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Detailing the Effects of Cannabidiol on Exosome Release in Ewing's Sarcoma

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Abstract

Ewing's sarcoma (ES) is a highly aggressive pediatric cancer found within bone that carries a low five-year survival rate within its patients. This is due, in part, to the cancer's high malignancy properties as well as the development of resistance to the chemotherapies used to combat this cancer. This project aims to determine if cannabidiol (CBD) has an effect on either exosome release or the protein, signal transducer and activator of transcription 3 (STAT3) within ES cells. Exosomes are vesicles that can carry proteins and other signaling molecules which are released by the cell and allow for cell-to-cell communication. Within cancer cells, these exosomes are hijacked and begin to communicate cancer proteins to other cancer cells or even healthy cells. Because of this, exosomes can allow for cancer migration, invasion, and immune escape which leads to making the cancer harder to treat. STAT3 is a protein that normally acts as a transcription activator but has abnormal functionality in 70% of all cancers, assisting in tumor-induced immunosuppression. This protein is associated with exosome release as it has an impact on exosome production as well as being able to be packaged into exosomes to be sent to other cells, causing more mutations and more cancer cells to be created. The effects of CBD on both mechanisms have been observed within other cancer types such as prostate cancer and hepatocellular carcinoma, showing that an increase in CBD concentration leads to a decrease in both cellular exosome release as well as STAT3 expression. CBD was found to have no statistical impact on ES cell viability allowing for exosome quantification. This data encourages further investigation of CBD as a therapeutic for both mechanisms of tumor progression.

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Introduction

Ewing's sarcoma (ES) is a highly aggressive primary bone cancer and is the second most common pediatric bone cancer (Fuchs et al., 2003). This cancer has a 70-80% survival rate within localized disease patients but has the ability to form secondary malignancies, which most often is found to be terminal, especially if hematopoietic or radiation-induced malignancies are formed (Fuchs et al., 2003). ES is termed as highly malignant, which means that the cancer is more aggressive than other types of cancer by being more likely to metastasize and create new tumors (Zöllner et al., 2021) with roughly 34% of patients with ES having signs of metastasis at diagnosis (Meshram et al., 2019). This bone cancer typically occurs within the long bones and pelvic girdle and can metastasize into the bone marrow, lungs, and other tissues (Ross et al., 2013). Primary treatments for ES include chemotherapy, surgery, and radiation therapy (Ozaki, 2015) (La et al., 2006). ES is often treated with chemotherapy initially in order to reduce the primary tumor volume to aid in surgical removal of the tumor (Ozaki, 2015).

A recurrent balanced chromosomal translocation that causes the fusion of the open reading frames of genes EWSR1 and FLI1 is the cause of Ewing's Sarcoma (Grünewald et al., 2018). This translocation occurs between chromosome 22 (EWSR1) and chromosome 11 (FLI1) (Tanner & Lessnick, 2015). EWSR1 in healthy cells is responsible for creating the EWS protein which participates in various processes such as gene expression, RNA processing and transport, and cell signaling (Law, 2006). The FLI protein's role in a healthy cell is to assist in cell proliferation, survival, and differentiation (Truong & Ben-David, 2000). Tumors that express the EWSR1-FLI1 fusion have also been found to have higher cell-to-cell adhesion switch to cell-matrix adhesion, causing an increase in migration and metastasis (Delattre et al., 1992). This specific fusion can be found in a majority of ES cases (Patócs et al., 2013). However, the fusion

of EWSR and the ETS gene family, of which FLI1 is a member of, can be found in all cases of ES (Patócs et al., 2013). Recent studies have suggested the role of the EWSR1-FLI1 upregulates the TENSIN3 protein which acts as an adhesion protein and assists with overall cell mobility (Ebegboni et al., 2024).

ES has also been found to become resistant to chemotherapy in recurrent cases of the cancer which leads to poor prognoses for patients (Heinen et al., 2016). The high rate of metastasis and resistance to chemotherapies leads to the need for new therapies that inhibit tumor migration. Inhibiting tumor migration would reduce the likelihood of recurrent cases as ES would be less likely to migrate before surgical removal. Surgery also has its limitations based on the tumor size, spread, and location within the body (Natarajan et al., 2010). Radiation therapy as a treatment option is ineffective as it carries a 50% five-year event free survival (Kersting et al., 2023). Due to these highly aggressive therapies being used and the cancer having a 20-30% 5-year survival rate, new therapies are needed to treat ES.

