CANNABIDIOL AS AN ANTICANCER AGENT IN GLIOMA CELLS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Sciences in Biological Sciences with Distinction

Spring 2018

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ACKNOWLEDGMENTS

I would like to thank my thesis advisor, Dr. Deni Galileo for the opportunity to learn from you for the past two years as well as Dr. Gary Laverty and Dr. Carlton Cooper for serving on my committee.

I would like to thank the University of Delaware Undergraduate Research for funding materials and for the summer scholars stipend.

Finally, I would like to thank lab mates Kyle Plusch, Alex Stubbolo, and Reetika Dutt for their assistance and collaboration.

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ABSTRACT

Because of the highly invasive nature of brain cancer glioblastoma multiforme (GBM), there has been little success with known treatment options. As a lab, we focus on trying to understand the factors that contribute to increasing the invasiveness of GBM. Specifically, we look at tumor initiating glioblastoma stem cells (GSCs), which have been shown previously to be resistant to common cancer treatments as well as a key component in tumor progression and recurrence, and adhesion protein L1CAM, which increases motility and proliferation in cancer cells, as major players.

Cannabidiol (CBD), a plant derived cannabinoid that lacks psychoactive effects, has been looked at as an alternative treatment for cancer. Previous claims have shown that CBD has antitumor properties and is able to induce death in glioma cells. Here I investigated CBD for its ability to limit motility of glioblastoma-derived cell lines T98G and GSCs at a concentration below lethality, whether CBD has a differential effect between L1CAM-positive and L1CAM-negative T98G cells, and if CBD can be used in conjunction with a small molecule inhibitor that is known to inhibit stimulatory the effects of L1CAM. Time lapse microscopy was used to collect images of cells over a 22 hour period to be measured for their velocity. Using this information, I hoped to determine if CBD could be a viable treatment for GBM.

In this study, the effects of CBD on GSCs and L1CAM-positive and -negative T98G cells was measured through time-lapse motility studies. Surprisingly, CBD appeared to have a stimulatory effect on GSCs. When CBD was tested on T98G cells, it had an inhibitory effect on L1CAM-negative cell motility, while the effect on

L1CAM-positive cells was not as clear. This could indicate that L1CAM is able to protect cancer cells from the effects of CBD. When coupled with a focal adhesion kinase (FAK) inhibitor, PF431396, that previously was shown to be able to inhibit cell motility in L1CAM-postivive glioblastoma cells, CBD did not potentiate its effects. These results suggests that CBD might be stimulatory for the cells that drive the growth and spread of GBM tumors (GSCs) and that continued research is necessary to test the capacity of CBD for treatment of GBM.

Chapter 1

INTRODUCTION

1.1 Glioblastoma

Glioblastoma multiforme (GBM), the most common and deadly form of brain cancer, accounts for about 70% of malignant gliomas in adults (Wen and Kesari, 2008). GBM is known for its high proliferation, infiltration through healthy brain tissue, and its resistance to apoptosis (Furnari et al., 2007). The invasiveness and heterogeneity of its tumors make GBM a difficult cancer to understand and resistant to treatments (Wen and Kesari, 2008; Furnari et al., 2007). Figure 1 shows how GBM tumors are able to recur and migrate over far distances resulting in a secondary tumor.

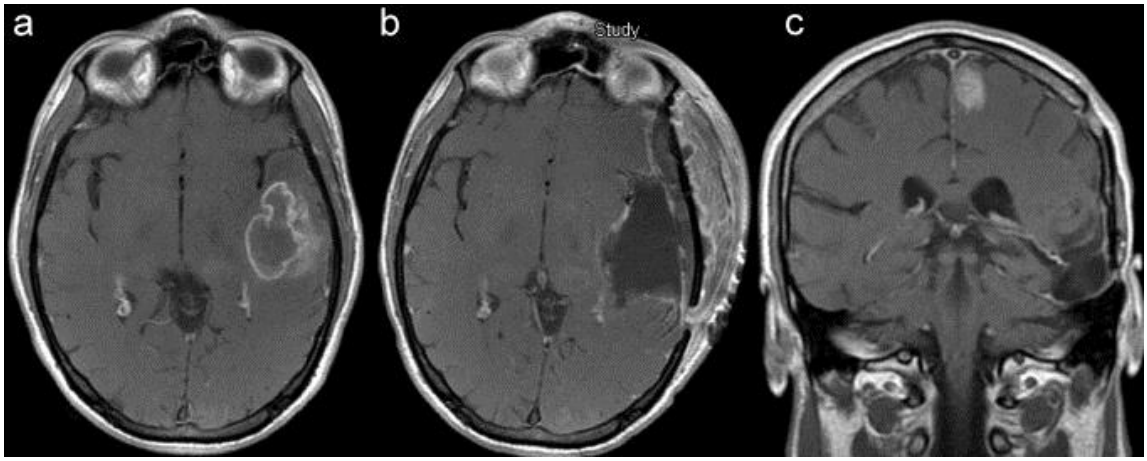


Figure 1. Left-temporal glioblastoma distant recurrence (a) before surgery, (b) after surgery, (c) 23 months after surgery (De Bonis et al., 2013).

The current standard treatment for GBM includes surgical resection (removal) when it is possible, followed by radiotherapy and chemotherapy (Wen and Kesari, 2008; Stupp et al., 2005). Adding temozolomide (TMZ), an oral alkylating agent, has increased the average patient's survival time by 2.5 months from the 12.1 month average from radiotherapy alone to 14.6 months (Stupp et al., 2005). Because of GBM's ability to invade through the brain, it is impossible to surgically remove the tumor completely making recurrence a common treatment problem. Two factors explored by the Galileo lab as key contributors to the invasiveness of GBM are the protein L1 cell adhesion molecule (L1CAM) and glioblastoma stem cells (GSCs).

1.2 Glioblastoma Stem Cells

Gliomas contain a small population of GSCs or tumor-initiating cells that drive GBM malignancy (Ignatova et al., 2002; Singh et al., 2003; Yuan et al., 2004; Bao et al., 2008; Varghese et al., 2008). They are a focus of tumor research because they are key contributors to radioresistance and chemoresistance (Wen and Kesari, 2008; Rich, 2007; Stanzani et al., 2017). It is suspected that because GSCs are located near blood vessels within the tumor (Lathia et al., 2011), they can contribute to the invasiveness of tumors and can travel over long distances within the brain, but their precise contributions to invasiveness are unknown. This invasiveness through the brain leads to GSCs being a source of tumor progression and recurrence. Tumor recurrence goes beyond the resected tumor margins (Di et al., 2010), and can extend into both hemispheres of the brain. Because of their contribution to tumor invasiveness, there is a great need for a better understanding of these cells and what treatments can be used to combat this effect.

1.3 L1 cell adhesion molecule

L1CAM is a transmembrane glycoprotein of the immunoglobulin (Ig) superfamily (Moos et al., 1988). L1CAM contains six Ig-like extracellular domains and five fibronectin type III repeats, a transmembrane region, and a cytoplasmic tail (Moos et al., 1988).

