Docoda J. Nunnery

901-709-442

1319 Park Street Apt 2

Huntington WV, 25701

Nunnery7@live.marshall.edu

910-876-0546

Determination of CB1 Receptor Activity for Emerging Synthetic Cannabinoid Compounds

MU Faculty Advisor: **Lauren Waugh, Ph.D.**

Assistant Professor of Forensic Chemistry, Marshall University

Richards18@marshall.edu

Internship Agency Supervisor: **Pamela Staton, Ph.D.**

Graduate Program Coordinator

Marshall University Forensic Science

Internship Technical Reviewer: **Richard Egleton, Ph.D**.

Associate Professor

Department of Pharmacology, Physiology and Toxicology

Joan C. Edwards School of Medicine

Marshall University

Abstract:

In 2006, a strange new psychoactive drug known as “Spice” was quickly gaining popularity. Starting in Western European countries, spice was declared as an incense and was advertised as “not for human consumption”, however it was consumed as herbal drugs (soaked in a synthetic cannabinoid solution) via smoking much like cannabis. Based on its reputation as a “legal high”, its popularity rapidly increased. Spice reached the United States sometime in 2008. The hype continued until finally in March 2011, the DEA took action by implementing temporary emergency scheduling on the five synthetic cannabinoids most associated with spice. Quickly following that, President Barack Obama signed the Synthetic Drug Prevention Act of 2012 (S.3187) on July 9th 2012 banning 15 synthetic cannabinoids in addition to 11 other synthetic designer drugs. Spice manufacturers, being financially motivated to stay one step ahead of the legislation, quickly switched to new synthetic cannabinoid structures that were not scheduled.

This arms race between the spice manufacturers and the slow moving legislation is the basis of the problem that this study hopes to alleviate. Based on The Synthetic Drug Abuse Prevention Act, synthetic cannabinoids have been put on to Schedule I of the Controlled Substances Act based on their structure and activity at the CB_1 receptor, therefore they must bind to the receptor and activate it. Through the years, synthetic cannabinoid structures have become more and more diverse to avoid illegal status, thus putting more emphasis onto the receptor binding and functionality characteristics. This project’s goal was to develop a method to determine if a compound is a CB_1 agonist through the use of functional and receptor binding studies. In this study, CB_1 receptor activity was investigated and known synthetic cannabinoid CP55940’s agonist activity at CB_1 was demonstrated by its ability to inhibit forskolin induced cAMP levels in GH4C1 cells.

The %B/B_o of each sample was calculated and plotted against the agonist concentrations of 0.2nM, 2nM, and 4nM. By using GraphPad ©, statistical differences of %B/Bo values amongst the agonist concentration ranges of 0.2 nM-4 nM and 2 nM-4 nM were found, and the overall goal of the study was accomplished. Future studies include method optimization and determination of receptor binding constants.

Introduction:

Synthetic cannabinoids are a large family of chemically unrelated structures functionally similar to the active compound of cannabis, Δ 9-tetrahydrocannabinol. These compounds bind to the same cannabinoid receptors all over the body as the endocannabinoid, anandamide. The cannabinoid receptors CB_1 and CB_2 were discovered in the 1980s. CB_1 was found to be in the central nervous system and is associated with psychoactive effects, while CB_2 is found in the immune system. Synthetic cannabinoids were initially developed over 40 years ago as pharmaceutical agents intended to regulate pain. Unfortunately, it proved to be a challenge to separate the desired properties such as pain management and appetite regulation from the unwanted psychoactive effects. (1)

The discovery of the CB_1 and CB_2 receptors indicated that endogenous cannabinoids may occur in the brain, which act as physiological ligands for CB_1. Two possible endogenous cannabinoids have been identified, anandamide and 2-arachidonylglycerol, adding further weight to the concept of a cannabinoid signaling system, otherwise known as the endocannabinoid system. Through detailed analyses of mammalian nervous systems including neuroanatomical and electrophysiological terminals, it has been revealed that the CB_1 receptor is targeted to the presynaptic terminals of neurons where it acts to inhibit the release of “classical” neurotransmitters such as dopamine. Based on these findings, it was presented that anandamide

is synthesized by post synaptic cells and acts as a retrograde messenger molecule to modulate neurotransmitter release from presynaptic terminals. It is this system that Δ 9-tetrahydrocannabinol and other exogenous cannabinoids take advantage of to produce psychoactive or therapeutic effects. (2)

In 2006, a strange new product known as “Spice” was quickly gaining popularity. “Spice” and similar products were sold as legal alternatives to marijuana. Not much was known about these new products, until analyses by gas chromatography-mass spectrometry and liquid chromatography- mass spectrometry analyses indicated that most of the products contained two major synthetic cannabinoids, cannabicyclohexanol and JWH-018. (3) Starting in Western European countries, Spice was declared as an incense and was advertised as “not for human consumption”, however it was consumed as herbal drugs (soaked in a synthetic cannabinoid solution) via smoking much like cannabis. Based on its reputation as “legal high”, its popularity rapidly increased. (4)

While synthetic cannabinoids originally emerged as a recreational drug in Europe in 2006, they did not reach the United States until sometime in 2008. The hype continued until March 2011, when the Drug Enforcement Agency (DEA) used its emergency scheduling authority to control the five compounds commonly found in “Spice” and similar products including JWH-018, JWH-023, CP-47,497, and CP 47497 C8 homolog. However, these compounds quickly replaced by new synthetic cannabinoids of the aminoalkylindole variety, which are still the most prevalent synthetic cannabinoids.

