and diluted BSA standards were pipetted onto a 96-well plate as triplicates at 25 µL per well. Then, the working reagent was prepared by mixing 50 parts of BCA Reagent A (7.5 mL) with 1 part of BCA Reagent B (150 µL), and then vortexing. 200 µL of working reagent was pipetting into each well involved in the assay. Once the plate was complete, it was incubated at 37°C for 30 minutes.

Following incubation, the BCA protein assay was read using a spectrophotometer set to 562nm. The data from the spectrophotometer was used to create a standard curve by plotting the average Blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/µL. Then, the standard curve was used to determine the protein concentration of the unknown CB1 membrane.

Ligand Binding Assay

In order to ensure we were running our competitive CB1 receptor binding screening at maximum efficiency, a ligand binding assay was performed to measure the binding of [3H]-CP55,940 to CB1 membrane. First, [3H]-CP55,940 serial dilutions were created. Ten microcentrifuge tubes were used, labeled 1-10. In vial 1, 1.6 µL of [3H]-CP55,940 was diluted in 1 mL of ROTH buffer, and it was then vortexed to ensure homogeneity before being used to create the 9 other vials. ROTH buffer contains 13.76 g (50 mM) Trizma HCl, 0.952 g (5 mM) MgCl, 2 g BSA, and 0.5 g NaOH (Roth 2018).

In vial 2, 500 µL of vial 1 was diluted in 500 µL ROTH buffer; vial 3, 500 µL of vial 2 was diluted in 500 µL ROTH buffer; vial 4, 500 µL of vial 3 was diluted in 500 µL ROTH buffer; vial 5, 500 µL of vial 4 was diluted in 500 µL ROTH buffer; vial 6, 500 µL of vial 5 was diluted in 500 µL ROTH buffer; vial 7, 500 µL of vial 6 was diluted in 500 µL ROTH buffer; vial 8, 500 µL of vial 7 was diluted in 500 µL ROTH buffer; vial 9, 500 µL of vial 8 was diluted in 500 µL ROTH buffer; and vial 10, 500 µL of vial 9 was diluted in 500 µL ROTH buffer.

After preparing the [3H]-CP55,940 dilutions, the assay controls were made. For the first control, 2 µL of the non-specific binding control, CP-55,940, was diluted in 248 µL of ROTH buffer. For the second control, referred to as no membrane, 8 µL of DMSO was diluted in 992 µL ROTH buffer. Then, the [3H]-CP55,940 dilutions and controls were pipetted onto a 96-well plate at 50 µL per well; each dilution was pipetted into eight wells, and each of the controls were pipetted into four wells.

Once all the compounds were plated, the membrane was prepared and plated. To prepare, 5273 µL of CB1 membrane was diluted in 6327 µL ROTH buffer, and then vortexed. Using a multichannel pipet, the CB1-buffer solution was then placed into a reservoir and pipetted into all wells included in the assay, excluding the no membrane control wells.

Then, the radioligand had to be added to the control wells. To prepare, 50 µL was taken from each of the 10 [3H]-CP55,940 dilutions and mixed in one microcentrifuge tube, creating a master solution. After vortexing the [3H]-CP55,940 master solution, 50 µL of it was added to the non-specific binding and no membrane wells.

After all compounds and controls were plated with membrane and radioligand, the buffer was added to some wells for a final volume of 200 µL per well. So, 50 µL was added to all [3H]-CP55,940 dilution wells and all no membrane wells. Then, the completed plate was incubated for 90 minutes at 37°C with gentle agitation. An hour into the incubation, a UniFilter GF/C plate was placed into 0.1% PEI at room temperature and allowed to soak.

Once the incubation was complete, the pre-soaked plate and cold wash buffer was transported to the radioactive room for washing. The plate was washed 14 times in order to filter out unbound [3H]-CP55,940, leaving only CB1 bound [3H]-CP55,940 on the UniFilter GF/C plate. The plate was then placed in the incubator to dry at 50°C for 15 minutes. Once dry, 50 µL of MicroScint20 was added to the entire plate, and it was sealed so that it could be read by the plate reader. The data from the plate reader was used to create a curve by plotting the CB1-bound [3H]-CP55,940 concentrations vs. the initial [3H]-CP55,940 concentrations per well. Then, the ligand binding curve was used to determine the Kd of [3H]-CP55,940.

cAMP-Glo Assay

In order to study cellular function, a cAMP-Glo assay was performed with the same cells. First, cAMP standard dilutions were created. Twelve microcentrifuge tubes were used, labeled 1-12. In vial 1, a 1.0 μM cAMP solution was created by adding 1.0 μL of 1 mM cAMP solution to 1000 μL HBSS/HEPES medium, and vortexing to ensure homogeneity before being used to create 11 other vials.

In vial 2, 100 μL of vial 1 was diluted in 100 μL medium; vial 3, 100 μL of vial 2 was diluted in 100 μL medium; vial 4, 100 μL of vial 3 was diluted in 100 μL medium; vial 5, 100 μL of vial 4 was diluted in 100 μL medium; vial 6, 100 μL of vial 5 was diluted in 100 μL medium; vial 7, 100 μL of vial 6 was diluted in 100 μL medium; vial 8, 100 μL of vial 7 was diluted in 100 μL medium; vial 9, 100 μL of vial 8 was diluted in 100 μL medium; vial 10, 100 μL of vial 9 was diluted in 100 μL medium; vial 11, 100 μL of vial 10 was diluted in 100 μL medium; and vial 12 contained 100 μL of plain HBSS/HEPES medium.

After preparing the cAMP dilutions, the assay control dilutions were made. Four microcentrifuge tubes were used, labeled A-D. In vial A, a 1.0 μM CP-55,940 solution was created by adding 1 μL CP-55,940 to 10 mL HBSS medium containing 30 μM Forskolin and 20 mM HEPES, and vortexing before used to create 3 other vials. In vial B, 1 μL of vial A was diluted in 900 μL medium; C, 1 μL of vial B was diluted in 900 μL medium; and vial D contained 1000 μL plain HBSS/Forskolin/HEPES medium.