Cannabidiol (CBD) is a key cannabinoid derived from the Cannabis sativa plant (O'Brien, 2022). Previous studies have shown that CBD can have a negative impact on tumor metastasis, viability, and proliferation. CBD has also been proven to affect exosome release within cancer cells (Kosgodage et al., 2018). Studies on CBD have shown that the compound triggers apoptosis within cancer cells by increasing production of oxygen species within the cell (Velasco et al., 2016). This effect is believed to be caused by cannabinoid receptors that have been identified within ES cells, allowing for binding of CBD to the cancer cells (Shoeib et al., 2021). CBD has also been linked to exosome release and signal transducer and activator of transcription 3 (STAT3) within other cancer types, such as breast and prostate cancers, by decreasing the number of exosomes produced by the cancer cells (Kosgodage et al., 2018). This

research is the basis for this study, investigating the effects of CBD on both exosome release and STAT3 expression within ES cells.

Exosomes are cellular vesicles that are secreted from all cells and transmit proteins and genetic material from one cell to another (Doyle & Wang, 2019). Exosomes exist as single membrane vesicles that range from 30 to 150 nm in diameter (Doyle & Wang, 2019). These vesicles have also been found to promote cancer cell growth through various means, such as migration, invasion, and immune escape (Zhang et al., 2020). Within cancers, exosomes are rapidly produced via mutations and have multiple different functions dependent on the cancer type (Han et al., 2022). Due to their role in cancer progression, exosomes have become a target for cancer therapeutics by attempting to either promote or inhibit exosome release from cancer cells (Tai et al., 2018).

The STAT3 protein is a member of a group of transcription factors within the cytoplasm that plays a role in cell proliferation, survival, differentiation, and angiogenesis (Zou et al., 2020). This protein family is produced within the mitochondria and not the nucleus, which is shown by the transcription factors being found within the cytoplasm and not within the nucleus (Lahiri et al., 2021). Within cancer cells, this protein is overactivated and causes tumor-induced immunosuppression, giving rise to becoming a target for therapeutic strategies (Zou et al., 2020). The abnormal functionality of this protein has been found to be present in nearly 70% of all cancers but there are multiple different pathways for this hyperactivation to occur (Tolomeo & Cascio, 2021). This is believed to be caused by hyperactivation of pro-oncogenic cytokine receptors and growth factors, increased activity of cytoplasmic non-receptor tyrosine kinases, loss of negative STAT3 regulation, or excessive stimulation by IL-6 or EGF cytokines (Tolomeo & Cascio, 2021). Previous research has shown that there is a relationship between CBD and

STAT3 expression within various cancers (Kosgodage et al., 2018). Specifically, CBD inhibits STAT3 expression by lowering levels of the protein tyrosine kinase 2 (TYK2) which acts as a promoter of STAT3 (Suryavanshi et al., 2022).

Therefore, the specific aims and hypotheses of this project are as follows:

- Specific Aim 1: Determine if CBD has the ability to impact levels of exosome release from ES cells. We hypothesize that CBD will decrease exosome release from ES cells.
- Specific Aim 2: Determine the ability of CBD to alter STAT3 expression levels within ES cells. We hypothesize that CBD will decrease STAT3 expression levels in the ES cells.

Methods

Treatments

Cannabidiol was purchased from Cayman Chemical, Ann Arbor, MI. The CBD used for treatment within A673 cells was obtained from a 100mM stock. The CBD used for treatment within MDA-MB-231 was diluted from a 100mM stock to a 10 mM stock. All treatments were kept in microcentrifuge tubes at -20 °C until used in experimentation.

Cell maintenance

MDA-MB-231 cells were fed RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 5% penicillin-streptomycin (Pen-Strep) solution. A673 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 5% penicillin-streptomycin (Pen-Strep) solution. The cells were grown in a humified incubator set to 37° C with 5% CO_2.

MTT assay

MTT assays were used to determine percent-cell viability after exposure to reagents but before exosome isolation. MDA-MB-231 and A673 cells were plated in a 24-well plate with a cell density of 3.8×10^5 cells/well. Both cell lines were plated using 10% FBS and 1% Pen-Strep media. After twenty-three hours of incubation, the wells were separated into three experimental groups. The control group was exposed to ethanol (EtOH) only, acting as a vehicle control as the CBD is dissolved in the EtOH. The other two groups were exposed to 1µM CBD or 5µM CBD for MDA-MB-231 cells while A673 cells were exposed to either 20µM or 40µM CBD. After one, four, or eight hours of exposure, the media was collected from the wells and 0.5mL of MTT

reagent diluted in DMEM was added to each well to incubate for one hour. The MTT reagent was then aspirated from the wells and 0.5mL of DMSO was applied to the wells and placed on a plate shaker for 15 minutes. The plate was then placed into a plate reader and the absorbance of the wells was read at 570nm. The measurements were recorded for each MTT.

Isolation of exosomes

Thermo Scientific Total Exosome Isolation Reagent was used to isolate exosomes from the conditioned media collected from the 1-hour and 4-hour MTT assays. This was done by applying Exosome Isolation Reagent to conditioned media at a 1:2 dilution and applying high-level centrifugation after overnight incubation at 4°C and collecting the supernatant to be used in further experiments. Isolation occurred according to the manufacturer's instructions (*Total exosome isolation reagent (from Cell Culture Media)*).

