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Cannabidiol as a Synergist in Chemotherapy

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Cannabidiol as a Synergist in Chemotherapy

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Abstract

Cannabidiol (CBD) is a non-psychoactive compound commonly found in hemp and non-hemp plants. It has a molecular effect on cells, but its effect in modifying cellular signaling pathways has not been thoroughly tested until recently. Experimentation was done to determine whether CBD could induce cell death in MCF7 breast cancer cells. Both normal MCF7 cells and Cisplatin resistant MCF7 cells were used for these experiments. The cells were dosed with 0, 0.5, and 1.0 µg/ml CBD dissolved in methanol. Later micrographs of the treated cells were collected and Annexin staining to determine the effect of CBD on cell death was conducted. Additionally, a Western blot and immunocytochemistry for Hsp27, a molecular indicator of cell stress, were performed. There were conflicting results across the tests that were conducted, but there was evidence that CBD had an effect on the viability and morphology of breast cancer cells. The Annexin staining showed no correlation between CBD dose and apoptosis, while Western blots showed no difference in Hsp27 expression across different doses of CBD with higher expression of Hsp27 in the chemoresistant MCF7 cell line. However, ICC imaging showed that higher CBD doses resulted in punctate Hsp27 localization. Light micrographs also showed vacuolization of the cells treated with CBD and a lower abundance of live cells in higher doses of CBD. Migration data supported CBD inhibited cell motility via a transwell migration assay. Further literature review was conducted in an effort to better understand these results in relationship to ovarian cancer cell lines. Since ovarian cancer is a highly aggressive, and highly metastatic form of cancer with little success in traditional treatment, a better understanding of the role of CBD in ovarian cancer research is warranted.
Introduction

Properties and Mechanisms of CBD

Cannabidiol (CBD) is a naturally occurring chemical from plants. The CDC indicates that CBD does not cause an impaired mental state in humans. It can be used in a variety of different products including food, cosmetics, and oils, and has recently been gaining ground as a medical intervention for a variety of different purposes including seizure disorders, chronic pain, and as a chemotherapeutic (CBD: What You Need to Know. 2022). Chemotherapeutic treatments can be used to address aggressive cancers like lung, ovarian, and breast cancers. The impact of novel types of chemotherapeutics are dependent on how the compound directly enters or binds to the cell membrane to induce signal transduction pathways. There are generally two cannabinoid receptors in the human body, CB1 and CB2. CB1 is expressed mostly in the central nervous system and generally under expressed in immune system cells while CB2 is primarily expressed in lymphocytes and only at low levels in the brain. Lymphocytes and the immune system are important factors in whether cells respond to CBD because they influence and control the systems responsible for creating inflammatory responses in the body (Gustafsson K et. al. 2008). Alongside these factors, CBD has been shown to increase vacuolization in breast cancer cells and was concluded to result from stress impacting the endoplasmic reticulum (Schoeman R, Beukes et. al. 2020). These receptors trigger many pathways in their respective domains. A better understanding of the mechanisms by which CBD impacts cell signaling and cell stress pathways may allow its use as a potential therapy for several human diseases.

The role of the cannabinoids in inflammatory disease and the stress response

Evidence supports that cannabinoids may be important in protection versus inflammation in animal models. One such study analyzed the effects of CBD and AEA for anti-inflammatory
properties in peripheral allergic reactions called allergic contact dermatitis. An allergen was presented to mouse keratinocyte skin cells from the ear, which typically elicits an immune response, triggering T cell activation, and causing local inflammation in hopes of destroying the allergen. The use of CBD and AEA had inhibited dermatitis in 6 hours in high doses, and 24 hours in low doses, doses ranged from 5 to 20 µM (Petrosino S, et. al. 2018). While this treatment is effective in subduing inflammation, it does not account for pain and irritation at the affected site. Medications like prednisone are traditionally administered to stop acute peripheral inflammation like dermatitis, as well as low severity airway inflammation like bronchitis, but this medication can take up to four days to take effect with potential side effects. To conclude, these cannabinoids may provide the same function as current medications over less time with little to no side effects.

The effect of cannabidiol has been investigated with Anandamide (AEA), a naturally occurring CBD ligand, to determine effects on levels of heat shock proteins (Hsps) in rat lungs, specifically Hsp25 and Hsp70. Anandamide is a natural cannabinoid ligand that can activate many of the present cannabinoid receptors (C1 - C3.) It was determined that AEA significantly upregulates expression of Hsp25 and Hsp70 in rat lungs as analyzed through a Western Blot. Since heat shock proteins are upregulated in inflammatory responses, it was concluded and theorized by this experiment that CBD and AEA may function efficiently as a pharmacological anti-inflammatory drug (Beata Kopczyńska, et. al. 2011).