Once all dilutions were complete, the original media was pipetted off of two rows of CB1 cells (24 wells). For the first row of 12 wells, 20 μL of each cAMP dilution was added in order from well 1 (left) to well 12 (right). For the second row of 12 wells, 20 μL triplicates of each

CP-55,940 dilution was added in order from left to right, with wells 1-3 containing 1 μM CP-55,940, wells 4-6 containing 0.1 μM CP-55,940, wells 7-9 containing 0.01 μM CP-55,940 and wells 10-12 containing 0 μM CP-55,940. Then, the plate was mixed by shaking for 60 seconds and incubating at room temperature for 15 minutes.

Next, 20 μL of cAMP-Glo Lysis Buffer was added to all 24 wells, and the plate was incubated with shaking at room temperature for 15 minutes. During the incubation period, the cAMP Detection Solution was created by combining 2.5 μL of Protein Kinase A to 1.0 mL of cAMP-Glo Reaction Buffer, and then mixing by inversion. 40 μL of the Detection Solution was then added to each well after the incubation was complete. Once added, the plate was mixed again by shaking for 60 seconds and incubated at room temperature for 20 minutes.

Once the third incubation was complete, 80 μL of Kinase-Glo Reagent was added to each well, and the plate was mixed a final time by shaking for 60 seconds and incubating at room temperature for 10 minutes. Following the final incubation, the luminescence was measured with a plate-reading luminometer. The data from the luminometer was used to create a curve by plotting the relative luminescence units vs. the cAMP standard concentration per well. Then, the cAMP-Glo curve was used to determine the EC50 of the CB1 cells.

GTPγS Assay

In order to further study cellular function, a GTPγS assay was performed using the same cell membranes that were isolated above. First, the test compound and CP-55,940 control

dilutions were created. For each, eleven microcentrifuge tubes were used, labeled 1-11 in different colors to stay organized. In vial 1 of the test compound dilution, a 10 μM compound solution was created by adding 2 μL of 10 mM compound to 498 μL of GTPγS assay buffer and vortexing to ensure homogeneity before used to create 10 other vials. In vial 1 of the CP-55,940 dilution, a 10 μM compound solution was created by adding 2 μL of 10 mM CP-55,940 to 498 μL of GTPγS assay buffer, and vortexing to ensure homogeneity before being used to create 10 other vials.

In vial 2 of each, 200 μL of vial 1 was diluted in 400 μL medium; vial 3, 200 μL of vial 2 was diluted in 400 μL medium; vial 4, 200 μL of vial 3 was diluted in 400 μL medium; vial 5, 200 μL of vial 4 was diluted in 400 μL medium; vial 6, 200 μL of vial 5 was diluted in 400 μL medium; vial 7, 200 μL of vial 6 was diluted in 400 μL medium; vial 8, 200 μL of vial 7 was diluted in 400 μL medium; vial 9, 200 μL of vial 8 was diluted in 400 μL medium; vial 10, 200 μL of vial 9 was diluted in 400 μL medium; and vial 11, 200 μL of vial 10 was diluted in 400 μL medium.

After preparing compound and CP-55,940 control dilutions, the assay vehicle, emax, non-specific binding, and no membrane tubes were created for assay controls. To prepare the assay vehicle, 500 μL of plain GTPγS assay buffer was added to a microcentrifuge tube. To prepare the assay emax, 2.5 μL of 10 mM CP-55,940 was diluted in 497.5 μL of GTPγS assay buffer. To prepare the assay non-specific binding, 20 μL of 10 mM GTPγS was diluted in 980 μL of GTPγS assay buffer. To prepare the assay no membrane, 50 μL of DMSO was diluted in 950 μL of GTPγS assay buffer.

	1	2	3	4	5	6	7	8	9	10	11	12
A	EMAX	EMAX	EMAX	EMAX	NSB	NSB	NSB	NSB	No Membrane	No Membrane	No Membrane	No Membrane
B	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	Vehicle
C	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	Vehicle
D	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	CMPD	Vehicle
E	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Vehicle
F	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Vehicle
G	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Vehicle
H	EMAX	EMAX	EMAX	EMAX	NSB	NSB	NSB	NSB	No Membrane	No Membrane	No Membrane	No Membrane

Figure 1: Plate layout example for a GTPγS assay.

After all compounds and controls were pipetted into the 96-well plate, the membrane and GDP dilutions were prepared by mixing 5273 µL of CB1 in 6327 µL ROTH buffer for the membrane and 30 µL of GDP in 3970 µL of assay buffer for the GDP before moving into the radioactive room. In the radioactive room, the radioligand was carefully prepared by mixing 2.5 µL of [^{35}S]-GTPγS to 50 mL of assay buffer, and the membrane, GDP and radioligand dilutions were added to the appropriate wells.

Then, the completed plate was incubated for 90 minutes at 37°C with gentle agitation. An hour into the incubation, a UniFilter GF/C plate was placed into 0.1% PEI at room temperature and allowed to soak. Once the incubation was complete, the pre-soaked plate and cold wash buffer was transported to the radioactive room for washing. The plate was washed 14 times in order to filter out unbound [^{35}S]- GTPγS, leaving only CB1 bound [^{35}S]- GTPγS on the UniFilter GF/C plate. The plate was then placed in the incubator to dry at 50°C for 15 minutes. Once dry, 50 µL of MicroScint20 was added to the entire plate, and it was sealed so that it could be read by the plate reader.