ExoELISA

A673 and MDA-MB-231 samples collected from exosome isolation were quantified for exosome release using the ExoELISA-ULTRA Complete Kit (CD63 Detection) from System Biosciences, Palo Alto, CA. This assay required the exosome media collected from previous experiments to be bound to the bottom of a well and exposed to a primary and secondary antibody in order to identify CD63 within the media. The wells are then exposed to ELISA substrate and stop buffer in order to create a yellow color for a positive product. The assay was run according to the manufacturer's instructions (*Exoelisa-Ultra Complete Kit (CD63 detection)*).

Creating Cell Lysates

MDA-MB-231 and A673 cells were plated onto a 6 well plate using a cell density of $9x10^5$ cells/well. Drug concentrations of 20µM and 40µM CBD were applied to the wells once at 80% confluency. After 24-hour incubation, the treatment groups were aspirated and Thermo Fisher Scientific Pierce™ RIPA Buffer or Lysis buffer #6 was applied to the cells. Thermo Fisher Scientific Pierce™ RIPA Buffer was applied to cells used for STAT3 Western Blot and Lysis buffer #6 was applied to cells used for STAT3 ELISA assay. The wells were scraped, and the supernatant was collected. The supernatant was then put on ice and centrifuged before being stored at -80°C for later use. Pierce™ RIPA Buffer was acquired from Thermo Fisher Scientific, Waltham, MA. Lysis buffer #6 was created from adding 5mM NaF and 6M Urea to Sample Diluent Concentrate 1 (5x), acquired from R&D Systems, Minneapolis. MN. The lysates were created according to manufacturer's instructions (*RIPA lysis and extraction* buffer) (*Human/mouse phospho-STAT3 (Y705) duoset IC ELISA*). Lysis buffer #6 was created according to manufacturer's instructions (*Sample diluent Concentrate 1 (5x)*).

Bradford Assay

Bradford assays were conducted on MDA-MB-231 and A673 lysates in order to determine overall protein abundance within each sample compared to a standard curve. This assay was used to dilute each sample to an equal amount of overall protein abundance for use in the STAT3 Western Blot and STAT3 ELISA. The Thermo Scientific™ Pierce™ Bradford Plus Protein Assay Reagent from Thermo Fisher Scientific, Waltham, MA. The Bradford assay was conducted according to manufacturer's instructions (*Pierce™ Bradford Plus Protein Assay Reagent*).

STAT3 Western Blot

A673 and MDA-MB-231 lysate samples collected were analyzed for STAT3 expression via western blot using Invitrogen NuPAGE Bis-Tris Gels. Sino Biological Human STAT3 protein (His Tag) was used as the protein control (*Recombinant Human STAT3 Protein (His Tag), HPLC-verified*). Invitrogen STAT3 Recombinant Polyclonal Antibody was used as the primary antibody and Invitrogen Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, AP was used as the secondary antibody (*Invitrogen STAT3 Recombinant Polyclonal Antibody*) (*Goat anti-rabbit IGG (H+L) cross-adsorbed secondary antibody, AP*). Western blots were run according to manufacturer's instructions (*NuPAGE™4 to 12%, Bis-Tris, 1.0-1.5 mm, Mini Protein Gels*.).

STAT3 ELISA

A673 and MDA-MB-231 lysates were plated on a 96 well plate along with a 6-fold standard dilution in order to quantify STAT3 presence within samples. Samples were bound to a capture antibody and were then exposed to a primary antibody specific to the STAT3 protein. A secondary antibody was then applied to the wells and bound specifically to the primary antibody. The secondary antibody had an enzyme bound to the opposite side of the structure that caused a color change to yellow within the well after substrate was added to the samples. The plate was then measured for yellow absorbance and compared each absorbance value to the standard curve in order to determine STAT3 abundance. IC Diluents #3 and #8 were created with Sample Diluent Concentration 1 (5x) acquired from R&D systems and created according to manufacturer's recommendations (*Sample diluent Concentrate 1 (5x)*). IC Diluent #1 and Block Buffer were created using Reagent Diluent Concentrate 2, acquired from R&D Systems,

according to STAT3 ELISA instructions (*Reagent diluent concentrate 2*) (*Human/mouse phospho-STAT3 (Y705) duoset IC ELISA*). STAT3 ELISA was conducted according to manufacturer's instructions (*Human/mouse phospho-STAT3 (Y705) duoset IC ELISA*).