One goal of this study was to determine the effects of Anandamide (AEA) on heat shock proteins Hsp70 and Hsp25, which are proteins found in organelles that respond to temperature changes, injuries and negative effects on the cell. Recent literature investigated how AEA is transported throughout the cell with assistance by Hsp70, and how this interaction forces AEA to
reuptake. This investigation questioned if AEA could enhance the cellular protection of Hsp70 and Hsp25 in rats that have sustained a traumatic injury to their lungs.

During this experiment, twenty-one male rats were kept in a controlled environment of light, temperature, and food source. The experiment divided experimental animals into two groups where in the first group had removal of the lungs just 2 hours after the injection of AEA, while the second group had removal of the lungs 24 hours after injection of AEA. Hsp70 and Hsp25 levels were analyzed in the lung tissue by immunohistochemistry and via Western Blot. The histological results show a significant increase of expression in both Hsp70 and Hsp25 when exposed to AEA in comparison to the control. With regard to temporal responses, lung removal at 24 hour shows a lower expression of both Hsps than those with lungs removed after 2 hours, but both experimental groups show increased Hsp expression as compared the control. In Hsp25 (the murine homolog of human Hsp27), this gap is bigger showing a 20-point difference in expression, while the difference in Hsp70 is only less than 5 points. Together, these results support that AEA may be promoting an early stress response in the cells. Therefore, the upregulation of Hsp70 and Hsp25 in a lung injury model may return the cell to homeostasis, clearing the AEA and sending the signal to nearby cells. This provokes a potentially cytoprotective response in the tissue in hopes to prevent subsequent tissue damage. This study supports that AEA may have potential pharmacologically as an anti-inflammatory drug; in this case, the injection of AEA is functions as a cellular stressor, causing an initial localized upregulation of Hsps. This signal is spread to other tissues functioning as a protective agent over surrounding systems (Kopczyńska B et. al 2011).
The role of cannabidiols as cancer therapeutics

While CBD has shown success as an antitumor synergist in some studies, it is not the only cannabinoid compound that triggers a response in the endocannabinoid system. Other compounds like delta-9-tetrahydrocannabinol (THC) and synthetic cannabinoids seem to also function in similar ways. In this case, synthetic cannabinoids may be more functional as a medicine because their structure can be more easily controlled and manipulated. A recent review of the literature on the role of cannabidiols as cancer therapeutics aimed to determine if pure CBD was significantly more effective than its counterparts in impacting proliferation of diverse tumor cell lines. CBD-treated cells showed decreased proliferation of leukemia cell lines. Additionally, CBD was found to have more favorable effects in a majority of cell lines over synthetic extracts in breast and prostate cancer. It was determined CBD was most effective at reducing glioma stem cell tumor proliferation, followed by THC, then lastly synthetic compounds. Furthermore, THC was found to be most effective in brain tumor cell lines (Nahler G. 2022) While this may seem contrary, this finding may be due to the fact CBD receptors are differentially expressed in specific cell types in the central nervous system than the rest of the body. Therefore, the effectiveness of different forms of CBD may be different in neuronal helper cells than the actual neurons.

Despite that cannabidiols were linked to the upregulation of heat shock proteins in inflammation studies, only one study has addressed the effect of CBD on expression of heat shock proteins. This experiment addressed the effects of CBD and THC on the expression of Heat Shock Proteins (Hsps) in gliomas and determined THC itself cannot act on CBD receptors. A cognate cannabinoid receptor is required for THC to function, while CBD can act independently of receptor-binding. CBD altered the expression of Hsps in glioma cell lines while
THC did not. In this experiment, T98G human glioblastoma cells were dosed every 48 hours over the span of 18-21 days. The results indicate a more marked upregulation of Hsps (Hsp-40, -60, -70 and -90) (~78% increase) in cells treated with CBD, with a more modest upregulation of Hsps (28%) with THC (Scott KA, et. al. 2015). Furthermore, this study also shows that inhibiting Hsps through pharmacological means increases the extent of cell death caused by CBD. This supports that CBD more strongly induces a stress response in cells, and as such, may support that CBD may increase expression of Hsps that can avert the successful treatment of diverse cancer types.

Another experiment investigated CBD in the lungs as a chemotherapeutic, and as a supporter of the natural immune system. In the first experiment, mice were injected with lung cancer cells and subsequently treated with CBD. They were specifically targeting cancer stem cells in this experiment, which are naturally more resistant. Both small cell and non-small cell lung cancers were investigated, and it was found that CBD caused activation of Caspase 3 and 7, enzymes that are activated as cells undergo cell death via apoptosis. Morphologically CBD also stopped spheroid formation of the cancer cells (Hamad H, et. al. 2011).