Competitive Receptor Binding Assay

To test the binding of a variety of ligands to the CB1 and CB2 membranes, a competitive receptor binding assay was performed using the same cell membranes that were isolated above. First, the 10 µM test compound solutions were prepared by mixing a 498 µL assay buffer to 2 µL compound for each. The total binding and no membrane solutions were prepared by mixing 992 µL assay buffer to 8 µL DMSO. Then, the non-specific binding control dilutions were made by mixing 248 µL assay buffer to 2 µL CP-55,940 for dilution 1 and doing a two-fold dilution for dilutions 2-4. The same is done for the second plate control which is AM-630 for CB1 and AM-251 for CB2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 1	Sample 1	Control 1 Dilution 1	Total Binding	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17	No Membrane
B	Sample 2	Sample 2	Sample 2	Control 1 Dilution 1	Total Binding	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18	No Membrane
C	Sample 3	Sample 3	Sample 3	Control 1 Dilution 1	Total Binding	Sample 11	Sample 11	Sample 11	Control 1 Dilution 3	Control 1 Dilution 3	Control 1 Dilution 3	No Membrane
D	Sample 4	Sample 4	Sample 4	EMPTY	Total Binding	Sample 12	Sample 12	Sample 12	Control 1 Dilution 4	Control 1 Dilution 4	Control 1 Dilution 4	No Membrane
E	Sample 5	Sample 5	Sample 5	EMPTY	Total Binding	Sample 13	Sample 13	Sample 13	Control 2 Dilution 1	Control 2 Dilution 1	Control 2 Dilution 1	No Membrane
F	Sample 6	Sample 6	Sample 6	Control 1 Dilution 2	Total Binding	Sample 14	Sample 14	Sample 14	Control 2 Dilution 2	Control 2 Dilution 2	Control 2 Dilution 2	No Membrane
G	Sample 7	Sample 7	Sample 7	Control 1 Dilution 2	Total Binding	Sample 15	Sample 15	Sample 15	Control 2 Dilution 3	Control 2 Dilution 3	Control 2 Dilution 3	No Membrane
H	Sample 8	Sample 8	Sample 8	Control 1 Dilution 2	Total Binding	Sample 16	Sample 16	Sample 16	Control 2 Dilution 4	Control 2 Dilution 4	Control 2 Dilution 4	No Membrane

Figure 2: Plate layout example for a competitive receptor binding assay.

Once all the compounds and controls were plated, the membrane and radioligand were prepared and plated. To prepare the membrane, 5273 µL of CB1 membrane was diluted in 6327 µL ROTH buffer, and then vortexed. Using a multichannel pipet, the CB1-buffer solution was then placed into a reservoir and pipetted into all wells included in the assay, excluding the no membrane control wells. To prepare the radioligand, 6.269 µL [3H]-CP-55,940 was added to the 5800 µL assay buffer and vortexed to obtain homogeneity.

Then, the completed plate was incubated for 90 minutes at 37°C with gentle agitation. An hour into the incubation, a UniFilter GF/C plate was placed into 0.1% PEI at room temperature and allowed to soak. Once the incubation was complete, the pre-soaked plate and cold wash buffer was transported to the radioactive room for washing. The plate was washed 14 times in order to filter out unbound [3H]-CP55,940, leaving only CB1 bound [3H]-CP55,940 on the UniFilter GF/C plate. The plate was then placed in the incubator to dry at 50°C for 15 minutes. Once dry, 50 µL of MicroScint20 was added to the entire plate, and it was sealed so that it could be read by the plate reader.

Western Blot

To test the expression of G proteins in the inactive CB1 membrane in comparison to the G proteins in the active CB2 membrane, a western blot was performed using the CB1 and CB2 membranes that were isolated above. First, electrophoresis was performed to electrophoretically separate the proteins by molecular weight. The precast gel was prepared by removing the comb and strip of tape at the bottom of the cassette, and the gel was inserted into the electrophoresis cassette holder. The well and front reservoirs were then filled with 1x MOPS (1x MES buffer with 10% gels) running buffer.

Prior to loading the samples, the wells were rinsed three times with running buffer. The protein ladder was loaded at 7 µL and the samples were loaded at 20 µL in the following order: 20 µg CB1, 10 µg CB1, 5 µg CB1, 2.5 µg CB1, 10 µg CB2, 5 µg CB2, 2.5 µg CB2. Once the samples were properly loaded, the electrodes were connected and the gel was run at 80 V at

room temperature until the dye fully integrated into the gel. Then, the voltage was increased to 120 V and the gel was run until the loading dye front was in the bottom ⅛ of the gel.

Next, the separated proteins were transferred to a PVDF membrane for detection. The membrane was presoaked in 100% MeOH for 0.5-1 minute and rinsed with ddH2O for 5 minutes prior to sandwich assembly. Four fiber pads and two pieces of filter paper were presoaked in the transfer buffer for 10 minutes. Once everything was finished soaking, the transfer cassette assembly was set-up in the following order: base of transfer holder, 2x fiber pad, filter paper, gel face down, PVDF membrane, filter paper, 2x fiber pad, lid of transfer holder. The assembly was then secured into the transfer electrophoresis holder and the outside of the system was filled with ice cold transfer buffer. Next, the electrodes were connected and the assembly was run at room temperature for 1 hour at 30 V. Finally, the membrane was removed from the transfer cassette and soaked in blocking buffer (5% BSA), shaken for 1 h at room temperature and then rinsed two times for 5 minutes with TBST.

Then, 10 mL of the primary antibody was prepared at a 1:1000 dilution in TBST with 1% blocking buffer. The primary antibody used in this study was recombinant anti-GNAI2 antibody purchased from Abcam. The membrane was placed in a container and the diluted primary antibody was added. Then, the membrane with primary antibody was incubated overnight at 4°C (1% blocking buffer) on a shaker. The primary antibody was recaptured and saved for future use before the membrane was rinsed as follows: 3 times quick in TBST, 3 times for 5 minutes in TBST while shaking at room temperature, and 1 time for 1 minute in blocking buffer.

Next, 10 mL of the secondary antibody was prepared at a 1:10000 dilution in TBST buffer. The secondary antibody used in this study was a goat anti-rabbit IgG antibody purchased

from ThermoFisher. The membrane was placed in a container and the diluted secondary antibody was added. Then, the membrane with secondary antibody was shaken for 1 hour at room temperature. After removing and discarding the secondary antibody, the membrane was rinsed as follows: 3 times quickly in TBST and then 3 times for 5 minutes in TBST at room temperature. Finally, western blot data was collected by obtaining images.

III. Results

CB1 and CB2 expressing cells were grown in cell culture for approximately 5 days prior to membrane isolation. The following assays were run using a membrane standard concentration of 10 μg/well, and calculations were made to maintain this standard based off of the membrane concentration determined using a bicinchoninic acid (BCA) assay. The concentration of one of the CB1 membranes used in testing was 0.22 μg/μL (**Figure 3**).

A [3H]-CP55,940 binding curve was established to test the validity of the concentration of radioligand being used in the competitive receptor binding assays. The original concentration being used in testing was 1.5 nM [3H]-CP55,940. Per the [3H]-CP55,940 curve, a Kd value of ~1.5 nM was found, which is near the known Kd value and therefore the desired standard concentration for testing (**Figure 4**).