Statistical Tests

The MTT, one-hour ExoELISA, and STAT3 ELISA assays were tested using One-Way ANOVAs as well as Dunnett's multiple comparisons test in order to test for statistical difference between the treatment groups and the control group within each experimental assay. The four-hour ExoELISA assays were tested using T Tests due to only having two experimental groups within the assay. Confidence interval >95%

Results

CBD's Effect on Cancer Cell Viability

This experiment was investigated which treatment groups at various time points have a significant effect on overall cell death. Because this experiment aims to establish which treatment groups have an insignificant effect, the ideal treatment groups were the groups that were shown to not have any statistical impact on overall cell viability. Cell viability for MDA-MB-231 and A673 cells treated with CBD was not significantly different between CBD treatment concentrations after a one-hour incubation period (**Figure 1**). In MDA-MB-231 cells, 1µM CBD treatment led to a 5.4% decrease in cell viability ($p = 0.1070$), and 5µM CBD treatment led to a 5.6% decrease in cell viability ($p = 0.0859$) compared to EtOH treated cells. In A673 cells, 20µM CBD treatment led to a 2.6% increase in cell viability ($p = 0.7031$), and 40µM CBD treatment led to a 4.3% decrease in cell viability ($p = 0.4140$) compared to EtOH treated cells. Because of this, both treatment groups (1µM and 5µM CBD for MDA-MB-231, as well as 20µM and 40µM CBD for A673, at a one-hour exposure period were used for exosome quantification.

Cell viability after four-hour treatment of CBD on MDA-MB-231 cells was shown to have a statistical difference between the 5µM CBD and the control group, and A673 cell viability showed statistical differences for the 40µM CBD group compared to the control group (**Figure 2**). In MDA-MB-231 cells, there was a 1.5% decrease in cell viability of the 1µM CBD group compared to the EtOH control group ($p = 0.9545$) and there was a 28.5% decrease in cell viability within the 5µM CBD treatment group compared to the EtOH control group ($p < 0.0001$). In A673 cells, there was a 9.1% decrease in cell viability of the 20µM CBD group compared to the EtOH control group ($p = 0.0701$), and there was a 44.1% decrease in cell viability within the

40μM CBD group when compared to the EtOH control group ($p \leq 0.0001$). Due to these findings, only media from the lower CBD concentrations for both drugs (1μM CBD for MDA-MB-231 and 20μM CBD for A673) at a four-hour exposure period were used for exosome isolation and quantification.

Eight-hour MTT assays were conducted on A673 cells and were found to have a statistical difference between both 20μM CBD and 40μM CBD treatment groups when compared to the control group (**Figure 3**). There was a 56.9% decrease in cell viability in the 20μM CBD group when compared to the EtOH control group ($p < 0.0001$) and a 62% decrease in cell viability in the 40μM CBD group compared to the EtOH control group ($p < 0.0001$). This experiment showed that neither CBD treatment group at an eight-hour exposure period should be considered or used for exosome quantification.

CBD's Effect on Cancer Cell Exosome Release

The ExoELISA used the protein CD63 to quantify the total exosome abundance within the conditioned media. The CD63 protein was targeted as it has been found to be in the membrane of intracellular vesicles, acting as a marker for the number of vesicles produced by cells. ExoELISA data was obtained for both MDA-MB-231 and A673 cells collected from one- and four-hour CBD treatments (**Figures 4,5**). For the one-hour ExoELISA, it was determined that there was no significant difference between the treatment groups for the A673 cancer cell line or between the EtOH group and the 1μM CBD group for the MDA-MB-231 cell line. However, there was a significant difference between the 5μM CBD group and the EtOH group in the MDA-MB-231 cell line. Within the MDA-MB-231 cell line, a 1.5% decrease between the EtOH group and 1μM CBD group ($p = 0.2864$) and a 3.8% decrease between the EtOH group

and 5µM CBD group (p = 0.0133) was observed. There was an increase of 6.1% in exosome abundance between the EtOH and 20µM CBD treatment groups (p = 0.8422) and a decrease of 3% in exosome abundance between the EtOH and 40µM CBD treatment groups (p = 0.9588) within the A673 cell line.

CBD's Effect on Cancer Cell STAT3 Expression

STAT3 expression levels after 24-hour treatment of one and 5µM CBD as well as 20 and 40µM CBD within MDA-MB-231 and A673 cell lines, respectively, were tested using a STAT3 Western Blot assay. This assay was conducted multiple times to identify the expression levels within each sample. However, protein bands failed to appear on the nitrocellulose paper (**Figure 6**). This assay should be conducted again in order to obtain data regarding STAT3 protein expression levels.

A STAT3 ELISA was also conducted to test for STAT3 abundance within each sample (**Figure 7**). This assay showed that there was no significant difference in STAT3 abundance between either CBD concentrations when compared to the EtOH group in both cell lines. In the MDA-MB-231 cell line, there was observed to be a 41.7% increase between the EtOH and the 1µM CBD groups (p = 0.6209) as well as a 20.3% increase between the EtOH and 5µM CBD treatment groups (p = 0.8764). There was also a 55.8% increase between the EtOH and 20µM CBD treatment groups (p = 0.8221) and a 46.3% decrease between the EtOH and 40µM CBD treatment groups (p = 0.8705) in the A673 cell line. Due to the high error bars, this assay should be repeated with a larger sample size to validate the data collected.

