5-1-2021

Meta-Analysis of Cannabigerol Effects on Breast Cancer Tissue Cells

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META-ANALYSIS OF CANNABIGEROL EFFECTS ON BREAST CANCER TISSUE CELLS

A Thesis

Submitted in Partial

Fulfillment for Graduation with Honors Distinction and

the Degree of Bachelor of Science

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College of Natural Health Sciences

MAY 2021

META-ANALYSIS OF CANNABIGEROL EFFECTS ON BREAST CANCER TISSUE CELLS

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RECEIVED BY THE UNIVERSITY THESIS/CAPSTONE PROJECT COMMITTEE ON:

May 8th, 2021

CBG Effects on Breast Cancer Tissue Cells

Abstract

Cannabigerol (CBG) is one of over 120 cannabinoids known that is produced by the *Cannabis sativa* plant and has become of recent therapeutic interest. Cannabinoids target primarily cannabinoid receptors 1 and 2 (CBR1/CBR2) and form ligand-receptor complexes that can activate many cell signal transduction pathways, explaining its wide range of beneficial effects across many conditions. Positive effects with the introduction of CBG have been shown in research for Huntington's Disease, appetite-consumption levels, antibacterial efforts, and numerous other areas. The impact of breast cancer is seen highest in the female population, and is a condition still searching for the best therapeutic treatment. Cell death is commonly seen as an immune response to target tumor aggregates in breast cancer. This study looked specifically into the effect of CBG on two types of breast cancer cells (E3 luminal and EWD8 basal). In order to determine if the CBG could affect the cells, primers were designed for genes that were hypothesized in previous research to respond to CBD. The results were measured by a PCR. To measure the effects of cell proliferation and cell death, a MTT and cell trace assays were performed which suggested decreased proliferation and increased cell death. Based on these results, it appears that CBG slows proliferation and potentially induces necroptosis in human breast cancer cell lines.

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Introduction

Cannabigerol (CBG) is one of over 120 cannabinoids known that is produced by the *Cannabis sativa* plant. These various cannabinoids have beneficial properties that could be useful therapeutically. Cannabinoids target primarily cannabinoid receptors 1 and 2 (CBR1/CBR2) and form ligand-receptor complexes that antagonize the alternative receptor complex. Many cell signal transduction pathways can be activated by CBG, explaining its wide effects across many conditions. Positive effects with the introduction of CBG have been shown in research for Huntington's Disease, appetite-consumption levels, antibacterial efforts, and numerous other areas. The impact of breast cancer is seen highest in the female population, and is a condition still searching for the best therapeutic treatment. Cell death is commonly seen as an immune response to target tumor aggregates in breast cancer. This study looked specifically into the effect of CBG on two types of breast cancer cells (E3 luminal and EWD8 basal). In order to determine if the CBG could affect the cells, primers were designed for genes that were hypothesized in previous research to respond to CBD. The results were measured by a PCR. To measure the effects of cell proliferation and cell death, a MTT and cell trace assays were performed which suggested decreased proliferation and increased cell death. Based on these results, it appears that CBG slows proliferation and potentially induces necroptosis in human breast cancer cell lines.

Review of Relevant Literature

Understanding CBG

This section will start the paper off understanding what Cannabigerol (CBG) is and the interactions that CBG has within a cell.

Cannabigerol (CBG) is one of over a hundred cannabinoids produced by the *Cannabis sativa* plant, which also produces Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Gugliandolo, Pollastro, Grassi, Bramanti, & Mazzon, 2018). These cannabinoids have shown to be beneficial anti-inflammatory and anti-bacterial agents (Gugliandolo et al., 2018). CBG has been brought to the forefront in the cannabinoid research of recent due to its potential benefits concerning human health. The current knowledge is limited in this area as these benefits are still being discovered; however, CBG shows promise in its positive effects across a wide range of conditions.