In July 2012, the Synthetic Drug Abuse Prevention Act of 2012 (S.3187) was signed into law by President Barack Obama to better control the synthetic cannabinoid compounds. This new law bans 15 synthetic cannabinoids in addition to 11 other synthetic designer drugs. It also increased

the amount of time an analogue can be temporarily scheduled from 1 year with the possibility of a 6 month extension to 2 years with the possibility of a 1 year extension. The spice manufacturers, being financially motivated to stay one step ahead of the legislation, quickly switched to a new synthetic cannabinoid that were not on the DEA's scheduling. (5)

This arms race between the spice manufacturers and the slow moving legislation is the basis of the problem that this study hopes to alleviate. Based on The Synthetic Drug Abuse Prevention Act, synthetic cannabinoids have been put on to Schedule I of the Controlled Substances Act based on their structure, whether they bind to the CB_1 receptor, and if they act as an agonist for the CB_1 receptor. Through the years, synthetic cannabinoid structures have become more and more diverse to avoid illegal status, thus putting more emphasis onto the receptor binding and functionality characteristics. In this study, CB_1 receptor activity will be investigated by measuring the ability of a known CB_1 receptor agonist to inhibit forskolin induced cAMP levels in GH4C1 cells.

Materials and Methods:

Cell Feeding and Preparation

The semi-adherent GH4C1 rat pituitary cancer cells were kept in a Thermo Napco Series 8000 DH CO_2 Incubator and was fed every Monday, Wednesday, and Friday. Using a filter vacuum funnel the media was prepared fresh every 3 to 4 weeks with 500 mL of Gibco F12 growth media, 90 mL of Horse Serum and 16 mL of FBS.

During cell feeding, the media was placed into a water bath at 37^oC for 30-45 minutes to warm to prevent damage of the cells. The cell flask was removed from the incubator and placed into a sterile biological hood. The suspended cells in the flask were pipetted into a sterile 50mL conical

Warmed growth media was placed into the cell flask to prevent damage to the remaining adherent cells.

The suspended cells in the 50 mL conical were placed into a centrifuge, and pelleted at 1000 rpm for 5 minutes. The 50 mL conical was placed back into the sterile biological safety hood. The supernatant was removed using a vacuum tube, while the avoiding the pelleted cells. A volume of 10 mL of new growth media was added to the 50 mL conical, sufficiently mixed to ensure redistribution of the suspended cells, and the cells were pipetted back into the original cell flask.

Reagent Preparation

The reagents of the Amersham cAMP Biotrak Enzymeimmunoassay (EIA) System were prepared according to manufacturer's recommended protocol for the intracellular cAMP measurement using the non-acetylation EIA procedure with novel lysis reagents. Assay Buffer (0.05 M acetate buffer pH 5.8 containing 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative) was prepared through quantitative transfer of the reagent bottle contents to a 500 mL graduated cylinder using distilled water, which was used to adjust the final volume to 500 mL mark. Lysis reagent 1a (2.5% solution of Dodecyltrimethylammonium Bromide in assay buffer) was prepared by transferring the contents of the bottle to a 100 mL graduated cylinder by repeated washing with assay buffer. It was dissolved in 60 mL of assay buffer using continuous stirring throughout. The final volume was adjusted to 80mL with assay buffer and mixed thoroughly. Lysis reagent 1b (0.25% solution of Dodecyltrimethylammonium Bromide in assay buffer) was prepared by transferring 10mL of lysis reagent 1a to a graduated cylinder and filling to 100 mL with assay buffer. Lysis reagent 2a was prepared by transferring the contents of the bottle to a 100 mL graduated cylinder and dissolving in 80 mL of assay buffer using continuous stirring throughout. The final volume was adjusted to 100 mL by adding assay buffer and mixing

thoroughly. Lysis reagent 2b was prepared by transferring 10 mL of lysis reagent 2a to a graduated cylinder and filling up to a final volume of 40 mL with assay buffer and mixing thoroughly. The cAMP standard (128 nm/mL cAMP in lysis reagent 1b) was prepared by adding 2 mL of lysis reagent 1b to the standard bottle and gently mixed until the contents are completely dissolved. The antiserum (anti-cAMP serum in lysis reagent 2b) was prepared by adding 11.0 mL of lysis reagent 2b to the antiserum original bottle and swirling until the solution is completely dissolved. Vigorous agitation and foaming was avoided. cAMP peroxidase conjugate (cAMP-horseradish Peroxidase in 0.05 M Acetate buffer pH 5.8, 0.02% (w/v) Bovine Serum Albumin and 0.01% (w/v) preservative) was prepared by adding 11.0 mL of diluted assay buffer into the cAMP peroxidase conjugate bottle and mixing until the contents are completely dissolved. The wash buffer (0.02M phosphate buffer pH 7.5, 0.05% (w/v) Tween 20.) was prepared by transferring the contents of the bottle to a 500 mL cylinder by repeated washing with distilled water. The final volume was adjusted to 500 mL by adding distilled water to the mark.