Discussion

This project shows that CBD at these concentrations, 1µM and 5µM for MDA-MB-231 cells and 20µM and 40µM for A673 cells, has little to no effect on cell viability within a one-hour treatment period but begins to impact cell viability as exposure time progresses. Using this information, we can focus on quantifying exosome concentration within a one-hour exposure time period for both CBD concentrations within each cell line as well as the 1µM CBD concentration for MDA-MB-231 cells and 20µM CBD concentration for A673 cells at a four-hour time period in order to determine if there is any effect on exosome release. This study sets up future work in exosome release by suggesting that these CBD concentrations can be used as treatments for both cell types for results to not be attributed to cell death but to the drug treatment's impact on exosome release.

The ExoELISA assay within this project presented results showing that there was no significant difference between treatment groups except between the 5µM CBD treatment and the EtOH group within the MDA-MB-231 cells at the one-hour time period. Due to this data not having a large sample size, this assay should be conducted again to validate its findings. This assay also suggest that the ExoELISA method lacks the sensitivity of the Nanoparticle Tracking Analysis used in previous studies to quantify exosomes due to the lowest value registered on the standard curve being roughly 1000x larger than the highest value registered by the NTA method (Kosgodage et al., 2018). To determine if sample volume collected has any effect on exosome abundance within the sample, this assay should be conducted again using larger volumes of conditioned media.

The STAT3 Western Blot failed to show any protein bands and therefore no data can be obtained from the assay in regard to STAT3 expression levels. However, due to the samples and

protein ladder running and transferring from the gel to the nitrocellulose membrane, it is assumed that this fault was not due to a loading or technique error. More optimizing of antibody concentrations should be considered as a possible option for obtaining a successful STAT3 Western Blot.

Analysis of the STAT3 ELISA assay suggests that there is no significant difference between the CBD treatment groups and the EtOH groups of both MDA-MB-231 and A673 cell lines. However, the sample size for both cell lines was small and could be the reason for the higher error bars found within each treatment group. Due to this, more replicates should be collected and analyzed by this assay to validate any findings obtained by this study.

CBD has been shown to be a promising therapeutic within various cancer types by inhibiting multiple cancer mechanisms and pathways. This project was unable to effectively show a significant effect, whether positive or negative, of CBD's effects on both exosome release and STAT3 expression levels within ES. However, this study suggests a methodology to conduct this study and obtain significant data, if present. More work needs to be conducted on refining each assay such as adjusting sample volume for the ExoEISA, antibody concentrations for the STAT3 Western Blot, and more replicates within the STAT3 ELISA. The refining of these protocols could lead to effective results within this study. However, this project suggests that there is a negative trend of CBD's effect on exosome release and STAT3 expression levels within ES. Due to this, the project should be examined further and conducted again using the suggested adjustments within each assay to determine if the negative trend observed is true. This study should also be expanded into other *Cannabis Sativa* extracts, such as cannabigerol (CBG), cannabichromene (CBC), and cannabinol (CBN) to determine if extracts related to CBD have a similar effect on exosome release as well as STAT3 expression. More types of cancer should

also be included in this study, such as rhabdomyosarcoma and osteosarcoma to determine if effects seen within ES could also be observed within other forms of cancer. This project highlights a potential therapeutic that could cause exosome release inhibition and reduction of STAT3 expression levels within ES based on previous research of CBD within breast cancer, prostate cancer, and hepatocellular carcinoma (Kosgodage et al., 2018). This research could lead to the reduced ability of ES tumors to metastasize, limiting their ability to spread through the body and potentially reduce the tumor's resistance chemotherapy treatments. Both would be important in treating ES within patients and hopefully increasing the cancer's overall survival rate.