Cannabigerol (CBG) is a non-psychotropic cannabinoid discovered in 1964 by Gaoni and Mechoulam (Gaoni, Mechoulam, 1964). In 1964, Gaoni and Mechoulam (1964) described *Cannabis sativa L.* as one of the most commonly used illicit narcotic drugs of that time. They were the first researchers to isolate the structure 3,4-trans-tetrahydrocannabinol, and partially synthesize the active constituent of cannabigerol (CBG) (Gaoni, Mechoulam, 1964). *C. sativa* originates from Central Asia and contains distinctive phytochemicals that further contribute to its therapeutic outcomes in various diseases (Zagozen, Cerenak, Kreft, 2020). The highest amount of cannabinoids within *C. sativa* are found within the "plants' inflorescences and glandular trichomes" (Zagozen, Cerenak, Kreft, 2020). Cannabinoids tend to bind and activate cannabinoid G-protein-coupled receptors-- CBR1 and CBR2 (sometimes denoted as CNR1/CNR2) (Navarro, Varani, Reyes-Resina, Sanchez de Medina et al., 2018). This ligand-

receptor interaction forms complexes where an antagonist system between the two cannabinoid receptors occurs -- CBR2 complex blocks CBR1 mediated effects (Navarro, Varani, Lillo, Vincenzi et al., 2020). CBG also targets transient receptor potential channels (TRP channels), COX1/2 enzymes, and alpha-2 adrenergic receptors (Borrelli et al., 2013). These few sources indicate the potential of CBG within therapeutic research, yet highlight the lack of concrete results and limited studies.

Prior CBG Research

Cannabigerol has been used in a plethora of research recently as its beneficial properties have become more apparent. Aqawi, Gallily, Sionov, Zaks, Friedman, and Steinberg (2020) studied cannabigerol's effects on prokaryotic *Vibrio harveyi*, as much of CBG's limited studies have been on eukaryotic cells. These researchers hypothesize CBG as an anti-biofilm and anti-quorum sensing catalyst for *V. harveyi*, preventing cell-to-cell communication and environment sensing (Aqawi et al., 2020). Their results supported their hypothesis as a potential anti-biofilm agent inhibiting quorum sensing with the use of DNA quantification, quantitative real-time PCR, and motility assays (Aqawi et al., 2020). Cannabinoids have been shown to have antimicrobial activity against certain species of gram-positive bacteria (Aqawi, Sionov, Gallily, Friedman, Steinberd, 2021). Using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), Aqawi et al. (2021) saw "intracellular accumulation of membrane portions" and swollen *Streptococcus mutans* after CBG introduction. Staining emphasized CBG's alterations to the membrane's structure and polarization state (Aqawi et al., 2021). Their hypothesis was supported that CBG exerts antibacterial properties against *Streptococcus mutans*

by increasing the bacteria's membrane permeability and subsequently preventing pH changes to the cell (Aqawi et al., 2021).

Within non-human research, rats/mice are readily used within CBG studies. Brierley, Samuels, Duncan, Whalley, and Williams (2016) study possible impacts on food intake with CBG introduction in rat populations. Cannabinoids devoid of the normal appetite-stimulating properties of THC still give rise to differences in eating behavior when introduced to eukaryotic organisms like rats (Brierley et al., 2016). The researchers observed feeding patterns through neuromotor tolerability testing, and regular meal monitoring. A range of 30-120 mg/kg of CBG doses were introduced to one experimental group, or a placebo was introduced to the control group; the researchers discovered the range of 120-240 mg/kg of CBG as the most impactful dosage, doubling the amount of food eaten by the rats (Brierley et al., 2016). This research showcases CBG's potential hyperphagic effects for the first time, with the direct relationship seen between food consumption and CBG dosage introduction (Brierley et al., 2016). Various plant-derived cannabinoids like CBG are used frequently in experimental models showing neuroprotective potential for Huntington's disease (HD) (Valdeolivas, Navarrete, Cantarero, Bellido, Munoz, Sagredo, 2015). CBG has already been shown to improve motor skills and antioxidant defense levels in mice, inspiring Valedolivas et al. (2015) to further understand the neuroprotective capabilities of the cannabinoid. This research utilized histological analyses and oxidative stress systems to discern that a series of genes are linked to HD and these genes' expression was partially normalized with CBG treatment introduction (Valdeolivas et al., 2015). Gene expression improved for the brain-derived neurotrophic factor and insulin growth factor-1 genes, but was reduced in the genes responsible for mutant huntingtin aggregates (Valdeolivas et al., 2015). Their hypothesis was partially supported, and the research opens up the possibility for