Finally, a review from Nahler, G aimed to identify the cytotoxic effects of CBD and other derivatives against many forms of cancer. They found CBD showed an effect on blood plasma levels when combined with Warfarin S and F separately. The result was an increase in blood plasma levels more than Warfarin alone (Nahler G. 2022). While this is not directly related to cancer, altering blood components and blood vessels may affect the way various cancers could metastasize in the body. For example, many cancers find their way to metastasize in the lungs. If CBD can improve the effect of blood thinning medications, blood and potentially cancer cells...
flow through the lungs more quickly. This gives cancer less time to leave the vessel and metastasize in a specific location.

Another common metastatic route that is particularly susceptible to cancer and tumor formation is via lymph fluid. CBD and AEA were also shown to activate natural T cells in the immune system to hinder tumor growth. In this study, CNR2 or cannabinoid receptor 2 r, which is expressed in lymph tissue was addressed directly. Using a FLAG-tagged (CNR2) knock in mouse model, it was determined that CBD inhibits tumor growth in cells that express CNR2. They concluded this activation helped lower the negative side effects often common with the use of traditional chemotherapeutics (Xiong, X., et al. 2022).

Cannabinoid receptors are expressed in lymphoma cells, a tumor type that needs a supportive tumor tissue microenvironment system. Understanding CBD receptor expression in Non-Hodgkin's Lymphoma was investigated (Gustafsson, 2008). Lymphomas impact the lymph system, which is a fluid system that cleans debris of the body and helps transport immune cells. Since lymph is a fluid that travels around the body, it is extremely prone to metastasis.

Expression of CB1 and CB2 in lymphomas grown in mice were investigated to determine if cannabinoids would suppress lymphoma tumor growth. This was investigated because previous studies had indicated there were potential pharmacological doses of cannabinoids that suppressed signal pathways related to cancer growth. MCL - mantle cell lymphoma - is a common type of lymphoma tissue line. The human MCL tumors were injected into female mice and allowed to grow for 3 days. The experimental cannabinoid drug R(+)-methanandamide (R(+))MA) was injected into the grown tumors, binding strongly to CB2 and weakly to CB1 cannabinoid receptors. Following treatment, expression of CB1a and CB1b was determined using PCR and was confirmed with a Western Blot analysis. The results of this study revealed that CB1 and
CB2 were over expressed in a majority of lymphomas, however there was a great variation among samples. The study revealed that lymphomas expressed CB1 and CB2 receptors and that the tumor size of R(+)methanandamide-treated mice decreased minimally supporting that this compound is not likely to be of clinical importance in lymphoma