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Appendix A

Figures

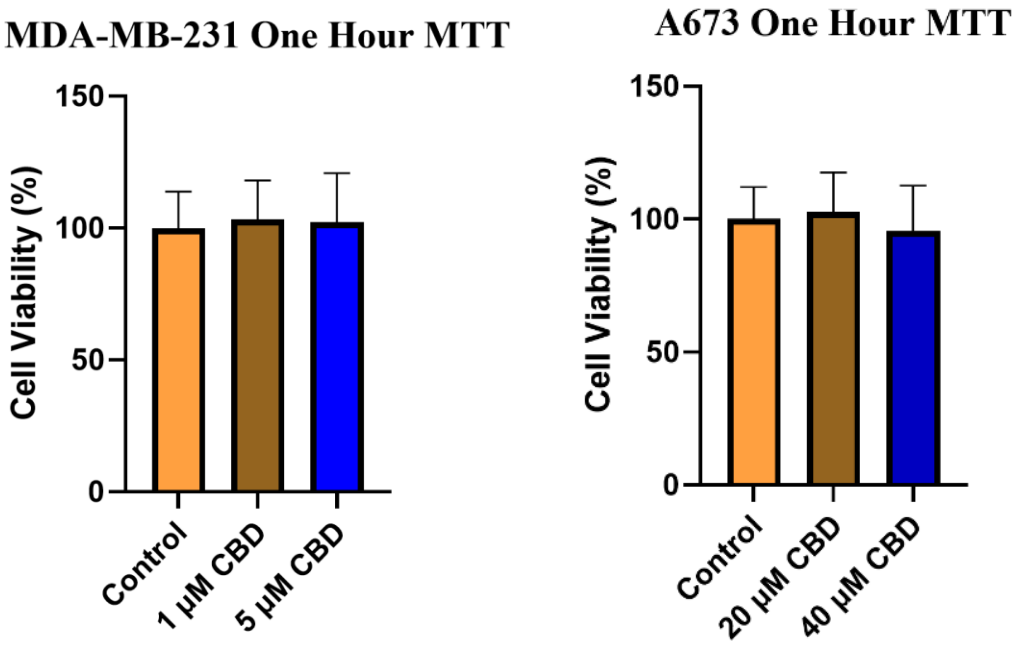

Figure 1: There is no statistical effect of CBD on cell viability in MDA-MB-231 and A673 cells after one hour treatment. MTT assays were used to analyze cell viability within both cell lines. Data is shown as mean ± SD.

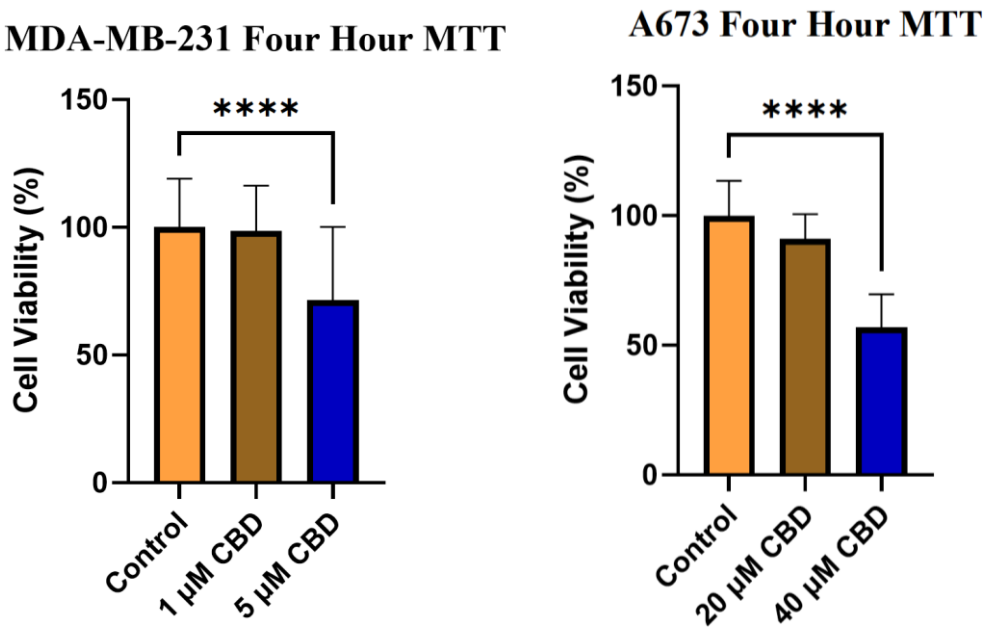

Figure 2: There is a statistical difference between the control and 5µM CBD treatment groups within MDA-MB-231 cells as well as between the 40µM treatment group and the control group within A673 cells. MTT assays were used in triplicate to analyze cell viability within both cell lines. Data is shown as mean ± SD. ****$p \leq 0.0001$.