Under normal circumstances, L1CAM functions in the developing nervous system to aid in neuronal cell adhesion (Keilhauer et al., 1985), neuronal migration (Lindler et al., 1983), neuron survival (Conacci-Sorrell et al., 2005), myelination of the peripheral nervous system (Wood et al., 1990), and axon outgrowth (Fischer et al., 1986). L1CAM can be proteolyzed by ADAM10 and ADAM17, members of the disintegrin and metalloprotease family, to release a soluble ectodomain fragment (Gutwein et al., 2003).

L1CAM has been shown to be upregulated in many human cancers including GBM (Moos et al., 1988). After being proteolyzed by ADAM10, L1CAM binds to cell surface receptors to promote cell motility and proliferation (Yang et al., 2009, 2011). It has been shown previously that L1CAM is expressed in GBM primary tumors and cell lines, and that this expression increases cell motility in glioma cells (Yang et al., 2009, 2011). This same increased motility was also seen when GBM-derived cell line U-118 was positioned on top of soluble L1CAM that had been adhered to a culture dish (Figure 2). When L1 expression was attenuated in a human GBM cell line it halted its invasion into brain parenchyma in chick xenografts (Yang et al., 2011; figure 3). This increased migration could be part of the reason for the failure of current therapeutic options.

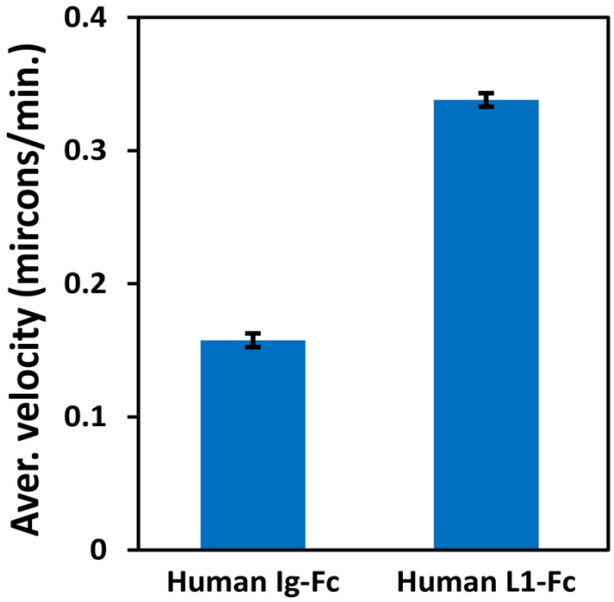


Figure 2. U-118 GBM cell motility was stimulated by immobilized L1-Fc. The average cell velocity increased by 50% when stimulated by soluble L1 that was adhered to the dish. P <0.001. (Stubbolo, 2018).

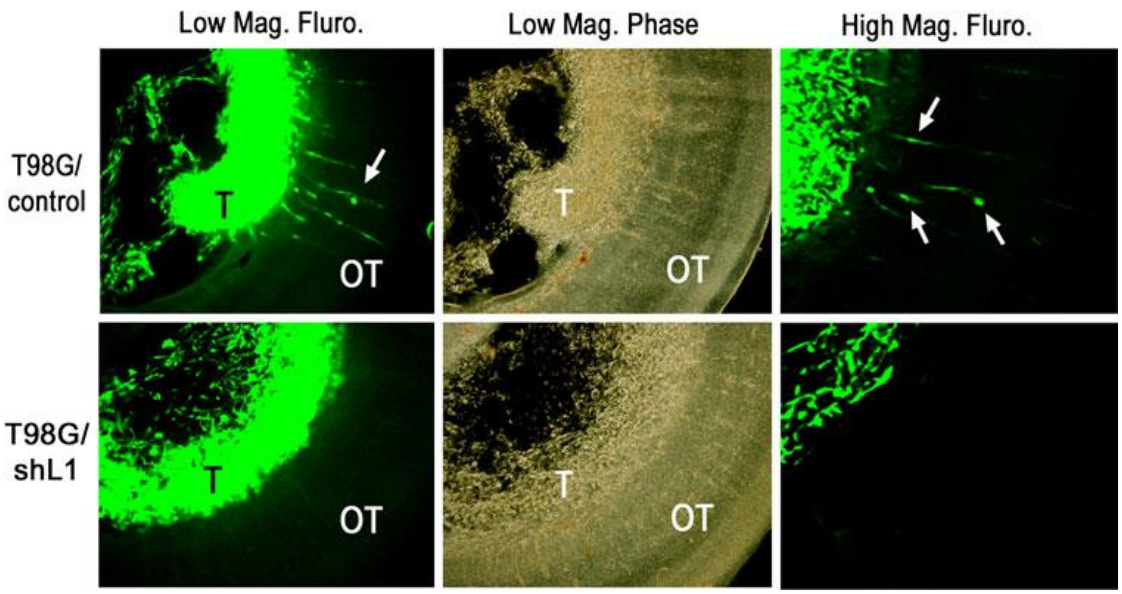


Figure 3. Previously, the Galileo lab has shown that L1CAM-positive T98G/pLKO.1 (top) have increased invasion in the brain compared to L1CAM-negative T98G/shL1 cells (Yang et al., 2011). Cells were labeled with a GFP retroviral vector, microinjected into chick embryonic brain, and analyzed in Vibratome sections. Both T98G cells formed tumors (T) inside the ventricle, but only the T98G/pLKO.1 cells showed infiltration inside the brain wall (OT).

Following the release of the L1CAM ectodomain, it binds to integrins to promote cell motility (Mechtersheimer et al., 2001). In an autocrine or paracrine fashion, L1 interacts with αvβ1, αvβ3, αvβ5, and α5β1 integrins, as well as the αIIbβ3 platelet integrin, through the Arg-Gly-Asp (RGD) motif in L1's sixth Ig-like domain (Mechtersheimer et al., 2001). Once bound to integrin, focal adhesion kinase (FAK), a non-receptor tyrosine kinase, is activated and undergoes autophosphorylation (Guan, 1997). FAK stimulates cell motility and proliferation (Mitra et al., 2005; Zhao and Guan, 2009), and have been shown to be highly expressed in GBM (Ding et al., 2010). L1CAM has also been shown to interact with fibroblast growth factor receptors

(FGFRs; Williams et al., 1994). It is likely that FGFRs bind to the motifs in the third and fifth FNIII domains of L1CAM (Kulahin et al., 2009). We have previously shown that by inhibiting the action of FAK, FGFR, or integrin, we were also able to inhibit the signaling of L1CAM (Anderson and Galileo, 2016). Motility and proliferation were decreased *in vitro* in GBM-derived cell lines that were positive for L1CAM expression (Anderson and Galileo, 2016). Of the three small molecule inhibitors previously shown to be effective against L1CAM (FAK, integrin, and FGFRs), a FAK inhibitor was chosen because it is able to inhibit cell velocity using multiple mechanisms, most notably by mediating the turnover of focal complexes (Mitra et al., 2005).