Cell Counting

The cell flask was removed from the Thermo Napco Series 8000 DH CO_2 Incubator and placed within a sterile biological safety hood. The suspended cells in the media of the flask was pipetted into a sterile 50 mL conical and centrifuged at 1000 rpm for 5 minutes. The supernatant media was vacuumed off and replaced with 1mL of new media to resuspend the cells. Fifty μL of the newly suspended cells were combined with 50 μL of Trypan Blue in a 1mL tube. The Trypan/cell solution was vortexed for 1 minute and allowed to incubate at room temperature for 5 minutes. Approximately 10 μL of the Trypan/cell solution was pipetted onto a hemocytometer. The number of cells were counted in four random squares of the same size and averaged

together. The number of cells/mL was calculated and appropriate dilutions made using cell media. .

cAMP Procedure

96 well plate

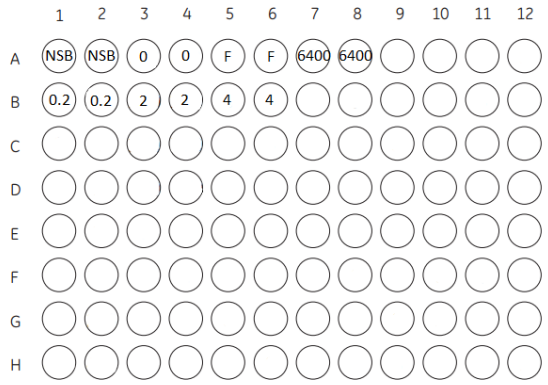

Figure 1: Plate setup

Protocol 3 for suspended cells of the Amersham cAMP Biotrak Enzymeimmunoassay (EIA) system was used as a guideline for the cAMP procedure. Cultured cells at ~100,000 cells/mL were added into a sterile standard 96 well micro plate at a volume of 100 μL. The plate was incubated at 37^{o}C for 24 hours.

After the 24 hour incubation, the wells were examined microscopically to determine if the cells had acclimated to their new environment and were all consistent amongst each other. A volume of 100 μL of 128pM/mL cAMP standard was added to the A7 and A8 wells. A volume of 50μL of forskolin was added to the A5 and A6 wells. A mixture of 50 μL of forskolin and 50 μL of the appropriate agonist concentration (0.2 nM, 2 nM, and 4 nM respectively) were added to wells B1 through B6 as depicted in figure 1. The cells were incubated at 37^{o}C for 10 min.

The microplate was centrifuged at 1500xg for 3 minutes to form a pellet in each well. The supernatant media was removed and the pellet was resuspended in 200 μL of lysis reagent 1b. The cells were agitated by placing them on a plate shaker for 10 minutes, removing them from the shaker and pipetting the media and cells up and down ~5 times, and then placing the plate back onto the shaker for 10 additional minutes. A microscopic evaluation using Trypan Blue was conducted to ensure the cells had lysed.

Microimmunoassay Plate

All reagents supplied with the Amersham cAMP Biotrak Enzymeimmunoassay (EIA) system were equilibrated to room temperature and mixed before use. The microimmunoassay plate was kept on ice unless otherwise noted. One hundred μL of lysis reagent 1b and 100 μL of lysis reagent 2b were pipetted into all NSB wells. A volume of 100 μL of lysis reagent 1b was pipetted into all zero standard wells. One hundred uL of each standard and unknown from the culture plate was transferred into the immunoassay plate. One hundred uL of lysis reagent 1a was added to the NSB wells and 100 uL of antiserum was added to each of the other wells. The plate was covered with the lid provided, gently mixed and incubated at 3-5^{o}C for exactly 2 hours, at which time, 50 μL of cAMP-Peroxidase conjugate was pipetted into all wells. The plate was covered with the lid provided and incubated at 3-5^{o}C for exactly 60 minutes. After the 60 minute incubation, all wells were washed four times with 400 μL of wash buffer. A volume 150 μL of enzyme substrate was immediately dispensed into all wells, the plate was covered, removed from the ice bath, and allowed to mix on a micro plate shaker for exactly 60 minutes at room temperature. A blue color developed.

The reaction was halted by pipetting 100 μL of 1.0M Sulphuric acid into each well and mixing. The optical density (absorbance) of each well was read in a Biotek Synergy 2 Multi-Mode Microplate Reader at 450 nm. The optical density determination was carried out within 20 minutes of the addition of the 1.0 M Sulfuric acid.

Protein Binding Normalization

The absorbance values a 450 nm were normalized to protein content to account for variability in cell content between wells. Thermo Scientific Pierce TM BCA Protein Assay Kit was used as a guide for this procedure. Standards of Bovine Serum Albumin (BSA) were prepared by serial dilution. A volume of 25 mL of each standard and unknown sample were pipetted in duplicate from the cells remaining in the 96 well microplate into a clean, sterile 96 well microplate. Working reagent was prepared by mixing 50 parts of BCA Reagent A and 1 part BCA Reagent B, and adding 200 μL to each well. The plate was mixed thoroughly on a plate shaker for 30 second and incubated at 37^{o}C for 30 minutes. The plate was cooled to room temperature and the absorbance was measured at 560 nm on a Biotek Synergy 2 Multi-Mode Microplate Reader.

Calculations

Protein Normalization

For the protein standards, repeat 1 and repeat 2 were averaged and plotted vs the standard protein concentration of each standard on Microsoft Excel ©. The linear equation was formulated and used later in cAMP calculations. For the samples and cAMP standards, the two repeats were averaged and used in conjunction with the protein standard curve linear equation to calculate the concentration of protein in each sample. If the standard curve did not encompass the sample signals, the sample was diluted by half and ran again with another standard curve and then

ultimately the protein concentration was multiplied by 2 to account for the dilution, as seen in Trial 2 with Figure 5 and Table 7.

cAMP Assay

For the cAMP standards and unknowns, the absorbance readings @ 450nm were used to calculate the %B/B_o using the equation below.

$$\left[\frac{(Sample\ or\ Std\ OD - NSB\ average\ OD)\ /\ (Sample\ \ protein\ concentration)}{(Zero\ standard\ OD - NSB\ average\ OD)/\ (Zero\ Std.\ \ protein\ concentration)}\right] \quad \text{X } 100$$