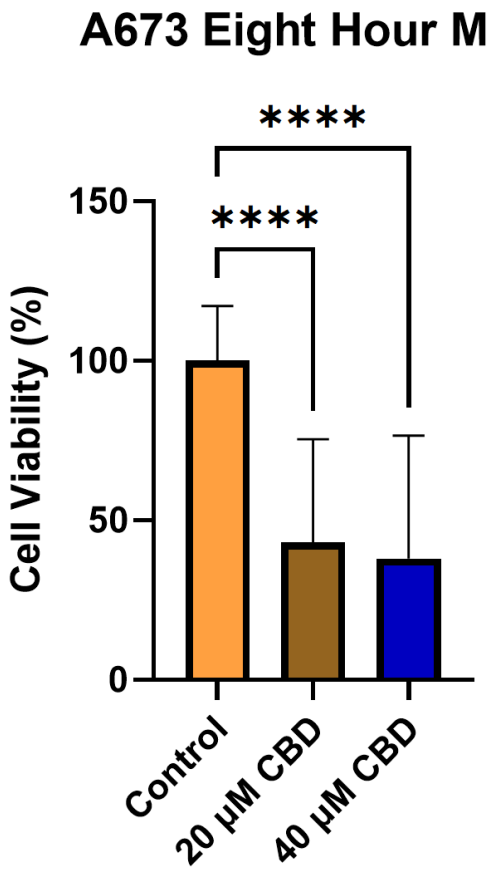

Figure 3: There is a statistical difference between the control both 20µM CBD and 40µM CBD treatment groups within A673 cells. MTT assays were used in triplicate to analyze cell viability within both cell lines. Data is shown as mean ± SD. ****$p \leq 0.0001$.

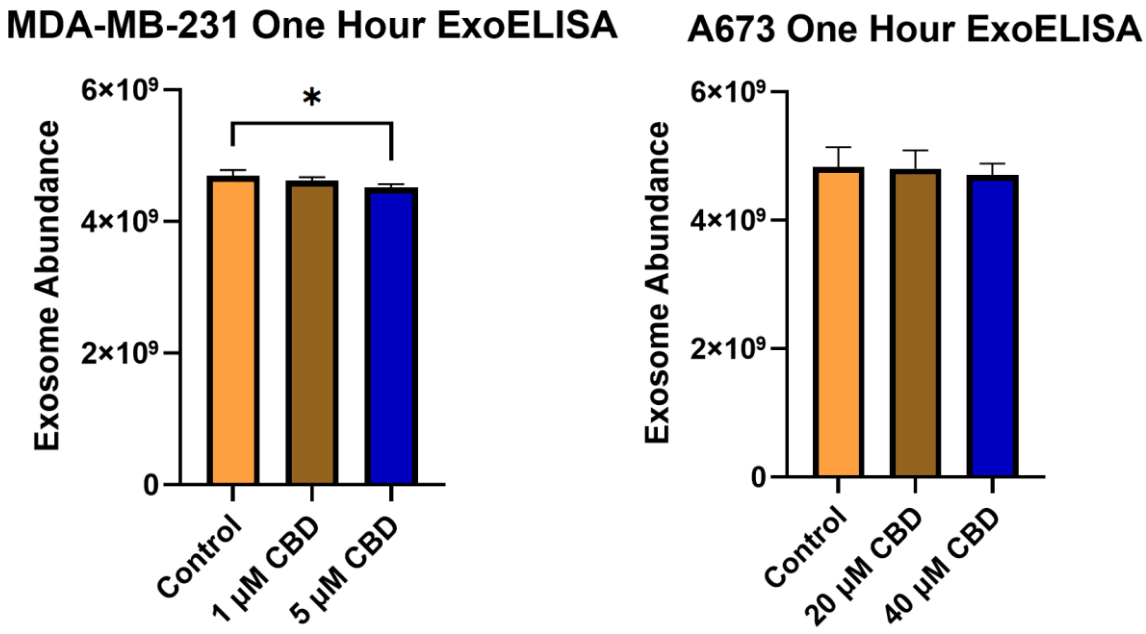

Figure 4: ExoELISA data suggests that there was no significant difference between the 1µM CBD and control group within the MDA-MB-231 cell line as well as either CBD concentrations compared to the control group for the A673 cell line at the one-hour time period. There was significant difference between the 5µM CBD and control groups in the MDA-MB-231 cell line. ExoELISA assay was conducted in triplicate to test for exosome abundance within each sample. Data is shown as mean ± SD. *$p \leq 0.05$