1.4 Cannabidiol

The limited efficacy of current therapies as well as the invasiveness of these tumor creates an urgent need for the investigation of new treatments for GBM. I decided to look at cannabidiol (CBD) as a possibility for combatting the effects of GSCs and L1CAM. Although treatment for cancer using cannabinoids has been mostly limited to its palliative (additional supportive) effects such as alleviating nausea induced by chemotherapy, inhibition of pain, and appetite stimulation, there has been increased research for their ability to promote cancer cell death and inhibit invasion and metastasis (Guzman, 2003; Pertwee et al., 2010; Velasco et al., 2012). Cannabinoids bind to and activate the G-protein coupled cannabinoid receptors CB_1 and CB_2. CB_1 is expressed in the brain and at many peripheral sites, whereas CB_2 is expressed in the immune system, microglia, and lowly expressed in the central nervous system (Pertwee et al., 2010; Howlett et al., 2002). It has been shown that cannabinoid receptors are able to modulate cell motility in different cancer cells with

CB_1 inhibiting breast cancer cell migration (Grimaldi et al., 2006), while CB_2 inhibited migration in bladder cancer cells (Bettiga et al., 2017). Both studies linked these decreases in migration to FAK signaling (Bettiga et al., 2017; Grimaldi et al., 2006). Another study showed that glioma cell migration was inhibited by a mechanism that was independent of the cannabinoid receptors (Vaccini et al., 2005).

Specifically, the cannabinoid CBD is being investigated because it lacks the psychoactive effects that Δ9-tetrahydrocannabinol (THC) has, while still showing the ability to inhibit tumor growth, cell viability, and migration in many cancers including gliomas (Vaccani et al., 2005; Marcu et al., 2010; Scott et al., 2014; Nabissi et al., 2012, 2015; Izzo et al., 2009). The mechanism of CBD's anti-cancer action has not been clarified yet, but it has been proposed that CBD-induced apoptosis is independent of the CB_1 and CB_2 receptors because CBD is known to lack significant binding affinities to these cannabinoid receptors (Nabissi et al., 2012; Izzo et al., 2009). There have been various proposed mechanisms of CBD-induced cell death in cancer including: (1) CBD's ability to generate reactive oxygen species and subsequently activate caspases (Izzo et al., 2009; Ligresti et al., 2006; Marcu et al., 2010), (2) CBD's ability to induce stress on the endoplasmic reticulum (Shrivastava et al., 2011), (3) CBD is able to upregulate expression and activation of Transient receptor potential vanilloid (TRPV) cation channels by increasing the concentration of intracellular calcium (Nabissi et al., 2012; Morelli et al., 2012), and (4) CBD can enhance the effectiveness of endocannabinoids or THC on the CB receptors (Marcu et al., 2010; Torres et al., 2011).

Not only has there been success with CBD as a solo treatment for GBM, but there is also promise using CBD in combination with other cancer treatments methods.

When CBD was combined with THC, it inhibited the growth of GBM-derived cell lines as well as enhanced the inhibitory effects of THC in cell growth (Marcu et al., 2010; Torres et al., 2011). CBD also was able to decrease the dose of THC necessary to inhibit tumor growth (Torres et al., 2011). The possibility of CBD being able to decrease the minimum effective dose in other cancer treatments could be promising for limiting toxic side effects. The combined administration of cannabinoids THC and CBD with other anticancer agents has been shown to act synergistically to inhibit tumor growth (Torres et al., 2011). The combination of THC, CBD, and TMZ exerts a strong anti-cancer action in xenografts generated with glioma cells (Torres et al., 2011). CBD and THC were also shown to enhance the anticancer effects of radiation (Scott et al., 2014). There has also been similar success without the addition of THC. When CBD was combined with chemotherapy drugs carmustine (BCNU), doxorubicin (DOXO), and TMZ on glioma cells, it showed a significant decrease in cell viability and induced apoptotic effects (Nabissi et al., 2012). Table 1 includes the concentrations of CBD used in previous studies on GBM that I referenced when deciding what concentrations I would use for my own studies on cell motility.

Study	Cell Line	CBD concentration	Result
Ligresti et al., 2006	-Human breast carcinoma cell MCF-7 -Human prostate carcinoma cell DU-145	25 µM	CBD significantly inhibited cell growth
Marcu et al., 2010	GBM cell lines SF126, U251, and U87	1.2 µM	CBD significantly inhibited cell growth and viability

Nabissi et al., 2012	GSC #83	25 µM	CBD increased GSC line differentiation
Nabissi et al., 2015	GSC #1, 30, 83	10 µM	CBD significantly inhibited GSC cell viability
Scott et al., 2014	GBM cell lines T98G and U87	10 µM	CBD significantly inhibited cell viability
Torres et al., 2011	GBM cell lines U87, T98G, and HG19	0.7 – 3.5 µM	CBD significantly inhibited cell viability
Vaccini et al., 2005	GBM cell line U87	3 – 9 µM	CBD significantly inhibited cell migration

Table 1. Summary of previous studies I used to determine what CBD concentration should be tested for GBM cell motility.

1.5 Research Goals

The potential for CBD to decrease the high rate of cell motility in GBM has yet to be explored more thoroughly. CBD's efficacy on L1CAM-positve and L1CAM-negative cell lines, GSCs, and as a component in a combination treatment with a FAK inhibitor was investigated in *in vitro* motility assays. Lentiviral vector techniques were used in T98G GBM-derived cells to shut down L1CAM expression. The hope would be to limit L1CAM-stimulated cell motility to in turn decrease invasion in GBM tumors and decrease tumor recurrence. CBD's effect on GSCs has yet to be explored, but because of the dangers that these cells pose, and a great need for treatment against them, it is important to test if CBD can decrease their cell velocity.

Because of CBD's success in increasing cell viability in many cancers, I wanted to use a motility assay to test CBD's effect at concentrations below that which caused lethality. I hypothesized that CBD could be used to inhibit cell motility and therefore combat the invasive nature of these tumors. My aims were as follows.

Aim 1: To determine what concentration of CBD is needed to kill my GBM cell lines, so that its effect on motility could be assessed at sub-lethal concentrations.

Aim 2: To determine if CBD can limit motility in GSCs.

Aim 3: To determine if there exists a differential effect of CBD on L1CAM-positive and L1CAM-negative T98G cell motility.

Aim 4: To determine if CBD can potentiate the effect that FAK inhibitor PF431936 had on L1CAM-stimulated T98G cell motility.

Chapter 2

MATERIALS AND METHODS

2.1 Cell Lines and culture

The human glioblastoma-derived cell line used, T98G, was obtained from the American Type culture collection (ATCC, Manassas, VA). T98G cells were previously transduced with a lentiviral vector encoding a shRNA that targets human L1CAM (TRCN 0000063917, cat. No. RHS3979-97052304) to create L1CAM-attenuated T98G/shL1. T98G cells were previously transduced with a non-targeting control vector pLKO.1 to create the L1CAM expressing T98G/pLKO.1 cell line (Yang et al., 2011).