CBD and Breast Cancer

Based on the literature, the role of CBD in chemoresistant cell lines was not widely studied. Historically, cannabis plant derivatives have been used to treat a variety of ailments, but lesser is known on cancer specifically (Turgeman I, 2018). In order to address the effectiveness of cannabinoids as antitumor agents, Shoeman et al, investigated CBD, as well as other derivatives of cannabinoids as a chemotherapeutic in triple negative breast cancer cells (Shoeman, 2020). Triple negative breast cancer (TNBC) lacks the receptors that other successful chemotherapeutics target (estrogen and progesterone receptors which respond to tamoxifen or aromatase inhibitors and HER2/Neu which responds to Herceptin). As such, TNBC is an extremely aggressive cancer type and is difficult to treat with conventional means. The researchers were motivated by recent literature that indicated cannabinoids were successful in inducing apoptosis and autophagy in cancer cells. Therefore, the goal was to better understand whether CBD holds promise as a new natural chemotherapeutic that would not only reduce traditional side effects present in many other anti-cancer treatments, but also be successful against TNBC. One specific area of study was a focus on investigation of the endoplasmic reticulum, as well as the finding that CBD increased the presence of vacuoles, lysosome size, and lipid content in cells. Finally, the glucose-regulated protein 78 (GRP78), a member of the Hsp70 family that is expressed in the endoplasmic reticulum was also investigated (Schoeman, R, et. al. 2020). The results of this experiment followed a cascade of testing. Initial testing compared two
different breast cancer cell lines, MDA-MB-231, and MCF7. Both of these cell lines were tested against each other multiple times with each CBD derivative included. Derivatives of CBD were tested in conjunction to determine synergistic responses, as well as on their own. The data indicates all the derivatives in both cell types had a range of 94-100% growth inhibition as compared to the control (DMSO, or vehicle) which had a 0.5-1.3% growth inhibition. After testing various derivatives on multiple cancer cell lines, results were analyzed to determine the most effective synergist. The most effective CBD derivative was C6, however, it was not clearly defined as to what this specific derivative was. Nevertheless, C6 was more successful in MCF7 lines, so the experiment further interrogated the effect of C6 on this cell line. Combinations of C6 with other CBD derivatives were further tested on cell proliferation, morphology, and function of MCF7 cells, with an overall success of this compound on inhibition of cell proliferation after two doses of C6. Indeed, while the control of DMSO shows ~ 30,000 cells are viable, a dose of 60 µm C6 results in only ~ 5,000 cells viable in the time course investigated. This C6 derivative showed antiproliferative properties in the cancerous samples, without reducing the viability of non-cancerous cell lines. The effect of CBD Derivative on cell cycle profiles was studied wherein cells were treated with C6 alone or in combination after which the quantity of cells in each phase of the cell cycle was scored. C6 decreases the percentage of cells in G1 and increases the percentage of cells arrested in G2 as the dose of C6 increases. This indicates that the cells cannot continue to enter into the cell cycle, but rather are halted in the cell cycle following treatment with C6. Furthermore, CBD causes cell shrinkage and imaging shows an increased lipid accumulation present in the cells. Finally, CBD causes upregulation of lysosomal and endoplasmic reticulum activity. This included decreased number but increased size of lysosomes as well as an increase in endoplasmic reticular activity due to stress. The
incidence of ER stress following C6 treatment is indicated by the upregulation of GRP78 protein.
The overall finding in this experiment defined the differences between the MCF7 and MDA-MB-231, where MCF7 are HER2 positive, and MDA-MB-231 lack any type of receptor, meaning MDA-MB-231 more closely resemble triple negative breast cancer. Results show that MCF7 cells were more easily affected by the CBD because they had more HER2 receptors, allowing CBD to manipulate them more efficiently than the MDA-MB-231 line. This is significant because CBD prevents the HER2 dimer formation with HER2 and CB2. Since CBD caused vacuolization of cells, these researchers further explored the nature of the circular structures in the cell lines. Following the Endoplasmic Reticulum Tracker allowed the team to determine the size and duration the vacuoles were present. They found that these vacuoles were present for a short duration of two minutes and ranged from 60-100 nm in size. They concluded that CBD treatment impacted progression of cells through the cell cycle in that cells were stuck in respective G1 and G2 phases and were unable to proliferate due to cell cycle arrest. They conclude that CBD and its derivatives decreased proliferation and induced cell death by either autophagy or apoptosis. In this case, CBD functions as an external stressor to the cell, causing a drift to autophagy. To compensate for this stress response, cells appear to upregulate endoplasmic reticulum function, which quickly fails, resulting in disruption of lysosomes and increased number and size of vacuoles within the cell (Shoeman, R, et. al. 2020). Overall, this study concluded and supported CBD as a synergist in chemotherapy in multiple forms of breast cancer.

**Effect of CBD on Cellular Morphology and Apoptosis**

Taken together, this review of the literature, much of which has been published since 2020, experiments were planned to test the effect of CBD on matched chemosensitive and
chemoresistant breast cancer cell lines. I was able to conduct my own laboratory experiments using the same MC7 cell lines investigating morphology, apoptosis, Hsp27 expression and cellular localization, as well as metastatic properties. The hypothesis addressed is that a dose response range of 0.5 to 20.0µg/ml CBD would decrease cell proliferation of both chemoresistant and chemosensitive MCF7 Breast Cancer Cells. To test the effect of CBD on breast cancer cells, chemoresistant and chemosensitive MCF7 breast cancer cells were treated with CBD in a methanol vehicle. The initial dosing investigated cell morphology and apoptosis of cells in response to CBD treatment. Morphology of cells supports less viable cells in micrographs of higher doses, which indicates a potential therapeutic effect in response to CBD. Micrographs taken show that CBD seems to cause increased vacuoles in cells (Figure 6) which supports the findings of Shoeman and colleagues. After initial testing, it was determined the doses chosen initially were too cytotoxic and were lowered to 0.5 to– 1.0 µg/ml CBD in methanol as compared to a control of a matched volume of methanol only, which was the vehicle in which CBD was dissolved.

In order to address the effect of CBD on apoptosis, cellular morphology, and protein expression, 200,000 cells/well were plated in a 6 well plate and treated with CBD for 48 hours after which cells were harvested for Annexin and lysate staining. Following treatment, cells were imaged via phase microscopy for morphological characterization and cells were then collected from each well and were divided with half of the cells analyzed for apoptosis via Annexin staining while protein was extracted from half of each sample to prepare lysates for Western blot analysis. . The Annexin staining allows cells that are engaged in apoptosis to be detected through green fluorescence while cells that are no longer viable are stained with a vital dye, propidium iodide.
After cell staining, cells are scored via Flow Cytometry to determine apoptosis vs live cell population.