The average %B/B_o of each standard and unknown was calculated. The resulting graph of plotting the average %B/B_o vs the concentration of the agonist CP55940 was used to interpret cAMP levels and the overall trend of the agonist's ability to inhibit cAMP production. The term B%/B_o refers to the amount of cAMP peroxidase conjugate that is bound to the antiserum. The microimmunoassay plate has donkey anti- rabbit antibodies, when the rabbit anti-cAMP antibody (antiserum) is added, any cAMP in the well will bind to the antiserum. The cAMP from the cells competes with the cAMP peroxidase conjugate for anti-cAMP antibodies. When the well is washed, it is washing away anything that is not bound to the well. The substrate (TMB) for the peroxidase is added, it binds to the peroxidase and creates a blue color. Therefore the darker the blue color, the more peroxidase there is in the well, the %B/Bo will be higher, which means the cAMP will be lower.

The equation for determining %B/B_o focused on subtracting out signal that is a result of the nonspecific binding of anything that is added into the well from both the sample and zero standard. Dividing the signal of a sample or standard by the maximum (zero standard) provides the percent of cAMP peroxidase that is bound. An increase in the amount of cAMP peroxidase

bound would lead to an increase in $\%B/B_o$ as the agonist concentration increases. Therefore a decrease in the total amount of cAMP was indicated as an increase in $\%B/B_o$.

Statistical Analysis

The data from all three trials was inputted into the statistical analysis software GraphPad ©. Using this software, various statistical tests were performed including: Collective statistics, D' Agostino & Pearson omnibus normality test, ANOVA test, Brown Forsythe Test, Barlett's Test, and Tukey's multiple comparison test. The aim of the statistical tests were to determine if the cAMP levels for the three different agonist concentrations (0.2nM, 2nM, and 4nM) were statistically different.

Results

Trial 1: Protein Normalization

Table 1: Trial 1 protein standard absorbance values

	Absorbance		
Protein (ug/mL)	Rep 1	Rep 2	Average Abs
0.00	0.09	0.12	0.10
25.00	0.14	0.15	0.15
50.00	0.15	0.15	0.15
100.00	0.22	0.23	0.22
200.00	0.34	0.34	0.34
400.00	0.58	0.59	0.59
800.00	0.97	0.89	0.93
1000.00	1.49	1.60	1.54
1500.00	1.81	1.97	1.89

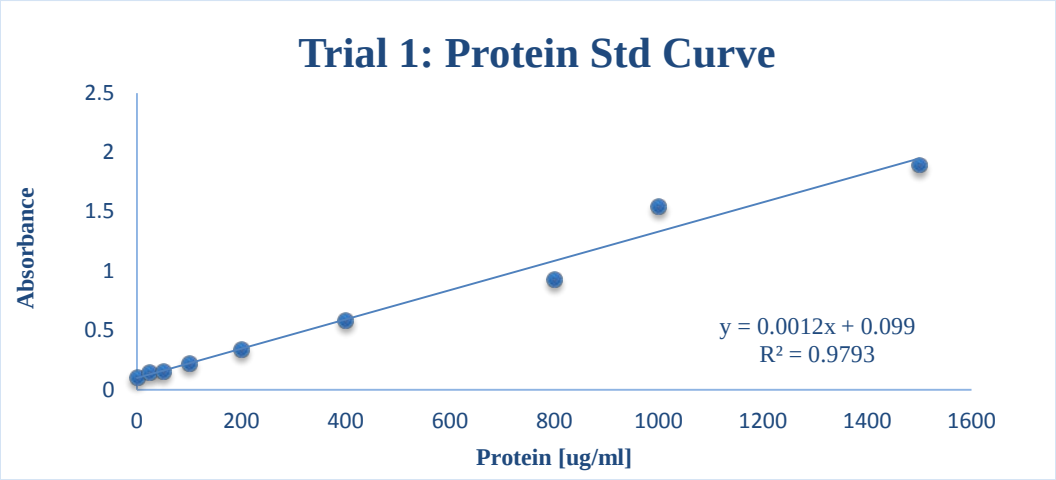

Figure 2: Trial 1 protein standard curve.

Table 2: Trial 1 protein concentrations

Sample	Absorbance		Average Abs	Protein (ug/mL)
Name	Rep 1	Rep 2		
NSB	1.83	1.76	1.80	1416.67
NSB	1.12	1.14	1.13	857.08
0 cAMP	1.72	1.04	1.38	1064.58
0 cAMP	0.85	0.83	0.84	619.58
6400 fmol cAMP	0.77	0.51	0.64	451.67
6400 fmol cAMP	0.69	0.75	0.72	515.42
Forskolin	0.91	1.05	0.98	735.83
Forskolin	0.98	1.00	0.99	739.58
0.2 nM CP55940	1.52	1.28	1.40	1081.67
0.2 nM CP55940	1.23	1.26	1.24	954.58
2.0 nM CP55940	0.91	1.25	1.08	814.17
2.0 nM CP55940	1.27	1.36	1.32	1015.83
4.0 nM CP55940	0.81	0.68	0.74	537.50
4.0 nM CP55940	0.79	0.79	0.79	577.92

Trial 1: cAMP Assay

Table 3: Trial 1 standards absorbance readings and protein concentration.

Standards				
Standard	Repeat 1	Repeat 2	Mean	Protein (ug/mL)
Zero	0.57	0.54	0.56	842.08
NSB	0.13	0.08	0.10	1136.88

Table 4: Trial 1 absorbance readings and %B/Bo values for samples.