Culture Conditions: Cells were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 100µg/mL penicillin-streptomycin. Cells were kept in an incubator at 37° C in 5% CO_2.

Our lab previously established five GSC lines from patient surgical specimens using the "Failsafe" culture method (Murrell et al., 2013). Because of a lack of definitive GSC markers, we define GSCs as GBM cells that grow in the Failsafe adherent culture conditions and media. Immunostaining GSCs for two known stem cell markers, Sox-2 and nestin (Mae et al., 2015), and analyzing expression by flow cytometry was used to show that GSCs expressed these markers. GSC lines 2015-2 and 2016-4 were used to test the efficacy of CBD.

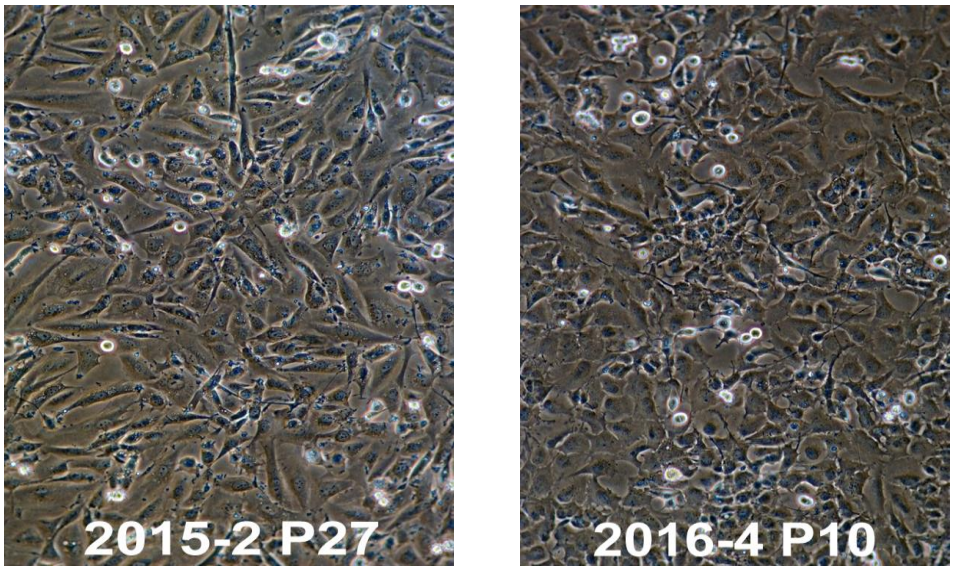


Figure 4. GSC lines # 2015-2 (left) and # 2016-4 in culture with passage number (P) indicated. Each GSC line has a unique morphology, and invade differently when injected into chick optic tectum (Plusch, 2018).

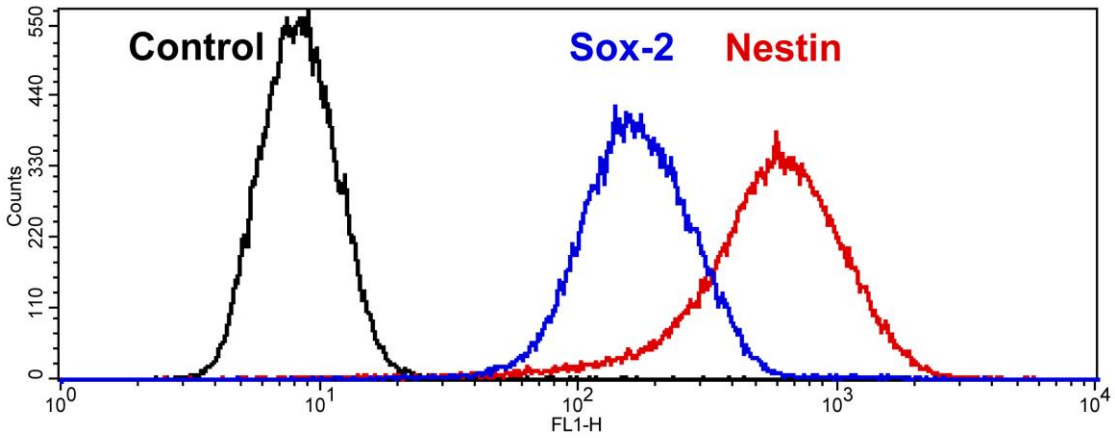
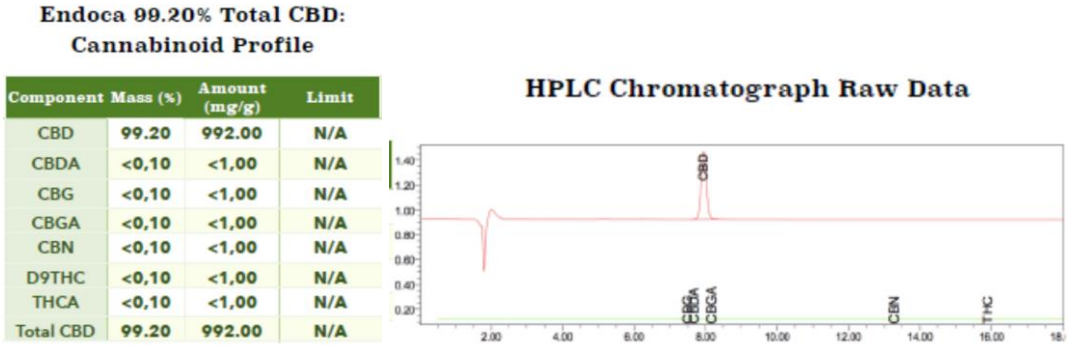


Figure 5. Flow cytometry histogram of GSCs 2015-2 stained for Sox-2 and nestin. This showed that GSCs express known stem cell markers Sox-2 and Nestin (Plusch, 2018). GSC line 2016-4 showed similar results.

2.2 Reagents and inhibitors

PF431396 (Cat. No. 4278; Tocris Bioscience, Bristol, United Kingdom) is a FAK inhibitor (IC50 of 2nM). Cannabidiol (CBD > 99% purity) was provided by Endoca Laboratory (Los Angeles, CA). CBD purity results are included in F[igure 6. PF431396 and CBD were dissolved in DMSO and stored at -20° C. DMSO was also used as control (carrier only) treatment in all experiments.



Endoca 99.20% Total CBD: Cannabinoid Profile

Component	Mass (%)	Amount (mg/g)	Limit
CBD	99.20	992.00	N/A
CBDA	<0,10	<1,00	N/A
CBG	<0,10	<1,00	N/A
CBGA	<0,10	<1,00	N/A
CBN	<0,10	<1,00	N/A
D9THC	<0,10	<1,00	N/A
THCA	<0,10	<1,00	N/A
Total CBD	99.20	992.00	N/A

Figure 6. Endoca Certificate of Analysis. The sample used was 99.20% CBD with no other terpenes or cannabinoids present.