The competitive receptor binding assays were successfully established for both the CB1 and CB2 receptor ligands. These assays were used to test a variety of natural product compounds, and their activity with CB1 and CB2 was compared **(Figure 5**). The identity of these compounds are not disclosed because they are currently still being tested and the findings have not been published.

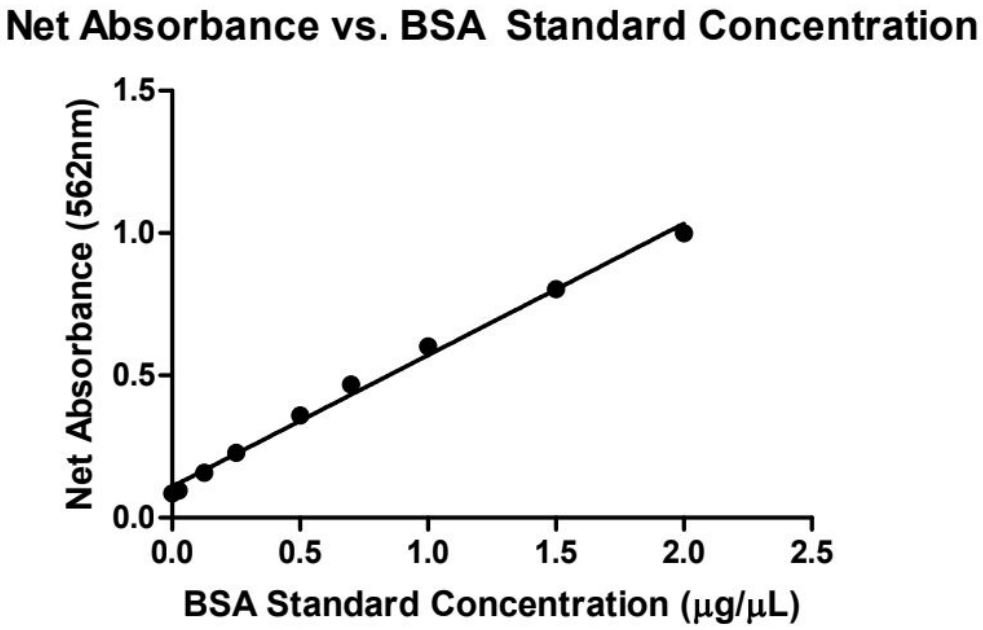

Figure 3: BCA curve showing the average blank-corrected 562nm measurement for each BSA standard vs. its concentration in μg/μL. The equation of the trendline is y = 0.4621x + 0.1091, which results in a CB1 membrane concentration of 0.22 μg/μL.

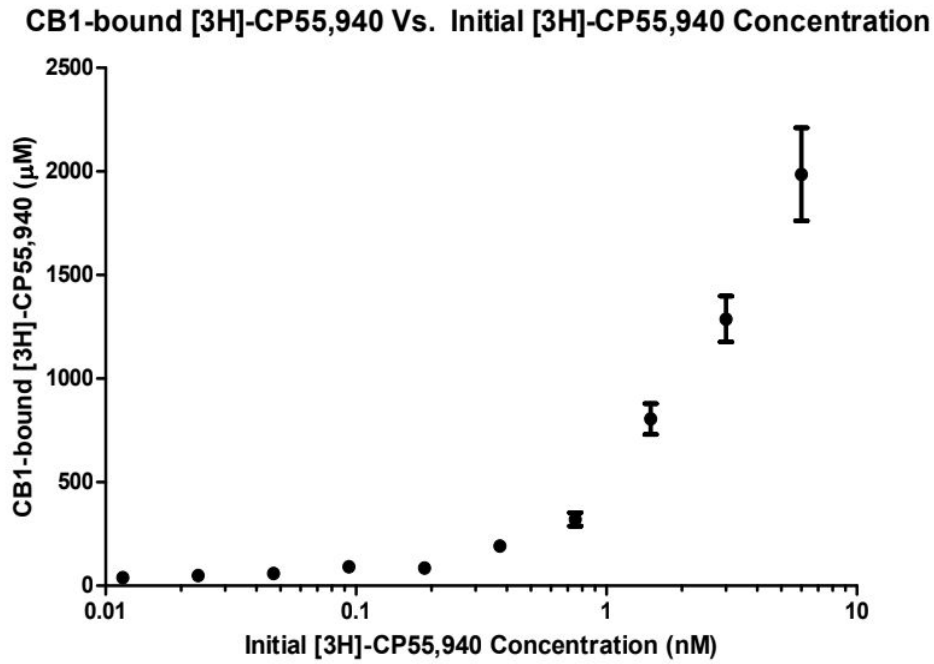

Figure 4: Ligand binding curve showing the CB1-bound [3H]-CP55,940 vs. its initial concentration in nM. Presents an equilibrium dissociation constant (Kd) value of the [3H]-CP55,940 agonist near its known Kd value of 1.5 nM.

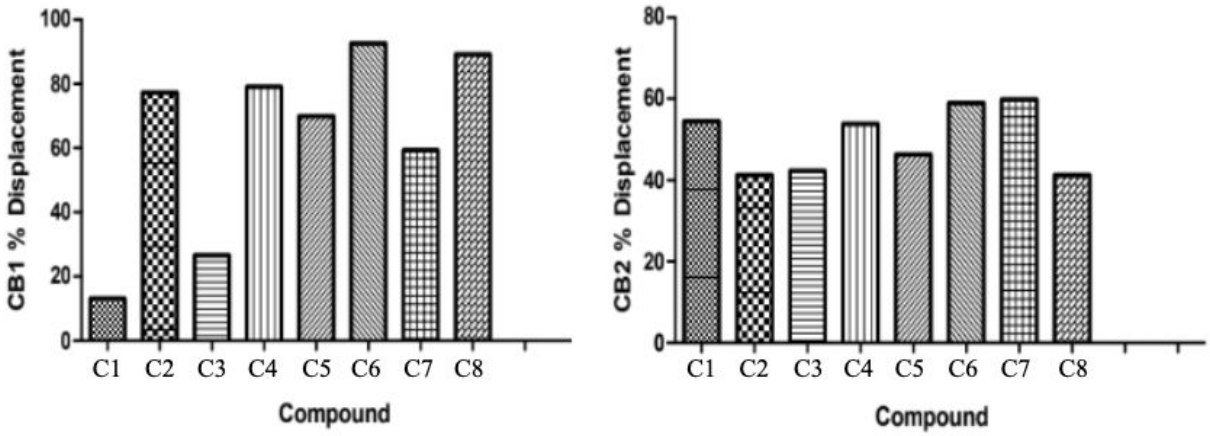

Figure 5: The comparison of competitive receptor binding of a variety of natural product ligands to the CB1 and CB2 receptors.