**CBD decreases survival in a dose-dependent manner**

The effect of CBD on cellular morphology and proliferation can be seen across increased doses (Figure 1). The standard 200,000 cells/well was used and cells were treated and observed over 48 hours. Testing incorporated proliferation from dose response, apoptosis vs. necrosis levels, cellular stress protein localization, and metastatic properties. The hypothesis that CBD would reduce cellular proliferation was supported in these MCF7 breast cancer cells. Initial testing only incorporated chemosenstive lines to find an ideal dose range. The results concluded some apoptosis rates in chemosensitive lines, but mostly necrosis was visualized. This may be due to the dose concentration as well as the administration. Cells were dosed all at once. If this was administered as a medication, either as a synergist to a chemotherapeutic or alone, it would be delivered slowly over time either intravenously, or ingested and absorbed slowly.

![Micrographs showing cell morphology at different doses of CBD](image)

**Figure 1.** In order to determine the ideal cytotoxic dose of CBD on MCF7 cells, micrographs were taken and show morphological characteristics following 48h of CBD Treatment at doses ranging from 0 to 7.5 μg/mL CBD. CBD in excess of 1μg/mL result in detachment of cells from plate.
CBD Causes Increased Cellular Vacuolization

During the course of this project my experimental design of dosing MCF7 Chemo-Standard and Chemo-Resistant Breast Cancer Cells was targeted at decreasing cell proliferation and determining potential metastatic properties. Even though the experimental results were somewhat inconclusive, the morphology of the cells were surprising. As the concentration of CBD doses increased, the size and number of vacuoles present in the cells also increased dramatically (Figure 2). This is something myself and my mentor did not predict. We found an experiment supporting this finding - Cannabinoid Combination Induces Cytoplasmic Vacuolation in MCF7 Breast Cancer Cells. These findings serve as a sort of control in the future indicating the experiments were done similarly to others. They indicated the highlighted regions were sections of increased vacuole production. The image on the right is a photo of my own results from the lowest dose of CBD administered (0.5 ug/mL). I chose this image because it had the highest proliferation to more easily see the increased vacuoles. The increased vacuoles can be observed in the center, the circular sections of lighter color indicate a higher number and size of vacuoles in a single cell. Both of these images are MCF7 cells with different doses of CBD. My doses were 0.5-7.5 ug/mL in methanol, which was suspended in DMEM solution for cell growth. The experiment used 40 and 60µM CBD in DMSO solution for cell growth.

![Figure 2: CBD causes vacuolization (red boxes) in both MCF7 and MCF7 CisR cells. This feature was shown by Schoeman R, et. al, 2020 (left) and is supported by our findings (Right, chemosensitive MCF7 treated with 0.5 µg/mL CBD).]
Analysis of Apoptosis via Annexin Staining

In order to determine the effect of CBD on apoptosis, cells were stained for externalization of phosphatidyl serine (using Annexin-FITC) as evidence by green fluorescence (scatter in the right lower and upper quadrants) and Propidium Iodide (a vital dye that leaks into all cells that are no longer viable as evidenced by red fluorescence (scatter in the upper right and left quadrants) (Figure 3). Once an ideal dose range was achieved of 0.0, 0.5, and 1.0 µg/ml. There was a negligible apoptotic response of cells in the chemosensitive MCF7 cells at 0.5 and 1 µg/ml, which we included here (Figure 3). Further testing as well as a standardization in CBD concentrations needs to be completed to better determine the impact of CBD on apoptosis.

However, the chemoresistant MCF7-CisR cells had too few cells to reasonably include this data. This data needs to be repeated using the less cytotoxic doses of CBD in order to determine if the chemoresistant cells are also secondarily resistant to CBD.
Effect of CBD on Heat Shock Protein Expression