2.3 Time-lapse microscopy

"Super Scratch" assays were modified from previously described protocols to test cell motility (Yang et al., 2011; Ding et al., 2011; Fotos et al., 2006). Cells were plated in 6-well plates and given 24 hours to grow to confluency. A horizontal scratch was made through the center of each 35 mm diameter well with a 1000µL pipette tip. The cell culture media in each well was replaced by 2mL of media containing DMEM supplemented with 0.5% FBS, 2mM L-glutamine and 100µg/mL penicillin-

streptomycin. Reduced serum media was used to decrease proliferation of cells. This insured that any changes seen in migration were due to the additives as opposed to FBS or the growth factors in it. Additives such as CBD, inhibitors, or the DMSO vehicle were added to the media prior to adding the media to the cells. All wells contained 0.5% DMSO, regardless of the concentration of drug. After changing media, the 6-well plate was taped, except for a small portion of one side, to seal the plate and prevent evaporation. The plates were placed in a culture chamber on an adjustable ProScan II automated stage (Prior Scientific, Rockland, MA) on a Nikon TE-2000E microscope. The plates were incubated for 1 hour in the chamber that was kept at atmospheric conditions of 5% CO_2/ 95% air before beginning. Atmospheric conditions were kept using a gas injection controller (Forma Scientific, Marietta, OH). The temperature was maintained at 37° C with a warm air temperature controller (Air Therm, World Precision Instruments, Sarasota, FL) and a temperature-controlled stage insert (Tokai Hit, Shizuoka-ken, Japan). Two separate locations on the scratch edge of each well were photographed every 10 minutes for 22 hours using a Retiga eXi CCD camera and a 20x Nikon Plan Fluor ELWD objective. MetaMorph Premier Software (Molecular Devices Corporation, Downingtown, PA) was used to operate the time-lapse system.

2.4 Cell Motility analysis

MetaMorph Premier Software was utilized following time-lapse experiments to track cells imaged from each well. The “Track Points” application tracked the migration over time for individual cells on the scratch edge. Fifteen cells per image or a total of thirty cells per scratch edge were tracked by their nuclei and analyzed for their velocity in an Excel spreadsheet generated by MetaMorph. The data was

converted from pixels per second to microns per minute , and an average for each scratch edge was calculated to determine the mean cell velocity for a given condition.

2.5 Statistical methods

Data is presented as mean ± standard error of the mean. The difference between data with a p-value ≤ 0.05 was considered significant. A one way ANOVA test or a two sample T-test was used to determine significance depending on the experimental setup.

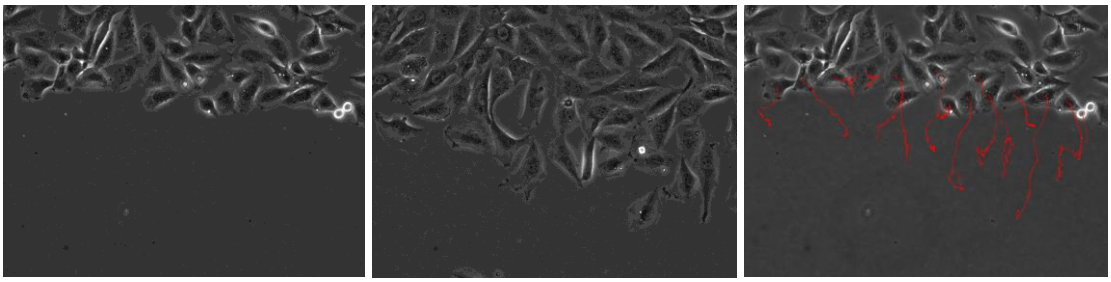


Figure 7. Time-lapse images were collected as described in the materials and methods section. The positions of 15 T98G/pLKO.1 cells along the scratch edge were tracked through the entire set of images. The left shows a scratch edge at time point 0. The middle shows a scratch edge at time point 133, which is the final image of the experiment. The right shows the tracked cell pathway lines created by marking the nucleus of a cell at each time point to show the extent of cell advancement.

Chapter 3

RESULTS

3.1 Determining an effective sub-lethal concentration of CBD

T98G/pLKO.1 cells (L1CAM-positive) were used to test a variety of concentrations of CBD. Concentrations ranging from 0.5-50 μM of CBD were tested to find the point where there was not any noticeable cell death or morphology changes. From these results, I determined that concentrations of 5 μM or less should be used to test cell motility. Because it is known that CBD can induce cell death in cancer cells, I wanted to investigate if CBD also has sub-lethal effects that could be beneficial against the invasive components, GSCs and L1CAM, that are studied in the Galileo lab.

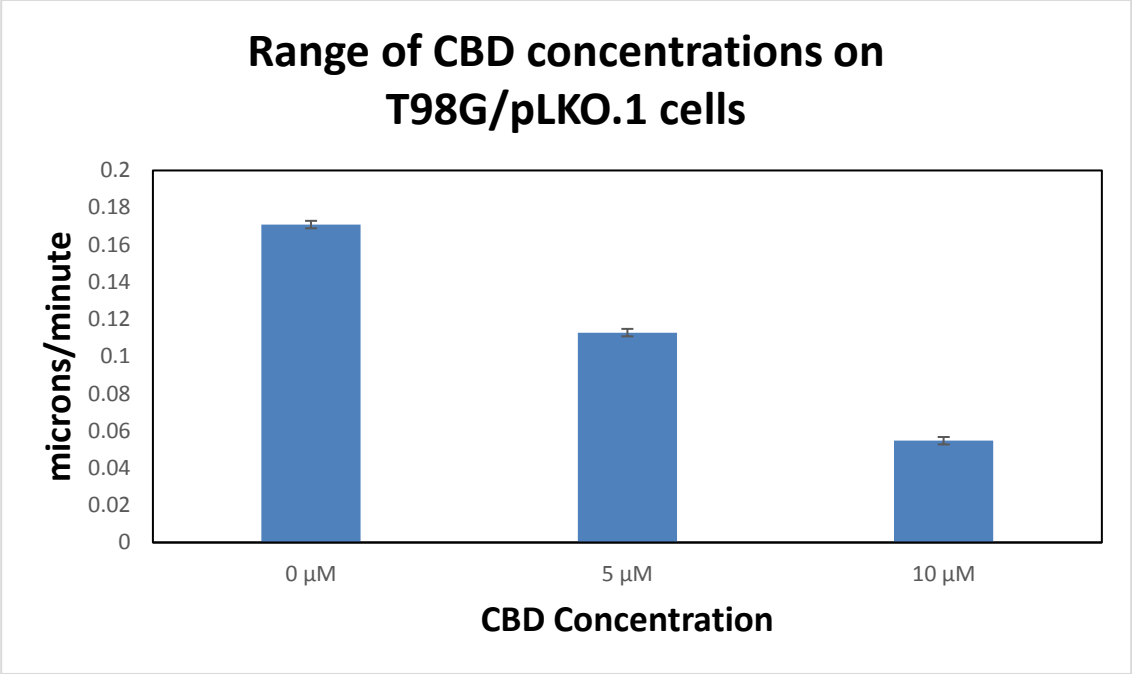


Figure 8. Concentrations tested in this trial were 0 µM, 5 µM, 10 µM, 25 µM, and 50 µM CBD. Cells treated with only the control vehicle had an average velocity of 0.171 microns/minute. The average velocities for 5 µM and 10 µM CBD were 0.113 microns/minute and 0.055 microns/minute. I was unable to track the 25 µM and 50 µM CBD conditions for motility because there was 100% cell death by time point 25 and time point 1 respectively. I did track 10 µM, but there was about 50% death by time point 30 and morphological changes from time point 1, so this concentration was deemed too high for the aims of this study.