A cAMP-Glo assay was established for the functional evaluation of CB1 and CB2 receptor ligands without the use of radioactivity. A cAMP curve was used as a control to test the concentration curves of ligands being tested in the assay (**Figure 6**). To test the validity of the cAMP-Glo assay for functional evaluation, a known agonist of the CB1 and CB2 receptor, CP-55,940, was administered to the cells for 20 minutes. A significant increase in CB2 receptor activity was observed (**Figure 7**), but the results of CB1 testing presented too much variation (**Figure 8**).

Given the failure of the cAMP assay for the functional evaluation of CB1 receptor ligands, we then utilized the GTPγS assay. Previous work in our laboratory has shown this assay successful for CB2 and opioid receptors, but it has not worked for CB1. In contrast to the cAMP-Glo assay, it requires radioactivity so it is not preferred. The variables of the GTPγS are known to be highly flexible, so GDP and [35S]-GTPγS curves were established to calculate the desired standard concentrations for testing. The [35S]-GTPγS curve presented a Kd of ~1000 pM (**Figure 9**), and the GDP curves had no notable findings (**Figure 10**). GDP was suggested to run at 30 μM in numerous academic texts, so that standard was used in testing. The GTPγS assay was also found unfit to produce reliable results for the CB1 receptor ligands (**Figure 11**).

To determine the reason for failure of the cAMP-Glo and GTPγS assay for the functional evaluation of CB1 cells, a western blot was run. The G proteins of the inactive CB1 cells were compared to the active CB2 cells. CB2 cells properly expressed both G2 and G3 proteins, but the CB1 cells showed a significantly decreased expression for concentrations at 10 mM, 5 mM and 2.5 mM (**Figure 12**).

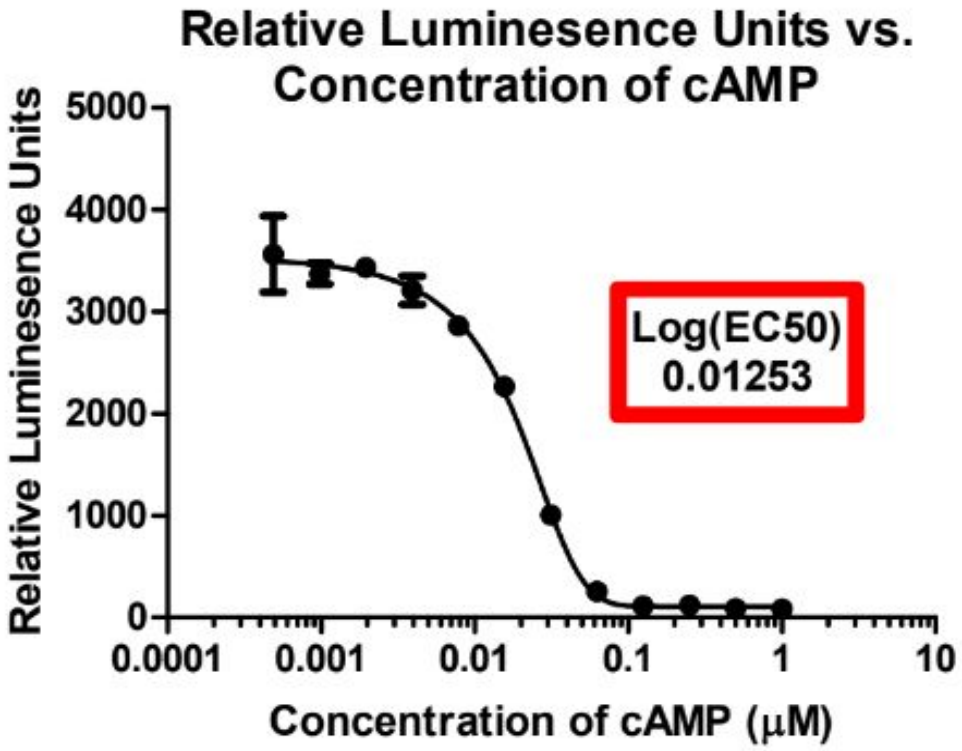

Figure 6: cAMP concentration curve showing the relative luminescence units vs. cAMP standard concentration in μM. Presents a log(EC50) value of 0.01253.

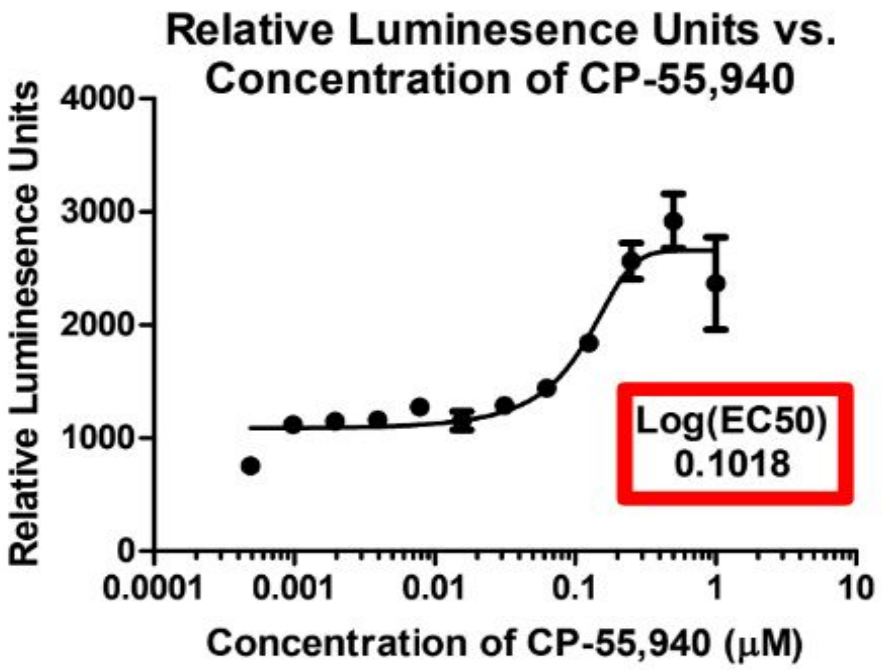

Figure 7: CP-55,940 concentration curve showing the relative luminescence units vs. CP-55,940 standard concentration in μM when testing CB2 receptor ligands. Presents a log(EC50) value of 0.1018.