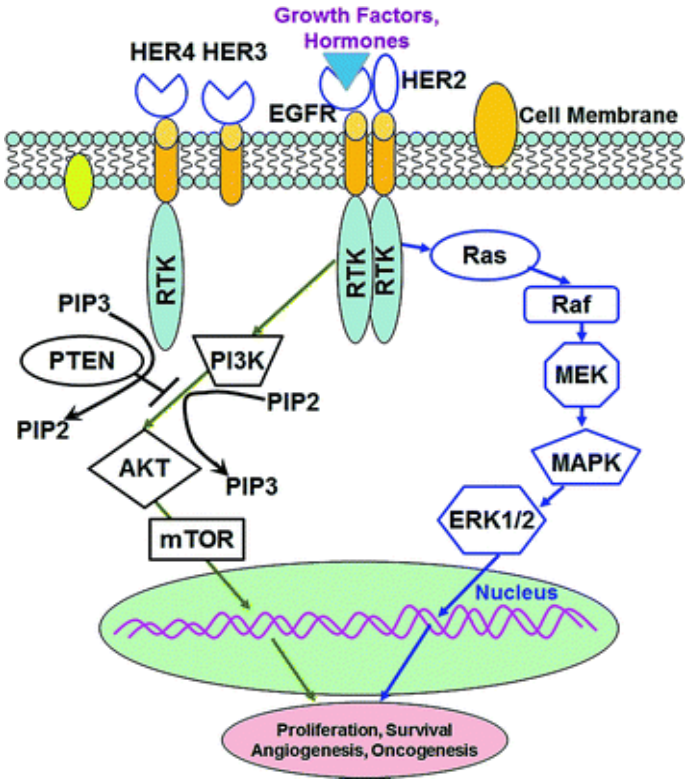
CBG therapy in treating neurodegenerative conditions like Huntington's Disease (Valedolivas et al, 2015).

Navarro, Varani, Reyes-Resina et al., (2018) study a different aspect of CBG in terms of its interactions with CBR1 and CBR2 receptors, and the subsequent effects seen from each complex activation (Navarro et al., 2018). This research is applicable to my research presented in this paper. Their team discovered that CBG showed competitive binding favoring CBR2 but not CBR1 in living CHO cells transfected with cDNA of human CBR1/2 receptors (Navarro et al., 2018). Further ERK phosphorylation, dynamic mass redistribution, and Beta 2 Arrestin recruitment assays showed CBG as a partial agonist for CBR2 (Navarro et al., 2018). The downstream cellular cascade of CBG on CBR1 still remains an open research topic, but this paper is successful in showing the partial potential of CBG regulating CBR2's signal cascade Navarro et al., 2018). Understanding CBG's interactions with and effects on the endocannabinoid receptors is essential in planning experiments targeting these receptors. These sources show the wide range of possibilities in using CBG therapeutically, which drives my curiosity towards CBG's effects on breast cancer tissue cells.

Breast Cancer Tissue Cells

Breast cancer is one of the most fatal cancer-related conditions among women across the world (S. Aziz, M. Aziz, 2012). There are 5 distinct subtypes of breast cancer, two of which are the luminal subtypes and basal-like subtypes (Badve, Turbin, Throat, Morimiya et al., 2007). Luminal cell subtypes make up >70% of tumors seen in breast cancer patients, primarily containing estrogen/progesterone receptors and cytokeratin (Haughian et al., 2012). Luminal cancers typically have a positive prognosis, but hormone resistance within recurring tumors is

highly likely (Haughian et al., 2012). Basal cell subtypes are defined by the presence/absence of estrogen/progesterone receptors, EGF1, EGFR, and/or CK5 (Haughian et al., 2012). Both cell types were used in the following research. Many major signaling pathways, like the one attached, show the complexity involved in understanding breast cancer and in targeting the best receptors for the patient's prognosis (Aziz, Aziz, 2012). In rats and mice, normal adaptive immune responses against these epithelial breast cancer cell types lead to an outgrowth of T-cells and HER2 antigen loss (Stein et al., 2019). Recognizing potential targets and indicators of breast cancer is still being investigated. Minimal work has been done toward CBG implementation in combating some of the detrimental impacts of breast cancer.