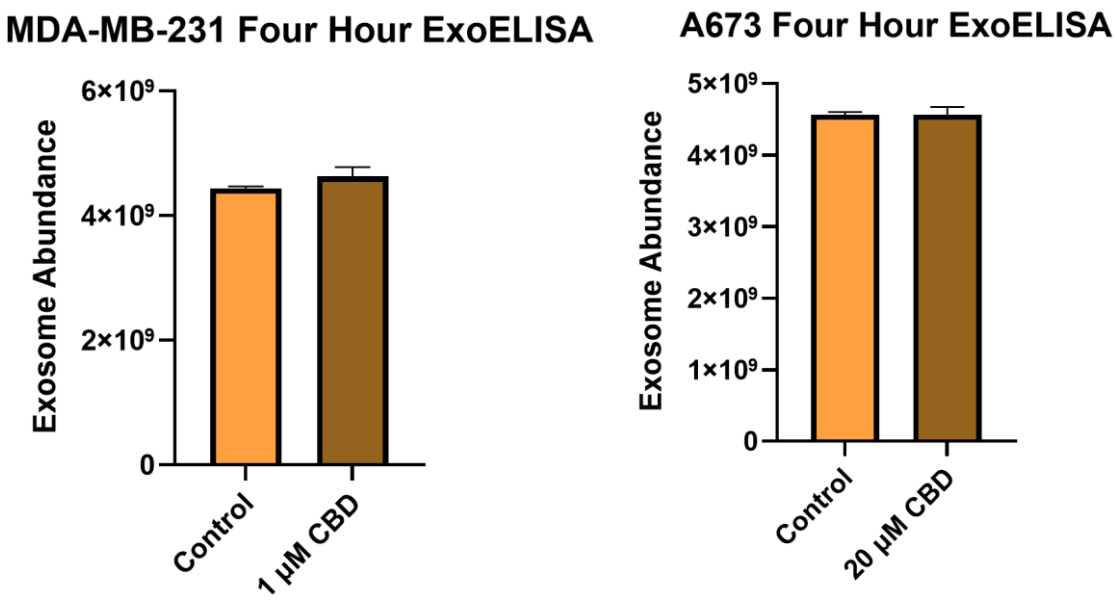

Figure 5: ExoELISA data suggests that there is no significant difference between the 1μM CBD and control groups within the MDA-MB-231 cell line at the four-hour time period. The same suggestion can be made regarding the 20μM CBD and control groups within the A673 cell line at the four-hour time period. Data is shown as mean ± SD.

Figure 6: The STAT3 Western Blot failed to show any protein bands in the 80 kDa size range. Due to this, STAT3 expression levels were not able to be visualized. This assay was conducted to visualize STAT3 expression levels and confirm STAT3 protein size.

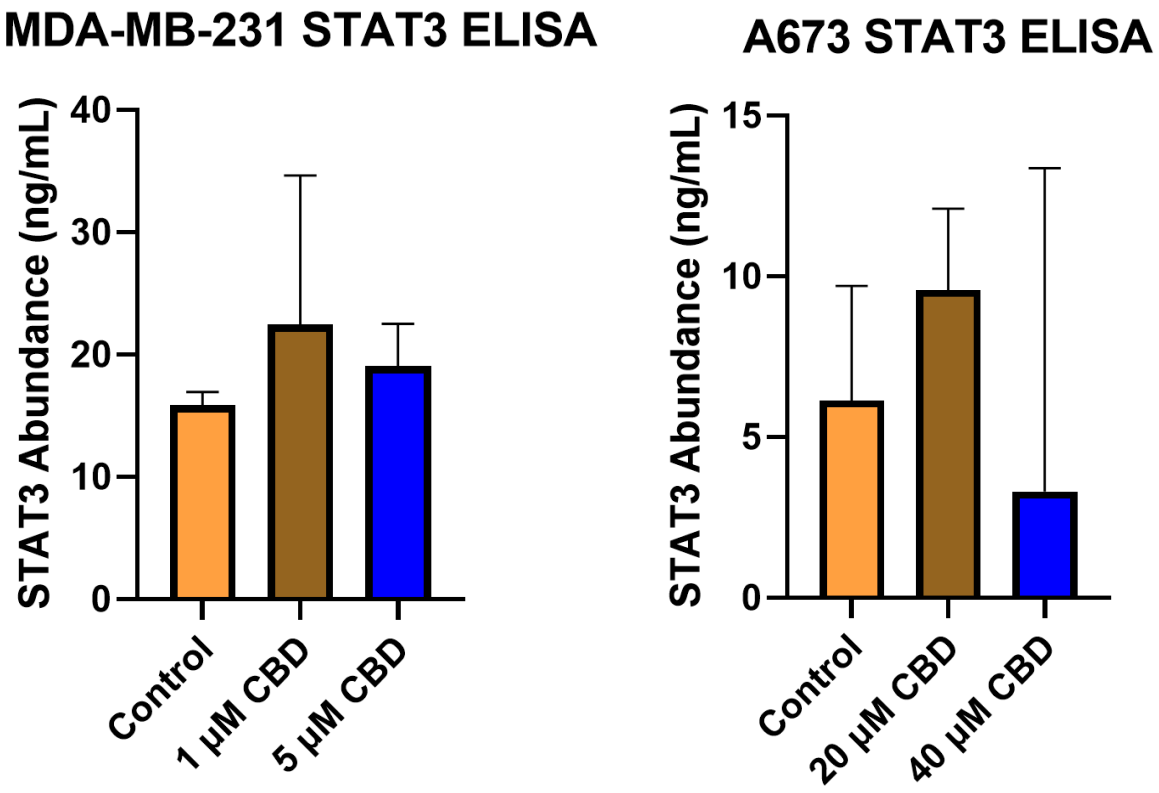

Figure 7: STAT3 ELISA data suggests that there is no significant difference in STAT3 expression levels after 24-hour treatment of either drug concentration when compared to the control group in both MDA-MB-231 and A673 cell lines. This assay was run in duplicate to test overall STAT3 expression within each sample. Data is shown as mean ± SD.