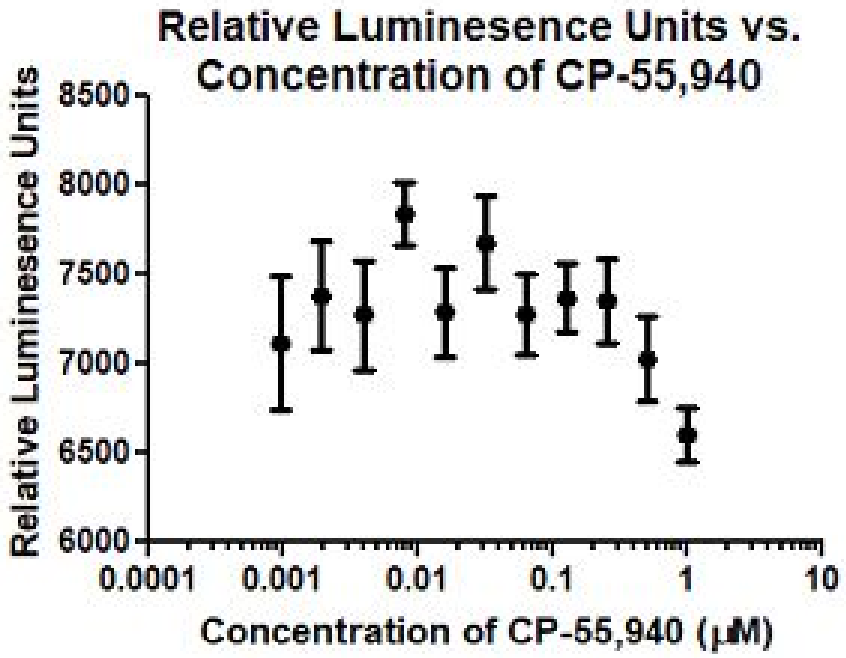

Figure 8: CP-55,940 concentration curve showing the relative luminescence units vs. CP-55,940 standard concentration in μM when testing CB1 receptor ligands.

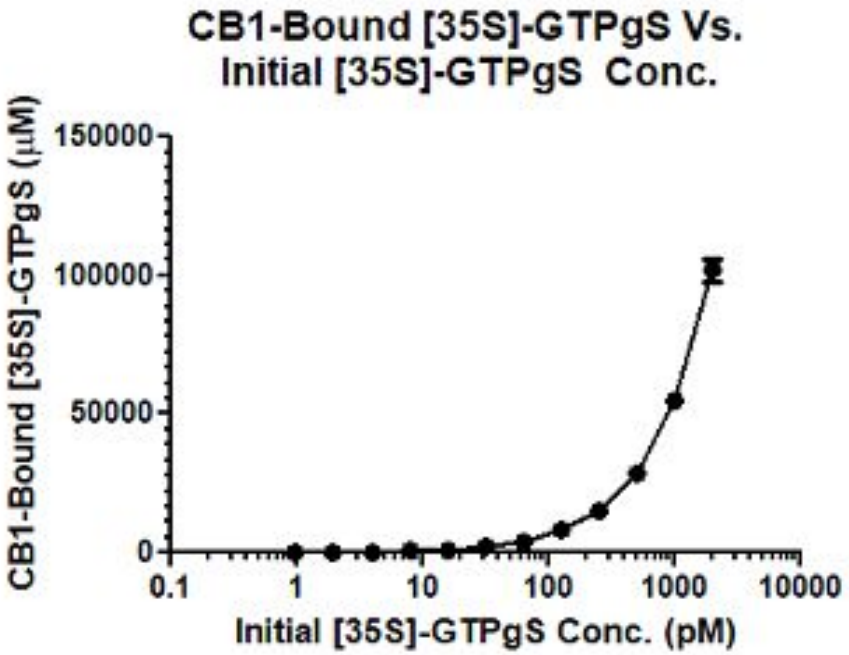

Figure 9: Ligand binding curve showing the CB1-bound [35S]-GTPγS vs. its initial concentration in nM. Presents an equilibrium dissociation constant (Kd) value of the [35S]-GTPγS at ~1000 pM.

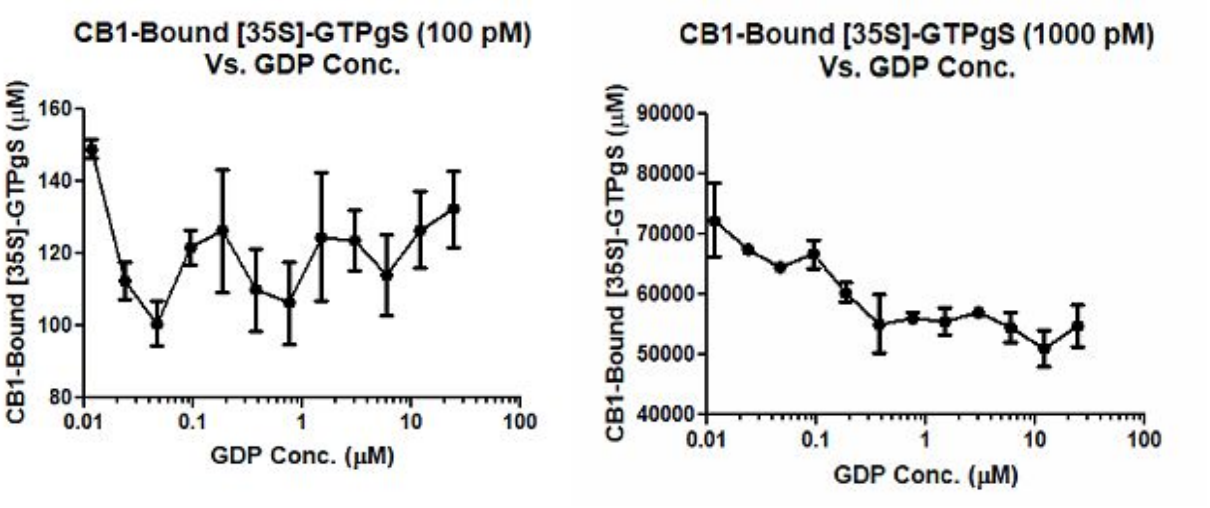

Figure 10: GDP curves at 100 pM and 1000 pM. No accurate Kd presented in either curve.