Aziz, S. W., & Aziz, M. H. (2012). Major signaling pathways involved in breast cancer. (pp. 47-64). New York, NY: Springer New York. doi:10.1007/978-1-4614-5647-6_4

Cell Death & Necroptosis

One method of therapeutics that are used to encourage our immune system to attack and eliminate cancer cells is immunogenic cell death (ICD) (Kaur, Johnson, Northcote-Smith, Lu, 2020). ICD allows the dying cancer cells to send protein signals out, stimulating tumor-associated immune cells to target invading tumors (Kaur et al., 2020). ICD is capable of being induced in breast cancer stem cells, and stem cells that have sustained through ICD have

potential as a vaccine to further initiate a stronger immune response (Kaur et al., 2020). This induction process is an insufficiently researched area of immunotherapy currently, but Kaur et al., (2020) reports of a “copper II complex (made of Schiff base and polypyridyl ligands)” as the first capable metal complex to induce ICD in these breast cancer stem cells (Kaur et al., 2020).

Another potential immune response breast cancer cells could induce is necroptosis, one type of programmed cell death associated with caspase 8 kinase, receptor-interacting serine/threonine kinase 1 (RIPK1), and RIPK3 (Sun, Pan, Ma, Chen, Zhao, Liu, 2021). Sun et al. (2021) investigates if 3-bromopyruvate (3-BP), an inhibitor for glycolysis, induces a form of cell death and if it affects breast cancer cell proliferation (Sun et al., 2021). 3-BP treatment was shown to inhibit cell proliferation in two cell types, causing a significant antitumor decrease correlated with necroptotic protein PpM1B (Sun et al., 2021). The preceding research relating breast cancer cell types and various forms of cell death indicate multiple potential pathways for targeting in treatment.

Following this review of relevant literature is my CBG research on breast cancer tissue cells from both subtypes. I investigated the effects of CBG on cell proliferation and subsequent immune responses, including cell death.

Research Design

This research was conducted in a team with Morgan Orand and Taylor Lupica, and utilizes procedures from UNCO's Cell/Molecular Lab course BIO442.

Research Question:

How will CBG media affect the gene expression of CNR1, CNR2, Ripk1, and MLKL? How will cell proliferation be impacted? In what way will cell death occur?

Hypothesis:

We predict an overall increase in CNR1, CNR2, Ripk1, and MLKL gene expression because the cannabinoid ligands present in the CBG should bind and activate downstream signaling of the CNR1 and CNR2 receptors within breast cancer cell lines.

We predict decreased breast cancer cell proliferation, and increased necroptosis cell death with introduction of cannabinoid binding from CBG agar due to CNR1 and CNR2 activation and dimerization, ultimately activating the downstream cell signaling pathway. This activated pathway induces cell death, and we hypothesize this to be necroptosis if Ripk1 and MLKL are present.

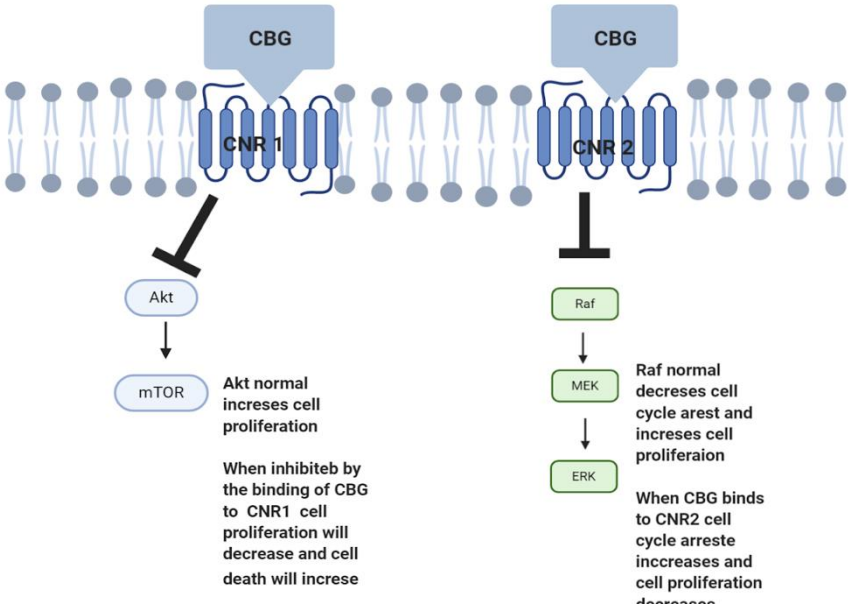


Figure 1 Kisková, T., Mungenast, F., Suváková, M., Jäger, W., & Thalhammer, T. (2019). Future Aspects for Cannabinoids in Breast Cancer Therapy. International journal of molecular sciences, 20(7), 1673. https://doi.org/10.3390/ijms20071673

Cell death frequency increases with the activation and dimerization of CNR2 and CNR1. This research aims to activate these pathways with the introduction of cannabinoids in CBG agar.