3.2 CBD did not inhibit motility in GSCs

The effects of CBD on GSC 2016-4 motility was analyzed by *Super Scratch* assay using time-lapse microscopy. Cells were incubated in 2 mL of low serum media that contained either DMSO only, 3 µM CBD, or 5 µM CBD. These concentrations were chosen because 0.5-2 µM CBD showed no significant decrease on L1CAM-positive T98G/pLKO.1 cells. Surprisingly, there was a significant increase in velocity between the control and 3 µM CBD of 46.5% ($P < 0.001$), and a significant increase

between the control and 5 µM CBD of 44.9% ($P < 0.001$). There was a non-significant decrease in velocity between the 3 µM and 5 µM conditions of 2.8% ($P = 0.623$).

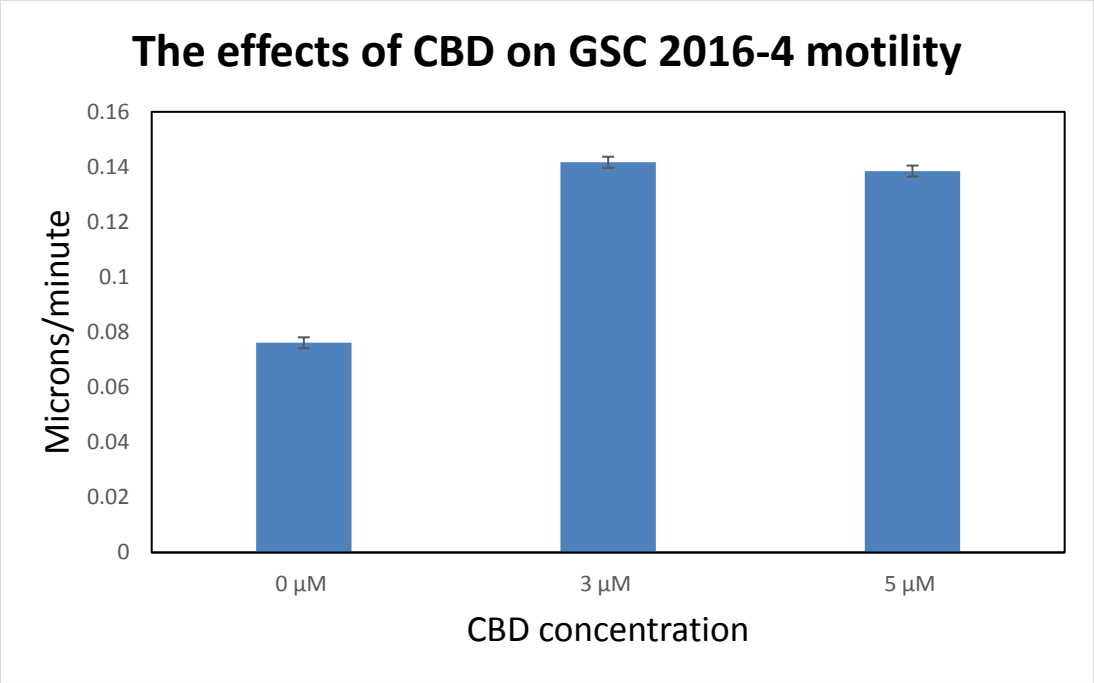


Figure 9. Effect of CBD on motility of GSC 2016-4 cells at varying concentrations. Cells treated with only the control vehicle had an average velocity of 0.076 microns/minute. The average velocities for 3 µM and 5 µM CBD were 0.142 microns/minute and 0.138 microns/minute.

The effects of CBD on GSC 2015-2 motility was also analyzed by *Super Scratch* assay using time-lapse microscopy. Cells were incubated in 2 mL of low serum media that contained either DMSO only, 3 µM CBD, or 5 µM CBD. There was a significant increase in velocity between the control and 3 µM CBD of 32.1% ($P < 0.001$), and a significant increase between the control and 5 µM CBD of 21.4% ($P < 0.001$). There was a significant decrease in velocity between the 3 µM and 5 µM

conditions of 13.6% ($P < 0.001$). These results on GSCs were surprising because it was shown previously that GSC viability was decreased when cells were treated with 10 µM CBD (Nabissi et al., 2015). I also expected GSCs treated with CBD to act similarly to the GBM cell lines.

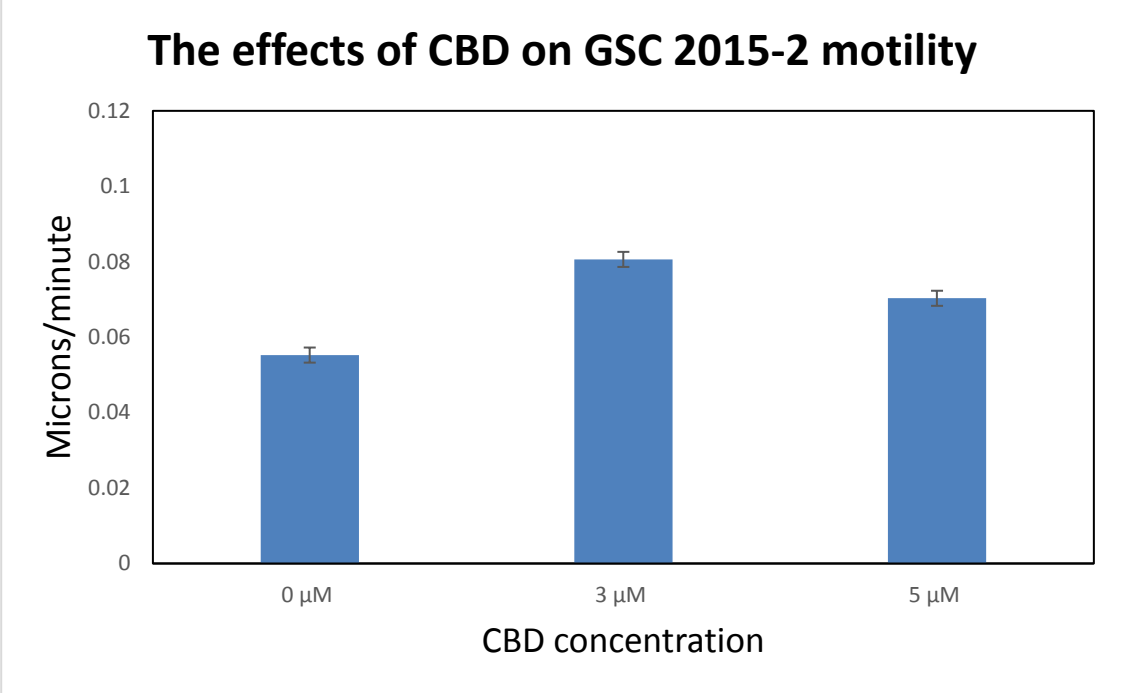


Figure 10. Effect of CBD on motility of GSC 2015-2 cells at varying concentrations. Cells treated with only the control vehicle had an average velocity of 0.055 microns/minute. The average velocities for 3 µM and 5 µM CBD were 0.081 microns/minute and 0.070 microns/minute.