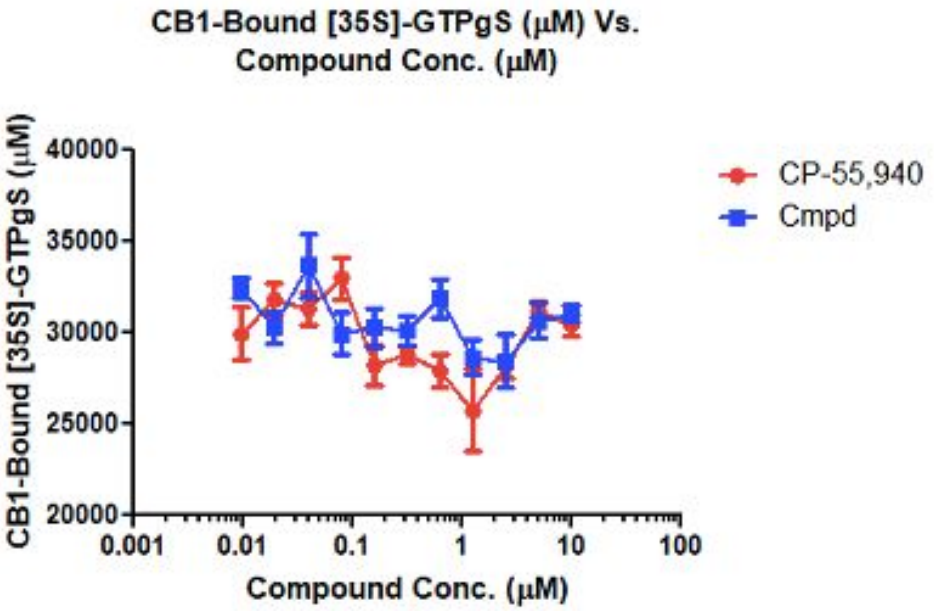

Figure 11: The results of a GTPγS assay comparing a CP-55,940 curve to one of a test compound. No defined curve shown.

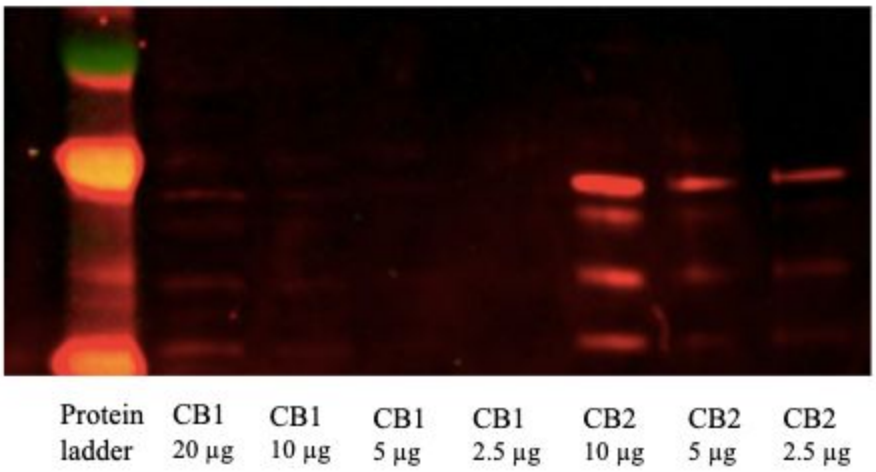

FIGURE 12: Western blot comparing the G protein expression in CB1 membrane to the Gi2 protein expression in CB2 membrane. Concentrations of 20, 10, 5 and 2.5 µg were tested for CB1. Concentration of 10, 5 and 2.5 were tested for CB2. The lanes are as follows from left to right: protein ladder, 20 µg CB1, 10 µg CB1, 5 µg CB1, 2.5 µg CB1, 10 µg CB2, 5 µg CB2, 2.5 µg CB2.

IV. Discussion

We observed that many natural product ligands produce competitive receptor binding to the CB1 and CB2 receptors *in vitro*. Of these, some seem to bind very similarly to CB1 and CB2, while others show great specificity between the two receptors. To ensure the accuracy of our competitive receptor binding assays, we validated the concentrations of the membranes and radioligands being used. The CB1 and CB2 membrane concentrations were validated using a bicinchoninic acid assay (BCA), and were run at a standard concentration of 10 μg/well. Other laboratories vary the amount of receptor proteins that are added, however we found this to be an effective window of binding and function of CB2.

The [3H]-CP55,940 radioligand was validated by establishing a [3H]-CP55,940 curve, and was run at a standard concentration of 1.5 nM/well. Per the [3H]-CP55,940 curve, a Kd value of ~1.5 nM was found, which was near the known Kd value and therefore the desired standard concentration for testing. However, there are other ligands that could have been used, such as THC and WIN55212. We selected CP-55,940 because it is a full agonist of both receptors.

In other studies, different agonists were used for the GTPγS assay. For example, Breivogel and colleagues used WIN55212 and found that it induced high affinity [35S]-GTPγS binding without affecting low affinity sites (1998). Govaerts and colleagues compared the ligand affinities and efficacies of HU210, CP55940 and WIN55212, and all three were confirmed at full agonist in the GTPγS assay (2004).