**Immunocytochemistry**

Another experiment that was conducted was an ICC using a 6 well plate in which cells were plated on glass coverslips. 200,000 cells/well were added each well. Following treatment with CBD, an ICC was used to visualize cellular components in color including Nuclear Staining, Actin, and Hsp27. Actin (red), Nuclei Staining (blue), and Hsp27 (green) stain via immunocytochemistry (ICC) are shown in control and CBD-treated cells (Figure 4). The ICC results which shows punctate cells dosed with CBD. Hsp27 appears to be localizing in cells dosed with CBD, while it is not specifically localized in control cells. Actin is stained to demonstrate the cytoskeletal structure of the cellular morphology. The same purpose is achieved with nuclei staining. The experimental stain in this case is Hsp27, determining its localization point is the purpose of this experiment. The ICC data supported the hypothesis of inducing cellular stress. The localization of Hsp27 displayed a punctate pattern demonstrating that it may localize to an organelle upon stress, and further investigation is warranted which may better identify the cellular localization of Hsp27. Next experiments would be to determine if Hsp27 localizes to specific cellular compartments following CBD treatment using such counterstaining of lysosomes, Golgi, and endoplasmic reticulum.
Western Blot Analysis of Hsp27

Control or CBD treated cells were collected 48 hours after treatment and total protein was isolated from cells, subject to SDS polyacrylamide gel electrophoresis, and transferred to a membrane for analysis of Hsp27 and tubulin. Tubulin was used as a positive control to ensure equal protein loading per well. The Western blot did not show variation in the amount of Hsp27 across different doses of CBD, but Cisplatin resistant MCF7s show increased Hsp27 expression overall (Figure 5). This finding is supported by other findings that heat shock proteins are upregulated in response to chemotherapy which seems consistent in cisplatin resistant MCF7 cells.

Figure 5: Western Blot Analysis of Hsp27 and Tubulin (loading control) from Chemosensitive MCF7 and chemoresistant MCF7 CisR Cells: Results show that Hsp27 is more highly expressed in MCF7 CisR than in MCF7 cells.
Effect of CBD on Cell Migration

*Transwell Migration Assay*

A final experiment that was conducted to determine the effect of CBD on the inhibition of cell migration. This assay uses a Transwell Migration assay to determine metastatic potential of cells. 75,000 MCF7 cells were plated in the upper chamber of the transwell plate. Cells were allowed to migrate from a chamber with 10% FBS toward a gradient of 20% FBS-containing medium for 48 hours. Top and bottom chambers were then washed twice with PBS and cells on the top insert were scraped off with cotton swab. Invaded cells were then fixed with methanol for ten minutes, followed by two PBS washes. Cells were stained with 0.4% crystal violet for ten minutes. Excess stain was washed off and plate air dried. Images were taken with a light microscope.

![Image of Transwell Migration Assay](image)

**Figure 6: Transwell Plate Migration Assays Show that CBD limits MCF7 cell migration.** 75,000 MCF7 cells were plated in upper chamber. Cells were allowed to migrate toward a gradient of 20% FBS-containing medium for 48 hours. These initial invasion assays show that migration of MCF7 cells decreased in the presence of CBD in a dose-dependent manner. Two of three wells (A, B) from one experiment is shown; Replication of this experiment is required.
microscope. These initial migration assays show that CBD decreased the migration of MCF7 cells in a dose-dependent manner.

Regarding the Transwell Migration Assay, there appears to be a potential correlation between increased dose of CBD and decreased cell migration chemosensitive cell types shown in Figure 6. Furthermore the tables show quantities of migratory cells, indicating a steep and sudden inhibition of metastasis. This assay data supports the hypothesis that CBD reduces migration in breast cancer cells. This hypothesis is supported by the literature including others’ investigations. Even though CBD and cancer treatment in its entirety a new field and is under investigated in the literature, CBD and cancer migration inhibition yields an even smaller pool of sources. One investigation used THC and another CBD derivative Cannabichromene (CBC) to investigate migration via a transwell assay. This study concluded that the CBD derivatives had a ~90% prevention of cell invasion compared to a methanol control (Anis O, et al., 2022). With such a low number of studies focused in this area, future study is warranted to confirm the hypothesis that CBD and its derivatives inhibit migration in cancer cell lines.

Overall, the testing of CBD on breast cancer cell lines yielded preliminary data which bear repeating in order to draw solid conclusions. Testing incorporated proliferation from dose response, apoptosis vs. necrosis levels, cellular stress protein localization, and metastatic properties. The hypothesis that CBD would reduce cellular survival was supported in both breast and ovarian cell cultures. The resistant cell lines displayed slightly less of a response. The hypothesis that CBD would increase apoptosis rates was somewhat inconclusive. The results concluded some apoptosis rates in chemosensitive lines, but mostly necrosis was visualized. This may be due to the dose concentration as well as the administration method in that cells were dosed all at once. If CBC is to be administered as a medication to a patient, either as a synergist
to a chemotherapeutic or alone, it would be delivered slowly over time either intravenously, or ingested and absorbed slowly. As such, animal studies will yield better *in vivo* results as to the effect of CBD on cancer cell migration, invasion and survival.