3.3 CBD decreases the motility of L1CAM-negative glioblastoma cells more than L1CAM-positive cells

The Galileo lab previously has shown that T98G cells express L1CAM (Yang et al., 2011). As described above, to create the cell line with attenuated L1CAM expression, T98G/shL1, T98G cells were transduced with a lentiviral vector of shRNA

for L1CAM [22]. L1CAM-expressing T98G/pLKO.1 control cells were created by transducing T98G cells with the empty lentiviral vector pLKO.1 containing no shRNA. L1CAM attenuated T98G cells have been previously shown to be slower than the T98G/pLKO.1 cells with normal L1CAM expression (Yang et al., 2011).

T98G cells were analyzed by *Super Scratch* assay. The below data represents the three repeats done for this experimental setup. Each well was treated with DMSO, 1 µM CBD, 2 µM CBD, or 5 µM CBD in 2 mL of low serum media. For T98G/pLKO.1 cells, the effect of CBD was not as clear. There was a decrease of 9.18% ($P < 0.001$) between the DMSO control and 1 µM CBD, followed by an increase of 10.1% ($P < 0.001$) between the control and 2 µM CBD, and a decrease of 5.11% ($P = 0.053$) between the control and 5 µM CBD. The average velocities across both cell types for 5 µM CBD differ by 47.3% ($P < 0.001$).

For T98G/shL1 cells, 1 µM, 2 µM, and 5 µM CBD had significant decreases in average cell velocity of 21.3% ($P < 0.001$), 18.9% ($P < 0.001$), and 59.8% ($P < 0.001$). There was a non-significant increase of 3.04% ($P = 0.395$) between 1 µM and 2 µM CBD, while 5 µM CBD had a significant decrease in cell velocity of 48.9% ($P < 0.001$) compared to 1 µM CBD, and a decrease of 50.5% ($P < 0.001$) compared to 2 µM CBD.

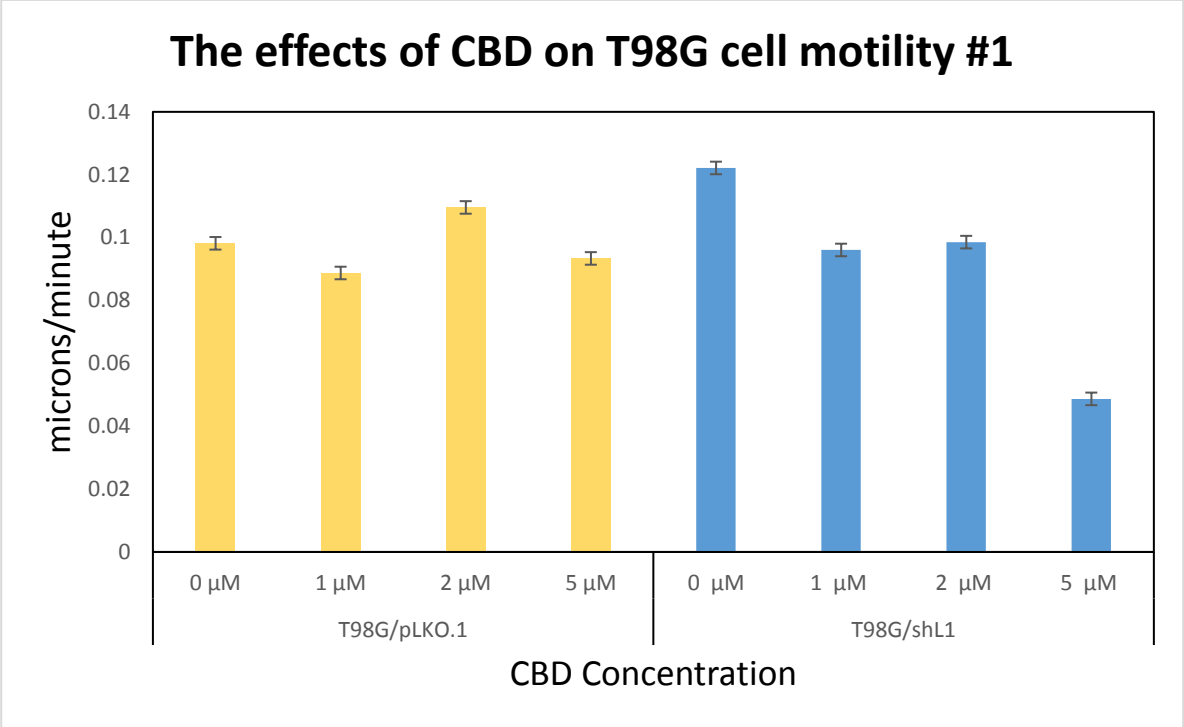


Figure 11. The effects of CBD on motility of T98G/shL1 and T98G/pLKO.1 cells. T98G/pLKO.1 cells incubated with DMSO only (control) had an average velocity of 0.098 microns/minute, with 1 µM CBD had an average velocity of 0.089 microns/minute, with 2 µM CBD had an average velocity of 0.110 microns/minute, and with 5 µM CBD had an average velocity of 0.093 microns/minute. T98G/shL1 cells incubated with DMSO only (control) had an average velocity of 0.122 microns/minute, with 1 µM CBD had an average velocity of 0.096 microns/minute, with 2 µM had an average velocity of 0.099 microns/minute, and with 5 µM CBD had an average velocity of 0.049 microns/minute.

In a second trial, both types of T98G cells were treated with either DMSO or 3 µM CBD. For the L1CAM-positive T98G/pLKO.1 cells, there was a decrease in cell velocity of 8.5% ($P < 0.05$) between the DMSO control and 3 µM CBD. For the L1CAM-negative T98G/shL1 cells, there was a decrease in cell velocity of 22.8% ($P < 0.001$).