Sample Name/Standard	Sample @ 450 nm	%B/Bo	Average %B/Bo
6400 fmol cAMP	0.61	210.18	191.75
6400 fmol cAMP	0.58	173.33	
Forskolin	0.44	83.90	83.05
Forskolin	0.43	82.21	
0.2 nM CP55940	0.46	60.52	63.87
0.2 nM CP55940	0.45	67.21	
2.0 nM CP55940	0.43	75.14	68.32
2.0 nM CP55940	0.44	61.51	
4.0 nM CP55940	0.43	113.81	116.77
4.0 nM CP55940	0.48	119.73	

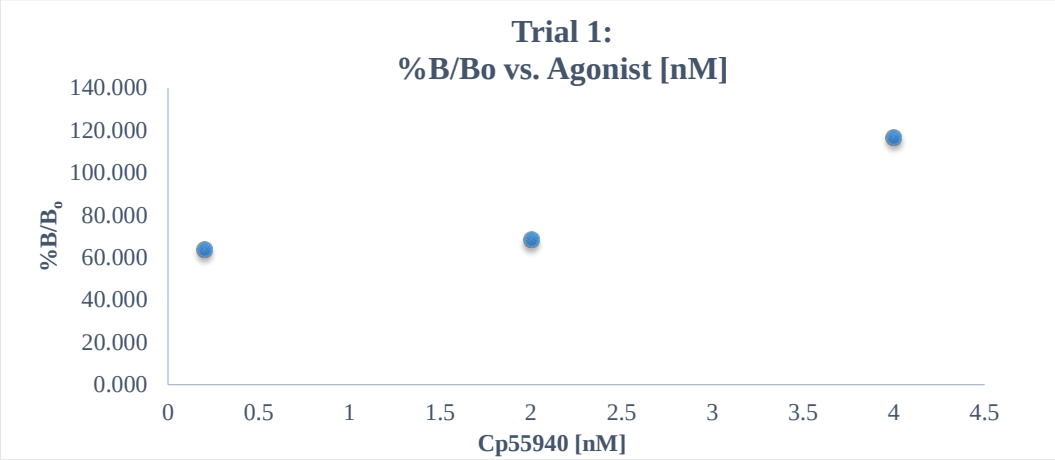

Figure 3: Trial 1 %B/B_o of Agonist concentrations of 0.2 nM, 2 nM, and 4nM.

Trial 2: Protein Normalization

Table 5: Trial 2 protein standards absorbance readings A.

Protein [ ] (ug/mL)	Absorbance		Average Abs
	Rep 1	Rep 2	
0.00	0.12	0.12	0.12
25.00	0.09	0.09	0.09
50.00	0.15	0.14	0.14
100.00	0.24	0.24	0.24
200.00	0.36	0.36	0.36
400.00	0.71	0.75	0.73
800.00	1.05	1.22	1.13

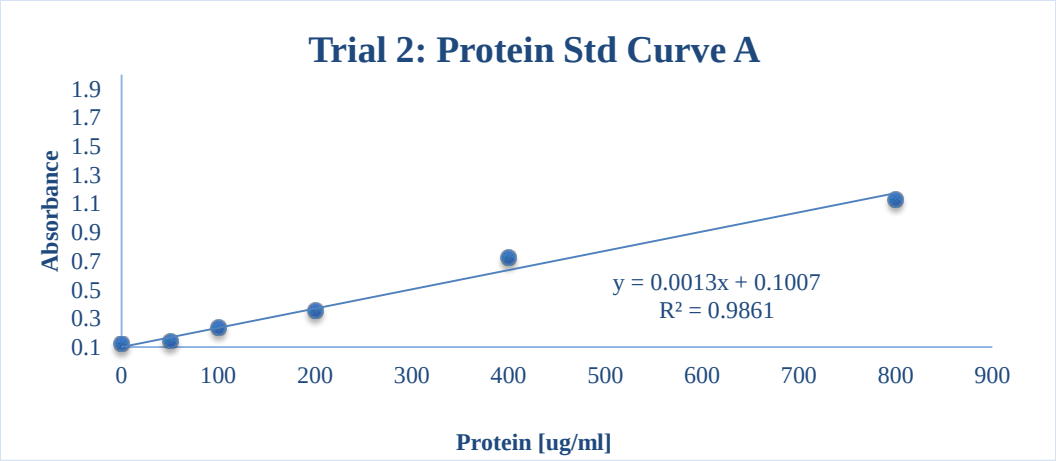

Figure 4: Trial 2 protein standard curve A.

Table 6: Trial 2 protein standard absorbance readings B.

Protein [ ] (ug/mL)	Absorbance		Average Abs
	Rep 1	Rep 2	
0.00	0.64	0.65	0.64
25.00	0.67	0.67	0.67
50.00	0.53	0.70	0.61
100.00	0.72	0.56	0.64
200.00	0.85	0.86	0.86
400.00	1.06	1.08	1.07
800.00	1.48	1.48	1.48