**Future Studies: Investigation of the Role of CBD on Ovarian Cancer**

Ovarian cancer is the fifth leading cancer related death in women. The normal anatomical location of the pelvic cavity and abdomen allow for many tumor forming cancers to grow without presenting symptoms until late-stage disease. This becomes exponential in gynecological cancers where menstruation causes constant epithelial division, which increases the chance of developing a cancer. Because of these factors, ovarian cancer traditionally develops unrecognized. When it does present, symptoms are vague including abdominal pain, irregular bleeding, and general cancer related symptoms such as weight loss and fatigue. These irregular symptoms pair with nonspecific tests. Normally tumors presenting in the ovaries can be palpated by a physician, however in some cases of ovarian cancer, this type of detection is not the case. Additionally, a transvaginal ultrasonography - which is an invasive procedure inserting an instrument that bounces sonic waves off the organs of the pelvic cavity, can be performed but is also nonspecific for ovarian cancer. Imaging using MRIs and PET scans can also be performed to show organs or malignancy can also be performed. For laboratory testing, a CA125 (Cancer Antigen) blood test can be performed, but this is also nonspecific for ovarian cancer. There are two major types of ovarian cancer, Type I and Type II. Type I originates in the ovaries, and is generally less fatal, while Type II originates in the fallopian tubes and is generally more fatal (Stewart C, et. al. 2019). Treatment can involve surgical intervention and/or chemotherapy. Surgical interventions depend on the severity of the prognosis. In the most severe cases, a total hysterectomy and bilateral salpingo-oophorectomy (removal of ovaries and fallopian tubes) can
be performed. Surrounding lymph tissue may also be removed in order to detect metastasis. Chemotherapeutics are also common, with the primary treatment choice being carboplatin, and a secondary choice is paclitaxel. Because of all these nonspecific symptoms, tests, and treatments, prognosis is generally poor. Patients typically present with developed stage III or IV ovarian cancer, with a five-year survival typically being under 50%. On the other hand, patients that present early and get diagnosed quickly generally have a very strong outcome, with survival rates being as high as 98% with early detected stage I development (Griffiths C, et. al., 2021)

Increased risk factors can include genetic predisposition, as well as late menopause and endometriosis (both of which increase epithelial cell division.) Decreased risk factors can include breastfeeding, early menopause, and a previous hysterectomy or tubal ligation (both of which decrease blood flow and hormonal exposure to the pelvic cavity) (Roett MA, Evans P.). Few studies have investigated the potential of CBD and its derivatives on ovarian cancer treatment. Therefore, further investigation of CBD as a synergist may help give insight into developing better prognosis in the future.

In order to understand if CBD may be advantageous in assisting treatment of ovarian cancer, it must first be determined if ovarian tumors express CBD receptors in their tumors. An investigation by A. Ronchi et. al. used Immunohistochemistry to determine if CBD1 receptor (Central Nervous System Receptor) was present in both Type I and Type II ovarian cancers. The study included benign, borderline, and malignant cancers. The study concluded malignant tumors had a moderate positive correlation with CBD1 receptor in Type I cancers, while having a weak negative correlation in Type II cancers. This indicates CBD1 receptor is present in both ovarian cancer types, but more strongly expressed Type I tissue versus the other (A. Ronchi et. al. 2021)
Further studies have also used CBD to decrease proliferation in cells lines. An investigation by Shalev N used HTB75 cell lines – a human ovarian cancer line, and a variety of CBD strains to test antiproliferative properties. Cells were treated with 20 µg/mL CBD after 48 hours resulting in 80% cell death (17% in control) as determined by an XTT assay. Furthermore, cells were treated with niraparib, an oral anticancer drug functioning as a PARP inhibitor, a DNA repair enzyme whose inhibition induces apoptosis. This singular administration of niraparib showed more necrosis when treated alone than when treated in coadministration with CBD (Dobovišek L et. al. 2020). However, apoptosis levels were similar in both CBD treated and untreated cells. They concluded that significant cytotoxic effects could be observed in as little as 24 hours. They indicated that CBD combined with niraparib as a chemotherapeutic may produce similar apoptotic effects and less necrosis than niraparib alone. (Shalev N et. al. 2022)