The cAMP-Glo and GTPγS assays were properly established for the functional evaluation of CB2 receptor ligands. The variables of the GTPγS are known to be highly flexible,

so GDP and [35S]-GTPγS curves were established to calculate the desired standard concentrations for testing. The [35S]-GTPγS curve presented a Kd of ~1000 pM, and the GDP curves had no notable findings. GDP was suggested to run at 30 μM in numerous academic texts, so that standard was used in testing.

The establishment of an efficient GTPγS assay for specific GPCRs can be difficult due to the varying levels of GDP, Mg^{2+} and Na^{+} required for observing stimulation of GTPγS binding. In GTPγS assays for the functional evaluation of CB1 and CB2 receptor ligands in other studies, vastly different concentrations of GDP and [35S]-GTPγS were used. Eldeeb and colleagues used 500 pM [35S]-GTPγS and 10 μM GDP (2016). Strange suggested the use of Eu-GTPγS as an alternative to [35S]-GTPγS because it is non-radioactive, but it has not been widely used (2019). Breivogel and colleagues varied the amount of GDP with the stimulation of [35S]-GTPγS binding by agonist found to be increased by increasing concentrations of GDP, up to a maximum of 125% at 30 μM GDP (1998).

When establishing the cAMP-Glo and GTPγS assays for the functional evaluation of CB1 and CB2 receptor ligands, we noticed a trend in the failure of only CB1 receptor ligand assays. Due to this, we ran a western blot comparing the G proteins in the inactive CB1 membrane in comparison to the G proteins in the active CB2 membrane. It was found that there was a decreased expression of G proteins in the inactive CB1 membrane in comparison to the active CB2 membrane at concentrations of 10, 5 and 2.5 mM.

The idea to run a western blot of the two membranes as a means to find a cause for the inactivity of the CB1 membrane was stemmed from research performed by Gettys and colleagues. Their research presented that in a recombinant system there may be more than one G

protein responding to the receptor, depending on the composition of the host cell membrane (Gettys et al., 1994). In the British Journal of Pharmacology, Strange suggested that it seems likely that GTPγS binding signals for a GPCR activating $G_{i/o}$ proteins will be to both of these G proteins. This could potentially lead to a complication in that if the agonist potency for stimulating GTPγS binding to the two G proteins were different this might lead to some flattening of the overall stimulation curve (2010).

Limitations of Experiment and Future Directions

Because this was an *in vitro* study, it is unknown whether the compounds tested will have an effect in living systems. For this reason, *in vivo* studies are necessary for the continued functional evaluations of these ligands on the CB1 and CB2 receptors. To expand the *in vitro* studies, agonists other than CP-55,940 could be used in testing and compared among each other. For example, THC and WIN55212 could also be tested. Furthermore, new cells should be purchased and tested for G protein expression via western blot prior to establishing effective cAMP-Glo and GTPγS assays for CB1.

In addition to *in vivo* studies of CB1 and CB2 receptor ligands, the evaluation of the orphan cannabinoid GPR55 is of interest. Given the complications of CB1, I would first run a western blot of the GPR55 membrane using the active CB2 membrane as a control to test the expression of G proteins. If active, I would then establish the cAMP-Glo and GTPγS assays for GPR55.

Overall Conclusions

The cAMP-Glo and GTPγS assays are effective methods in the functional evaluation of CB2 receptor ligands. The improper expression of G proteins in the CB1 membrane isolated for these experiments may be the cause of the inability to use cAMP-Glo and GTPγS assays for its functional evaluation. To properly establish cAMP-Glo and GTPγS assays for CB1, new cells should be purchased and tested for G protein expression via western blot before their establishment is attempted.

LIST OF REFERENCES

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ CGTP Collaborators. The Concise Guide to PHARMACOLOGY 2013/14: G protein-coupled receptors. Br J Pharmacol. 2013a;170:1459–1581.

Breivogel CS, Selley DE, Childers SR. Cannabinoid receptor agonist efficacy for stimulating [35S]GTPgammaS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. J Biol Chem. 1998;273(27):16865–16873

Cheng Y, Hitchcock SA (2007) Targeting cannabinoid agonists for inflammatory and neuropathic

pain. Expert Opin Investig Drugs 16:951–965

Eldeeb K, Leone‐Kabler S, Howlett AC (2016). CB1 cannabinoid receptor‐mediated increases in cyclic AMP accumulation are correlated with reduced Gi/o function. *J Basic Clin Physiol Pharmacol* 27: 311– 322.

Gettys T. W., Sheriff-Carter K., Moomaw J., Taylor I. L., Raymond J. R. (1994). Characterization and use of crude alpha-subunit preparations for quantitative immunoblotting of G proteins. *Anal. Biochem.* 220 82–91. 10.1006/abio.1994.1302

Gilissen J, Geubelle P, Dupuis N, Laschet C, Pirotte B, Hanson J. Forskolin-free cAMP assay for Gi-coupled receptors. Biochemical Pharmacology 2015; 98: 381–391. pmid:26386312

Govaerts SJ, Hermans E, Lambert DM. Comparison of cannabinoid ligands affinities and efficacies in murine tissues and in transfected cells expressing human recombinant cannabinoid receptors. *Eur J Pharm Sci*. 2004;23:233‐243.

Ji H.F., Li X.J., Zhang H.Y. Natural products and drug discovery. Can thousands of years of

ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia? EMBO Rep. 2009;10:194–200. doi: 10.1038/embor.2009.12.

Pertwee, Roger (Professor of Neuropharmacology, School of M.) *Handbook of Cannabis*. Oxford University Press, 2016

Roth, Bryan L. *National Institute of Mental Health Psychoactive Drug Screening Program: Assay Protocol Book.* Vol. 3, University of North Carolina at Chapel Hill, 2018.

Sandeep S. Vansal & Dennis R. Feller (1999) An Efficient Cyclic AMP Assay for the Functional Evaluation of β-Adrenergic Receptor Ligands, Journal of Receptors and Signal Transduction, 19:5, 853-863, DOI: 10.3109/10799899909042877

Strange PG (2010) Use of the GTPγS ([35S]GTPγS and Eu-GTPγS) binding assay for analysis of ligand potency and efficacy at G protein-coupled receptors. Br J Pharmacol 161:1238–1249. https://doi.org/10.1111/j.1476-5381.2010.00963.x