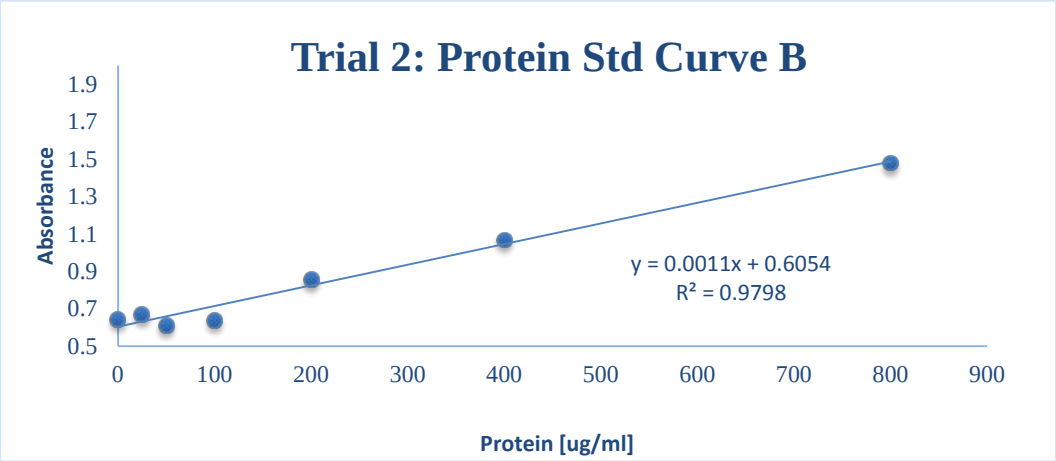

Figure 5: Trial 2 protein standard curve B.

Table 7: Trial 2 Sample protein concentrations.

Sample	Absorbance		Average Abs	Protein [ ] (ug/mL)	
	Rep 1	Rep 2			
NSB	0.53	0.52	0.52	323.69	
NSB	0.47	0.58	0.52	325.62	
0 cAMP	0.75	0.75	0.75	501.77	
0 cAMP	0.62	0.62	0.62	399.85	
6400 fmol cAMP	0.81	0.87	0.84	568.69	
6400 fmol cAMP	0.54	0.41	0.48	289.85	
Forskolin	0.52	0.58	0.55	342.92	
Forskolin	0.80	0.82	0.81	546.77	
0.2 nM CP55940	1.31	1.18	1.25	583.27	1166.55
0.2 nM CP55940	1.23	1.15	1.19	532.82	1065.64
2.0 nM CP55940	1.14	1.04	1.09	438.73	877.45
2.0 nM CP55940	0.81	0.86	0.83	206.45	412.91
4.0 nM CP55940	1.00	1.08	1.04	391.00	782.00
4.0 nM CP55940	1.18	1.16	1.17	509.64	1019.27

Trial 2: cAMP Assay

Table 8: Trial 2 standards abosrbance readings andprotein concentration

Standards				
Standard	Repeat 1	Repeat 2	Mean	Protein (ug/mL)
Zero	0.43	0.71	0.57	450.81
NSB	0.08	0.07	0.08	324.65

Table 9: Trial 2 sample absorbance readings and %B/Bo values.

Sample Name/Standard	Sample @ 450 nm	%B/Bo	Average %B/Bo
6400 fmol cAMP	0.43	55.95	81.62
6400 fmol cAMP	0.42	107.28	
Forskolin	0.62	144.00	116.25
Forskolin	0.61	88.49	
0.2 nM CP55940	0.37	23.16	26.13
0.2 nM CP55940	0.42	29.09	
2.0 nM CP55940	0.65	59.68	63.33
2.0 nM CP55940	0.38	66.98	
4.0 nM CP55940	0.60	60.83	110.86
4.0 nM CP55940	1.89	160.88	

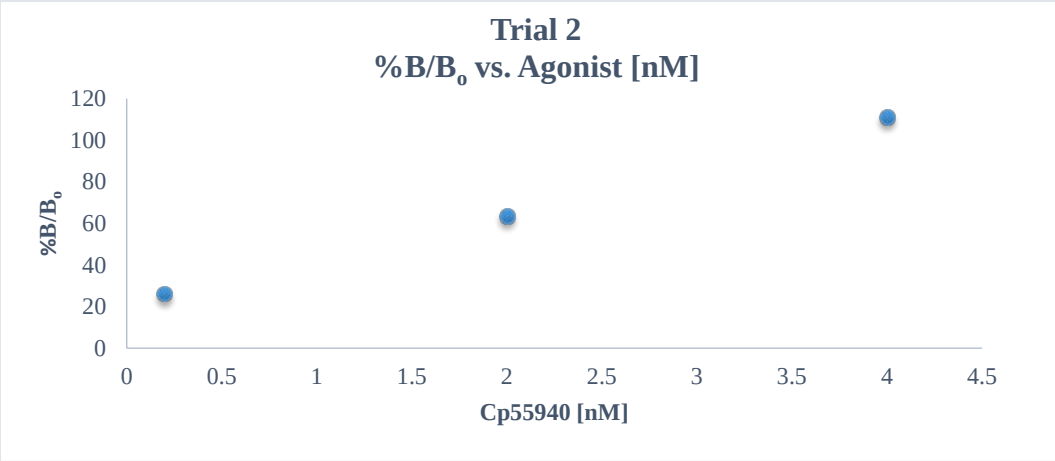

Figure 6 : Trial 2 %B/Bo of Agonist concentrations, 0.2 nM, 2 nM, and 4 nM**.**

Trial 3: Protein Normalization

Table 10: Trial 3 protein standard absorbance values.