Another investigation supports the concept of CBD enhancing a traditional chemotherapeutic by pairing CBD with paclitaxel (Taxol), doxorubicin, and cisplatin. Additionally, this study supports the concept that the CBD1 receptor is overexpressed in some ovarian cancers. Cell line SKOV3 was used and treated with 10, 15, and 20 µM CBD delivered as a microparticle. The results determined that treatment of CBD when combined with common chemotherapeutics decreased cell viability by 20% at 10 µM, with maximally decreased cell viability of 25% at 20 µM They also determined that pretreating cells with 10 µM CBD prior to the administration of anticancer drugs can decrease the cell viability by another 20%. This study concluded CBD treatment with cisplatin and doxorubicin was not as effective. Treatment with CBD in this case has been shown to be successful paired with Taxol. Taxol is a nonspecific cancer treatment, meaning it causes the traditional undesirable side effects of chemotherapy. Administering CBD with Taxol may allow a smaller dose of Taxol to achieve the same result.
This would reduce patients' side effects and could improve overall mood/outlook on treatment. This experiment also tested CBD administration on chicken embryos. Cancer was induced in the chick embryos via injection due to natural immune deficiency. Daily CBD was administered into chicken eggs and tumor growth was tracked. Tumor growth was decreased by about 25% when treated with CBD daily and decreased by about 10% with a single dose of CBD. (Fraguas-Sánchez AI, et. al. 2020)

It is important to remember this treatment concept is not universal and still in early investigation. An experiment investigated CBD with OVCAR-5, an ovarian cancer cell line, in a mouse model wherein different numbers of cancer cells were injected into female immunosuppressed mice in 1, 3, and 10 million cells per injection. The study concluded tumor volumes had a disparity after 60 days of AEA administration. When tumors were initiated with similar tumor volumes, treatment with AEA daily resulting in increased tumor volume of 1.7x that of the control (Blanton HL, 2022) This study concluded that chronic administration of CBD derivatives may actually be harmful in treating ovarian cancer. Linking to former work in the Krevosky Lab alongside the finding of others that CBD upregulates Hsps, this may support that CBD treatment leads to a more chemoresistant and aggressive tumor type (Carmichael, et al, 2023). Future investigation is needed to determine the success and validity in a pharmacological usage.

**Future Investigation**

Since it is known that ovarian cell lines express CBD receptors, a future investigation treating ovarian cancer cell lines would be beneficial in developing new treatments. Ovarian Cancer Stem Cells (OCSC) are a specific subtype of cells present in a large percentage of ovarian cancers. This cell type demonstrates plasticity, and promotes tumor properties and
metastasis, making them difficult to target and treat. These cells constantly change their phenotypic expression, as well as upregulate survival mechanisms including DNA repair (Koltai H, 2022). Treating these cells with CBD as a synergist with niraparib and investigating the cell’s Bcl-2 and heat shock protein expression levels will add to the current knowledge in the field.

Previously it was supported that treating HTB75 cell lines, an ovarian cancer line, with niraparib and CBD as a synergist was effective in keeping consistent apoptosis levels and reducing necrosis (Shalev N, 2022). I would like to improve this experiment treating OCSC’s with a similar administration as well as tracking Bcl-2 levels in these cells. Bcl-2 is a protein located in the mitochondrial membrane that activates cascades to inhibit apoptosis. This is a good target for the stem cells because their morphology may allow for dramatic increases of Bcl-2 expression compared to normal cell lines (Lui GYL, 2020). I have recently investigated Bcl-2 as a protein of interest in an embryo model expression. This may also be a strong target for study on the effect of CBD on Bcl-2 in cancer investigation based on its multiple domains including extracellular receptor activation, and intracellular mitochondrial mediation and regulation (Singh P, Lim B. 2022.)

Human Ovarian Cancer Stem Cells would be used for this experiment, and a first line of investigation would be to determine the expression of specific CBD receptors. Previous investigations have confirmed the presence of CBD receptors in ovarian lines, however, an analysis of expression levels of CBD receptors in a panel of cell lines and humor tumor sections would be valuable. Cells would be initially treated with 20 µg/ml CBD and 6 µg/ml niraparib over 48 hours. It would then be possible to perform Flow Cytometry and confirm success with apoptotic levels similar to the previous experiment. Although I would expect the apoptosis levels to be slightly lower due to the natural resistance of cancer stem cells. To further this investigation, Bcl-2 and Hsp 27 levels would be investigated via Western Blot.
treatment was successful, I would hypothesize Bcl-2 levels would be downregulated while Hsp27 would be upregulated in treated cells vs untreated with Tubulin as a positive control. This may allow for targeted treatments on these cancer stem cells to create a functional treatment for ovarian cancer.

Since administering CBD as a synergist has shown success in cell lines previously, the next step of this process would be following up in a mouse model before pharmacological support can be created. As it is known currently there is no specific treatment for ovarian cancer, so targeting points of overexpression could be key in improving prognosis. The administration of CBD will also help improve symptoms as mentioned earlier.
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