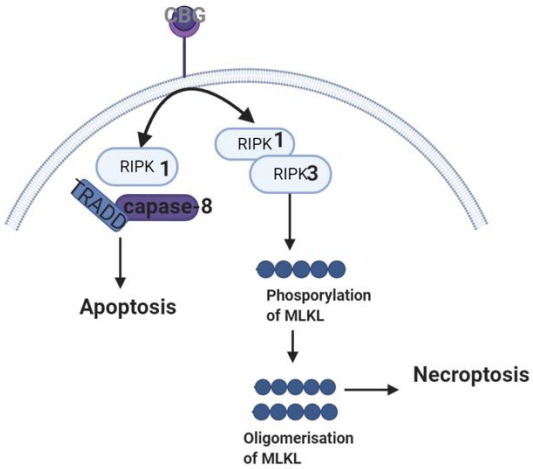


Figure 2 Dhuriya, Y. K., & Sharma, D. (2018). Necroptosis: A regulated inflammatory mode of cell death. Journal of Neuroinflammation, 15(1), 199. doi:10.1186/s12974-018-1235-0

This is the predicted pathway downstream of CNR1 and CNR2 that would lead to the hypothesized necroptosis.

Experimental Design

Methods:

Culture of Experimental Cell Lines

Control: E3 Cell lines on MEM media

Experimental: E3 cell lines on MEM media with CBG

- Concentration 1: 2 micromolar
- Concentration 2: 20 micromolar
- Concentration 3: 200 micromolar

We cultured E3 (luminal) cell line cells in T25 flasks for assays. Cells were split to promote growth by first removing E3 cells from original flask to pellet. The cells were centrifuged at 300rpm for 15 minutes. 5mL of PBS was added after the first supernatant was removed, and the cells were centrifuged again, but at 400rpm for 10 minutes. The supernatant was removed again, and this was repeated once more. After the third supernatant had been removed, 1.5 mL of trypsin was added and allowed to incubate to interrupt the cell's natural adhesion to the flask. The trypsin was removed, and 1mL of MEM was added to the cells, then the sample was centrifuged at 400rpm for 5 minutes. 4mL of cells were put into one flask, and the other 4mL were put in a different flask.

The cells were split into experimental and control flasks. The control flask was left untreated. Three different doses of CBG were tested in this experiment-2uM, 20uM, and 200 uM. The vehicle for this drug had 10 uL Tween-80, 37.5 uL DMSO, and 750 uL PBS. Cells were counted

with a hemocytometer on 1st, 3rd, and 7th days to evaluate effects on proliferation after enough cells had grown.

CellTrace Violet Cell Proliferation Kit (*CellTrace™ Violet Cell Proliferation Kit Procedure*)

This assay was used to monitor numbers of cell divisions of proliferating cells using diluted dye. With flow cytometry, different cells have different peaks on the graph assuming the cells have various numbers of cell divisions. Cells from both the experimental and control flasks were spun down, and the supernatant was removed, leaving a pellet. CellTrace Violet (1:1000 dilution) staining solution was added the same day, and cells were resuspended in solution. The flasks were incubated at 37 Degrees Celsius for 20 minutes in the dark. Complete culture medium (MEM) was mixed in and the cells were incubated again at 37 Degrees Celsius for 5 minutes. Cells were spun down again, supernatant removed leaving a pellet, and resuspended in fresh, pre-warmed media. Flow cytometer analysis was performed 4-5 days after the addition of the dye and treatments. This was repeated 3 different times.

MTT Assay

The MTT assay was used to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. This assay was set up in triplicate according to Table 1. The colorimetric assay is based on the reduction of yellow tetrazolium salt into purple formazan crystals by metabolically active cells. Only E3 cells were plated in a 96 well dish, and 100,000 cells were plated in each well. The cells were left 1 week in the incubator (37 C) and then 45 ul of media and 5 ul of MTT was added to each well. They were incubated for 4 hours. After 4 hours, purple crystals formed and were completely dissolved in solubilization solution. The absorbance reading was taken from the plate and the data was normalized for analyzing the effect of the different amounts of CBG. (Cellular/Molecular Biology Protocol Guide, 2020) .