Protein (ug/mL)	Absorbance		Average Abs
	Rep 1	Rep 2	
0.00	0.11	0.11	0.11
25.00	0.12	0.12	0.12
50.00	0.15	0.16	0.15
100.00	0.20	0.20	0.20
200.00	0.35	0.35	0.35
400.00	0.59	0.61	0.60
800.00	0.99	1.02	1.01
1000.00	1.18	1.22	1.20
1500.00	1.77	1.87	1.82
2000.00	2.41	2.42	2.41

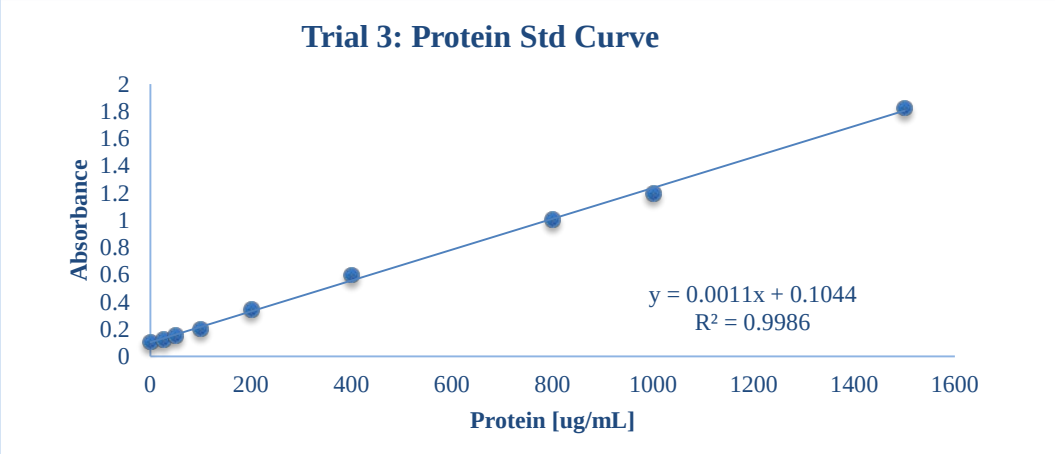

Figure 7: Trial 3 protein standard curve.

Table 11: Trial 3 protein concentrations for samples.

Sample	Absorbance		Average Abs	Protein (ug/mL)
	Rep 1	Rep 2		
NSB	0.44	0.44	0.44	307.36
NSB	0.68	0.70	0.69	528.73
0 cAMP	0.48	0.51	0.50	355.09
0 cAMP	0.55	0.57	0.56	411.91
6400 fmol cAMP	0.33	0.35	0.34	215.09
6400 fmol cAMP	0.53	0.54	0.53	390.09
Forskolin	0.48	0.47	0.47	335.09
Forskolin	0.38	0.45	0.42	282.82
0.2 nM CP55940	0.37	0.41	0.39	259.64
0.2 nM CP55940	0.46	0.52	0.49	346.91
2.0 nM CP55940	0.48	0.50	0.49	352.82
2.0 nM CP55940	0.51	0.56	0.54	393.73
4.0 nM CP55940	0.44	0.47	0.46	320.55
4.0 nM CP55940	0.48	0.51	0.49	354.64

Trial 3: cAMP Assay

Table 12: Trial 3 standard absorbance readings and protein concentration.

Standards				
Standard	Repeat 1	Repeat 2	Mean	Protein (ug/mL)
Zero	0.52	0.56	0.54	383.50
NSB	0.09	0.10	0.10	418.05

Table 13: Trial 3 Absorbance readings and %B/Bo values for samples.

Sample Name/Standard	Sample @ 450 nm	%B/Bo	Average %B/Bo
6400 fmol cAMP	0.46	145.12	121.00
6400 fmol cAMP	0.49	96.89	
Forskolin	0.32	57.99	63.96
Forskolin	0.33	69.93	
0.2 nM CP55940	0.42	106.97	84.22
0.2 nM CP55940	0.34	61.47	
2.0 nM CP55940	0.37	65.80	65.44
2.0 nM CP55940	0.39	65.08	
4.0 nM CP55940	0.48	102.47	93.06
4.0 nM CP55940	0.44	83.65	

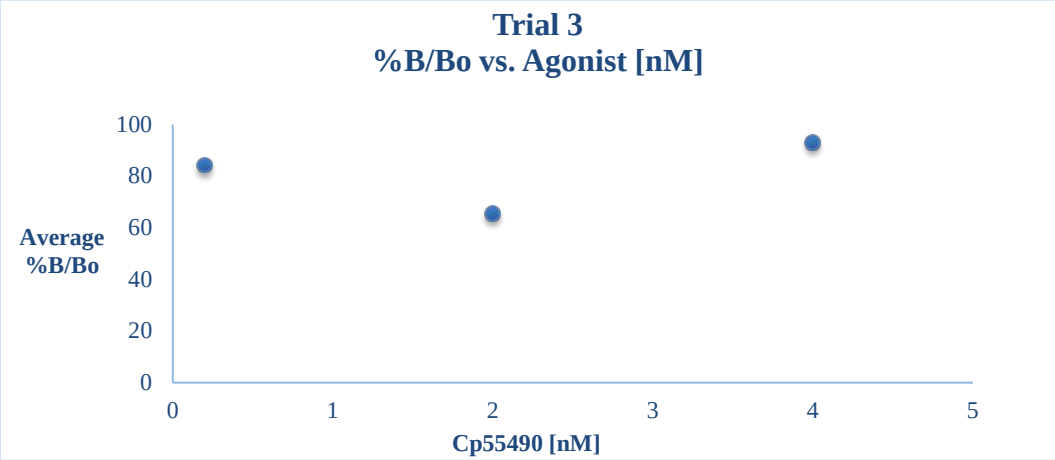

Figure 8: Trial 3 %B/Bo of Agonist concentrations of 0.2 nM, 2 nM, and 4nM.