Control (+)	Control (-)	2uM CBG	20uM CBG	200uM CBG
Control (+)	Control (-)	2uM CBG	20uM CBG	200uM CBG
Control (+)	Control (-)	2uM CBG	20uM CBG	200uM CBG

Table 1. Treatment groups in MTT Assay

RNA Isolation

We isolated total RNA from the E3 and EWD8 cell lines without CBG treatment following protocol from *Pure Link Mini Kit*. This was done very carefully as RNA is fragile and prone to degradation by RNases. Once the E3 and EWD8 RNA were isolated, we tested to see if it was pure enough to continue transforming the RNA to cDNA. Using a Nano-drop spectrophotometer that was first blanked, a sample may be applied, and readings can be taken. The readings observed come from the ratio of absorbance at 260nm and 280nm. A ratio of ~2.0 is considered acceptable for the purity of RNA.

cDNA Synthesis

The next step was ensuring the RNA quality, and electrophoresis on a denaturing agarose gel. The denaturing gel was prepared with 1% agarose water, 10x MOPS buffer and formaldehyde. The RNA samples were mixed with formaldehyde- containing loading dye and

were denatured at 65 degrees Celsius for five minutes. We performed a native (non-denaturing) agarose gel electrophoresis which allowed us to judge the overall integrity of a totally RNA preparation by inspection of the 28S and 18S rRNA bands. The secondary structure of RNA altered the migration pattern in the gel so that it did not migrate according to true size. The intact RNA should have a sharp 28S and 18S rRNA band.

Primer Design

Primers were designed to amplify a 100-200 bp fragment of each gene of interest. Two primers were designed for each gene (a forward and a reverse) corresponding to the 5'/3' boundary (respectively). Using the basic rules of thumb for primer design, and NCBI, mRNA sequences of each gene were obtained and run through Primer Blast. The following were the obtained sequences for each gene with the best qualifications for ideal primers:

CNR1_F- AGTGGAGGTGGCAGAATGTG

CNR1_R- GCATCATGTAGGTGGGGAC

CNR2_F- CAGAGGAGCCTAAAACAGCCA

CNR2_R- AGCCTGGTCATGTTTATGAGAT

MLKL_F- AGTTTACAACGAGGGGTGGT

MLKL_R- CCTCTGTGGATGGTAGGGTTC

Ripk1_F- CATCTCCTACGGCTCGCAAT

Ripk1_R- AGCCCCACTTCCTATGTTGC

Primers were then ordered and used for future assays.

PCR

Once the RNA was found to be intact, it was then synthesized into cDNA in order to be stored and manipulated to be used in PCR. To synthesize the RNA, 1 ug of RNA was put in a

nuclease free PCR tube and the volume was adjusted to 11ug with the autoclaved DEPC treated water. 1 ul dNTPs and 2 uL anchored oligo dT22 were added and spun down, denaturing the RNA. Afterwards, 4 ul 5X First strand buffer, 1.2ul DEPC-treated water, 1ul DTT, 05.uL RNase-out and 0.3 ul Superscript III were added to the previous PCR tube. The tube was spun down again and ready for PCR and storage. This was done for both the E3 and EWD8 cell lines. With the obtained cDNA and the previously designed primers (CNR1, CNR2, RIPK1, MLKL) a polymerase chain reaction was performed. Primers were diluted depending on their 'nmol' value (ex. 19.34 nmol= 193.4 uL of water added). There was a total of 11 PCR tubes. Eight experimental PCR tubes contained 18 ul of the master mix (1 ul E3 or EWD8 cDNA, 10 ul GoTaq and 7 ul nuclease free water) and 1ul of the forward primer and 1 ul of the reverse primer. The negative control contained 18 ul master mix without cDNA and with one (any) of the primers-- 1 ul forward and 1 ul reverse. Two known positives were also introduced that had beta-actin forward and reverse primers (following the same amounts as the test PCR tubes). All tubes were run in the thermocycler: A. 1 cycle at 94 C for 2 minutes B. 40 cycles of: 1. Denaturing: 94C for 30 seconds 2. Annealing 52-68 C for 30 seconds 3. Elongation 72 C for 30 seconds. After 40 cycles, there was a 10-minute extension for final elongation at 72 C, then a hold at 12 C. Once this was done, 10 ul of each of the 11 samples were mixed with 2 ul of sybr green dye and loaded into a 1% agarose gel. The gel then ran at 100 volts for 45 minutes. The gel should hopefully show bands at all of the different primers, indicating that the primers worked and that the genes we targeted were present. Once these results were obtained, different concentrations of CBG (2mM, 20 mM, and 200 mM) were added to the cDNA of the E3 and EWD8 cell lines to see if there was an increase in gene expression (i.e. a brighter band) with the introduction of CBG.

Necroptosis assays were unable to be conducted due to the COVID-19 pandemic.

Results/Discussion

MTT Assay

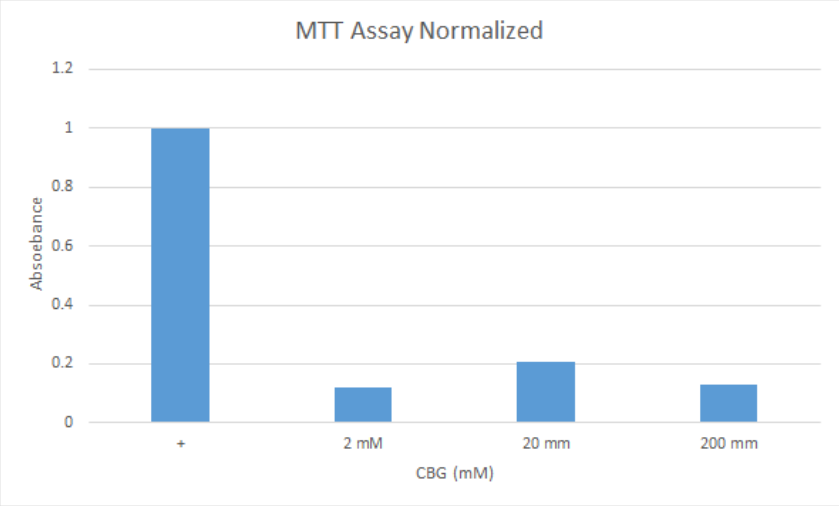


Figure 3 MTT Assay Results (Normalized) - Micromolar dosages of CBG

The MTT assay assessed the viability of the cells when introduced to different amounts of CBG. The data that was collected was normalized, meaning it was compared to a series of so-called "normal forms" in order to reduce data redundancy and improve data integrity. Normalization of the data made sure that all the data looked and read the same way across all different doses of CBG. According to Figure 3, 2 uM allied for the least amount of cells to survive and 20 uM allowed for the most amount of cells to survive when treated. Unfortunately, no statistic could be run on this data because time only allowed for one trial of the MTT assay to take place. To confirm these results and draw conclusions, the MTT assay would need to be repeated, and combined with the other assays. From this data, we may begin to hypothesize that 2 uM may be the most effective of increasing death/decreasing viability in E3 and EWD8 human breast cancer cells.

PCR

The PCR gave some very interesting results as we had expected all of the genes we hypothesized to be expressed, but found that only the CNR 2 gene was expressed. The data also suggests that the expression of CNR2 is greater in EWD8 cells than E3 cells.