Statistical Data

Table 14: ANOVA statistical summary

ANOVA Summary	
F	5.91
P value	0.01
Are differences among means statistically significant? (P<0.05)	YES
R square	0.44

Table 15: Collective statistics

Col. Stats	0.2nM	2.0nM	4.0nM
Number of Values	6.00	6.00	6.00
Minimum	23.16	59.68	60.83
Median	61.00	65.44	108.10
Maximum	107.00	75.14	160.90
Mean	58.07	65.70	106.90
Std. Deviation	30.17	5.38	34.09
Std. Error of Mean	12.32	2.20	13.92

Table 16: Tukey statistical test

Tukey's Multiple Comparisons Test	Mean Diff	95% CI of Diff	Significant?
.2 vs. 2	-7.63	-47.31 to 32.06	No
.2 vs 4	-48.82	-88.51 to -9.139	YES
2 vs 4	-41.20	-80.88 to -1.512	YES

Discussion/ Interpretation:

The overall trend from the data for Trial 1 and Trial 2 is an increase in $\%B/B_o$ as the agonist concentration increases. This shows a decrease in cAMP production, as more agonist is added; which supports our hypothesis. Trial 3 had an increase of %B/Bo from the 2 nM to the 4 nM, which does support our hypothesis. However, Trail 3 also had a decrease of %B/Bo from the 0.2 nM to the 2 nM CP55450, which is not consistent with our hypothesis. This decrease in %B/Bo is be a result of the high absorbance value of the first 0.2nM reading, and would most likely be averaged within the general trend with a greater sample size and more optimized procedure.

From the data it can be seen that the forskolin alone does not produce as large as an effect on the cells as expected, but instead only produces an effect when coupled with the agonist. This can be explained by the concentration of forskolin (3μM) not being optimized for the cells used in this procedure. The concentration of forskolin was obtained from literature that used overly expressed Chinese hamster ovary cells. (6) A dose response study of forskolin on the cells used in this procedure is needed.

The 6400 fM cAMP standard for all three trials did not work as expected, the %B/Bo for all three trials should have been close to zero and from the data it can be seen that is not the case. One explanation is that the 128 nM/mL cAMP standard that came with the kit was not of top quality. Trials with fresh cAMP is needed.

Another aspect from the data is that the %B/Bo values are not consistent and tend to overlap. Once the forskolin is optimized to this specific cell line, it would be beneficial to compare forskolin only to forskolin plus agonist to investigate the decrease %B/Bo from a maximum and to determine a better ratio of forskolin/agonist to apply to cells to get more consistent data.

The results of statistical tests demonstrated that $\%B/B_o$ values of the agonist concentrations of 0.2 nM-4 nM, and 2 nM-4 nM were significantly different and can be seen in Table 16. However the $\%B/B_o$ values of the agonist concentrations of 0.2 nM-2 nM were not significantly different, which as previously stated is a result of a high reading from the 0.2nM CP55450 and may be overcome by an increase in sample size and procedure optimization.

Conclusions:

With the exception of the 0.2 nM-2 nM drop in $\%B/B_o$ in Trial 3, all other data resulted as expected, the %B/Bo increased as the agonist concentration increased, which means an overall decrease in cAMP production. The statistical analysis data showed that the differences between the %B/Bo values of the 0.2 nM-4 nM and 2 nM-4 nM were significantly different, however the %B/Bo values were not significantly different between the 0.2 nM and 2 nM concentrations.

Based on the data and statistical analysis and in lieu of the 0.2 nM and 2 nM %B/Bo values not being significantly different, the goal of the study was accomplished. It is possible to demonstrate CB_1 agonist activity by inhibiting forskolin induced cAMP levels in GH4C1 cells. With method optimization and a greater sample size, we hope to spread significant difference of %B/Bo values amongst all concentration ranges and develop the method to be more precise and accurate.

Acknowledgements:

The author thanks Dr. Lauren Waugh for her patience and kind guidance, Dr. Richard Egleton for cellular supervision and troubleshooting solutions/interpretations, and Evan Thompson for technical assistance.

References:

1. *Understanding the "Spice" Phenomenon*. European Monitoring Centre for Drugs and Drug Addiction. Print.

2. Elphick, Maurice, and Micheala Egertova. "The Neurobiology and Evolution of Cannabinoid Signaling." *The Royal Society: Biological Sciences* 356.1407 (2001): 381-408. Print.

3. Uchiyama, Nahoko, Ruri Kikura-Hanajiri, Jun Ogata, and Yukihiro Goda. "Chemical Analysis of Synthetic Cannabinoids as Designer Drugs in Herbal Products." *Forensic Science International*198.1-3 (2010): 31-38. *Forensic Science International*. Web. 24 Aug. 2014. <http://www.fsijournal.org/article/S0379-0738(10)00010-1/abstract>

4. Lindigkeit, Rainer, Anja Boehme, Maike Luebecke, Marion Wiggermann, Ludger Ernst, and Till Beuerle. "Spice: A Never Ending Story?" *Forensic Science International* (2009): 58-63. Web. 24 Aug. 2014. <www.elsevier.com/locate/forsciint>.

5. O.Crews, Bridgit. "Synthetic Cannabinoids: The Challenges of Testing for Designer Drugs." *Clinical Laboratory News* 1 Feb. 2013: 8-10. Print.

6. Carmona, Murielle R., Anne Le Duigou, Didier Oustric, Francis Barth, Monsif Bouboula, Pierre Carayon, Pierre Casellas, and Gerard Le Fur. "Modulation of CB1 Cannabinoid Receptor Functions after a Long-Term Exposure to Agonist or Inverse Agonist in the Chinese Hamster Ovary Cell Expression System." *The Journal of Pharmacology and Experimental Therapeutics* 287 (1998): 1038-047. Web.