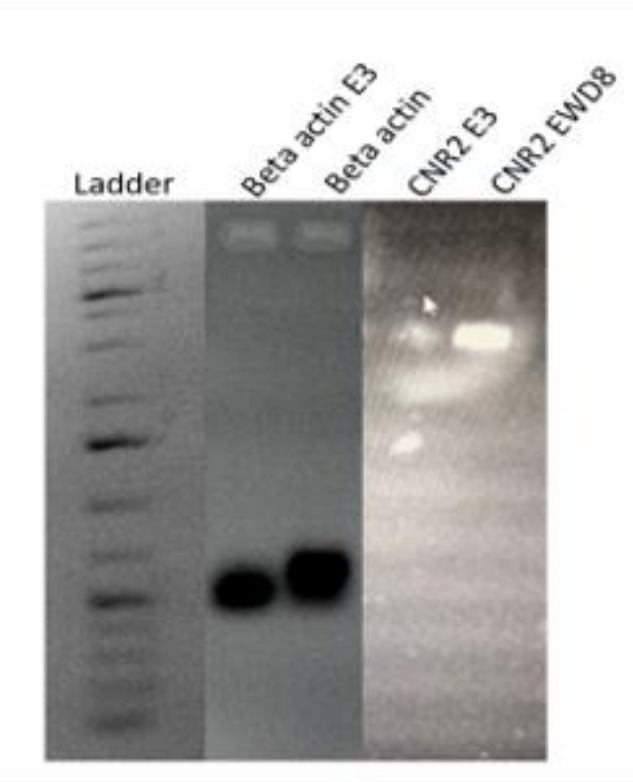


Figure 4 PCR Gel Images

Cell Trace

Cells	Intensity of Dye 1st Trial	Intensity of Dye 2nd Trial	Intensity of Dye 3rd Trial
Control (No CBG)	316.227766	100	199.526231
2 uM CBG	1258.92541	1258.92541	630.957344
20 uM CBG	3162.27766	2511.88643	4466.83592
200 uM CBG	5011.87234	12589.2541	7943.28235

Table 2. Raw Data of Concentrations of CBG vs Intensity of Dye (Three trials)

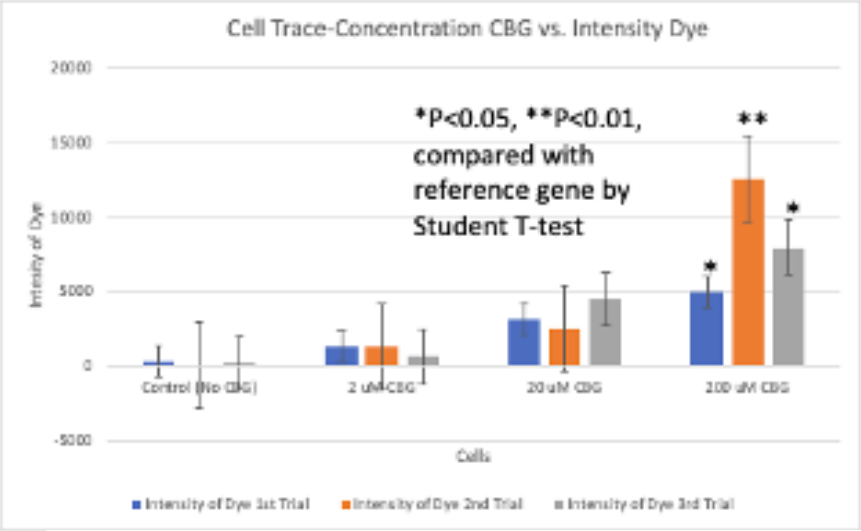


Figure 5 Cell Trace-Concentration of CBG vs Intensity Dye (Three trials)

The cell trace data showed decreasing intensity of dye with increased dosages of CBG (Figure 5). Further cell proliferation statistical analysis was not possible due to COVID-19, but we predicted that a lower intensity indicates greater rates of cell proliferation which was shown to be accurate from the results. 200uM of CBG saw greater intensity between the three trials stipulating a decreased rate of cell proliferation.

Discussion of Research

Based on the findings from the literature and the research conducted, CBG has potential as a therapeutic agent in helping slow cell proliferation in breast cancer cell lines. CBG has been useful in many other conditions and is on the rise in the research industry as more benefits are discovered. There are large gaps in exact signal transduction pathways affected with CBG, but research like the one conducted help contribute to the understanding of CBG's targets.

The slowing of cell proliferation and preferential binding of CNR2 did not surprise me. The review of relevant literature supports the results of competitive binding toward CNR2 than CNR1 (Navarro et al., 2018). Better gene targets could have been chosen to further emphasize this, like the ones listed in other research studies studying eukaryotic cells. Cell necroptosis was unable to be investigated due to the COVID-19 pandemic, but a western blot and necroptosis assay would be useful in determining the exact immune response seen between CBG and breast cancer cells.

In conclusion, CBG appears to slow the proliferation of human breast cancer cells, activate CNR2, affect cells at different doses, and affect the E3 and EWD8 cell lines in different ways. This research indicates there is potential to help lessen tumor severity in breast cancer with CBG, and demands further investigation be done with this cannabinoid. Other studies should be conducted to test for the presence and activation of CNR1 in these cells. In addition, more research should be done to investigate the inflammatory effects of this drug on the breast cancer cell subtypes to further understand side effects associated. This is just the surface of CBG's research and progress continues to be made as shown through the research collected since 1964.

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