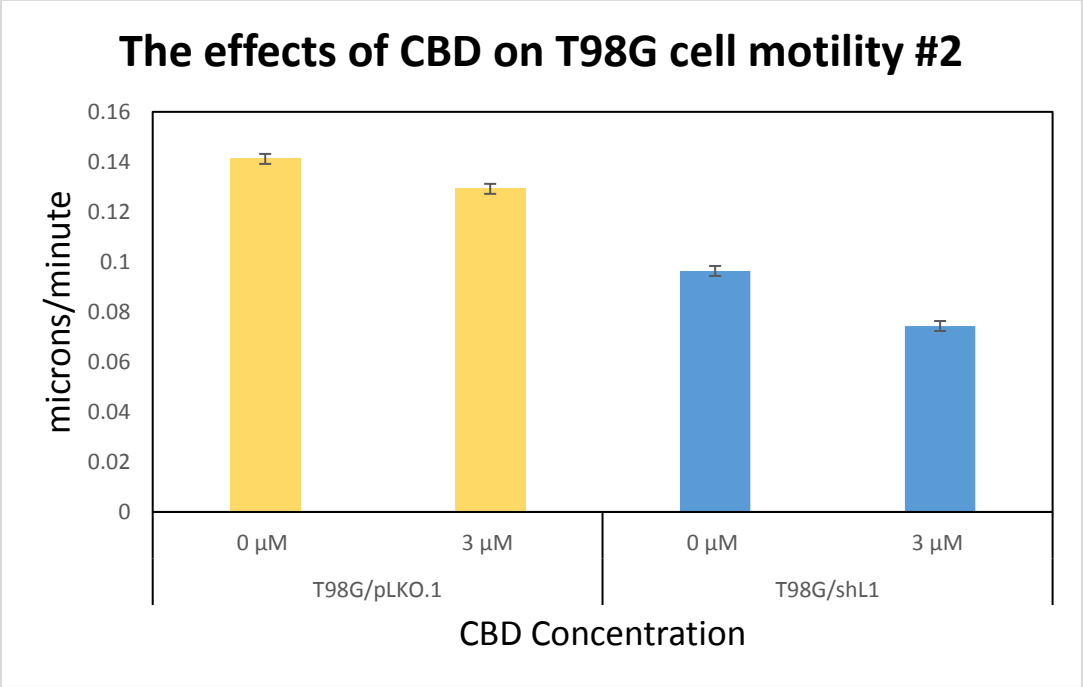


Figure 12. The effects of CBD on T98G cell motility. T98G/pLKO.1 cells incubated in DMSO only (control) had an average velocity of 0.141 microns/minute, while T98G/pLKO.1 cells incubated in 3 µM CBD had an average velocity of 0.129 microns/minute. T98G/shL1 cells incubated in DMSO only (control) had an average velocity of 0.096 microns/minute, while T98G/shL1 cells incubated in 3 µM CBD had an average velocity of 0.074 microns/minute.

In a third trial, both types of T98G cells were incubated in DMSO, 3 µM CBD, and 5 µM CBD. For the L1CAM-positive T98G/pLKO.1 cells, the decreases in average velocity were by 15.1% ($P < 0.001$) when treated with 3 µM CBD and by 30.2% ($P < 0.001$) when treated with 5 µM CBD. For the L1CAM-negative T98G/shL1 cells, the decreases in average velocity were by 31.7% ($P < 0.001$) when treated with 3 µM CBD and by 42.3% ($P < 0.001$) when treated with 5 µM CBD. When comparing the results at a given concentration between cell types, T98G/shL1

was 21.5% slower than T98G/pLKO.1 with 3 µM CBD and was 19.3% slower with 5 µM CBD.

Although the specific velocity values varied from trial to trial, there was a general trend of T98G/shL1 cells having a CBD dose-dependent decrease in cell velocity. The T98G/pLKO.1 cell velocity was more variable between trials; in one trial, CBD decreased cell velocity, but in a lesser extent than the T98G/shL1 cells, while in other trials, CBD had no effect on cell motility.

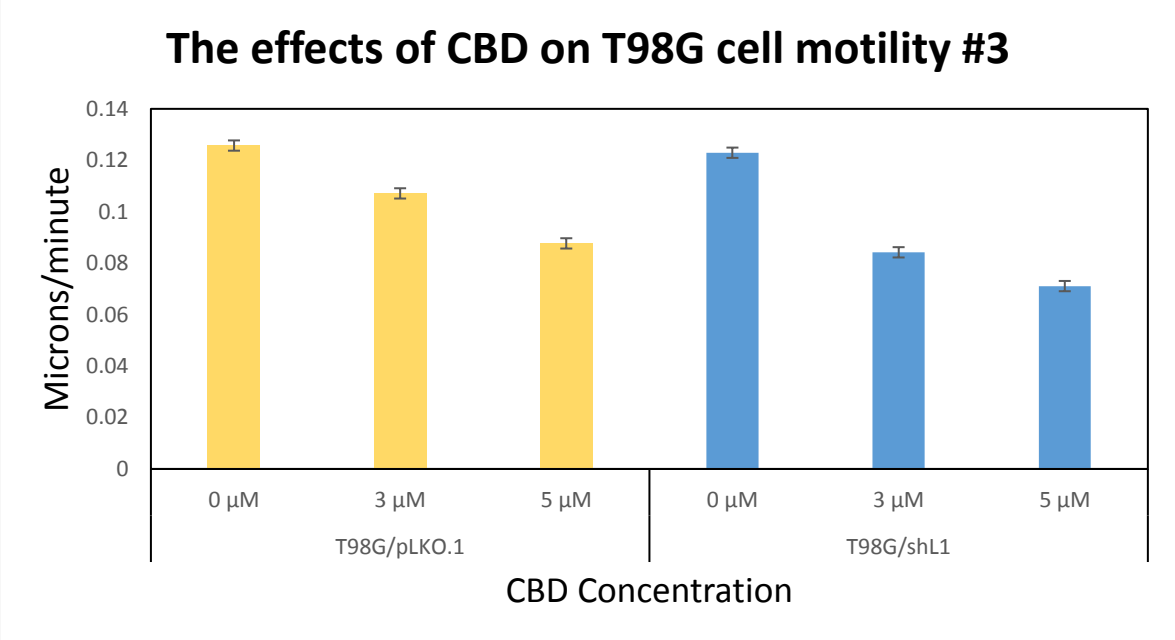


Figure 13. The effects of CBD on T98G cell motility. The average cell velocity of L1CAM-expressing cells incubated with DMSO only (control) was 0.126 microns/minute, with 3 µM CBD was 0.107 microns/minute, and with 5 µM CBD was 0.088 microns/minute. The average cell velocity of L1CAM-attenuated cells incubated with DMSO only (control) was 0.123 microns/minute, with 3 µM CBD was 0.084 microns/minute, and with 5 µM CBD was 0.071 microns/minute.

3.4 Effects of CBD combined with the FAK inhibitor PF431396 on motility of L1CAM-positive and L1CAM-negative glioblastoma cells

The Galileo lab has previously shown that T98G/shL1 cells with attenuated L1CAM-expression had 93% of the phosphorylated FAK^{y397} levels compared to L1CAM-positive T98G/pLKO.1 cells (Yang et al., 2011). We have also shown that inhibitors of FAK have a greater effect on L1CAM-expressing glioblastoma cells (Anderson and Galileo, 2016). A dual FAK/PYK2 inhibitor, PF431396, was chosen because the mechanism by which CBD potentially works against cancer cells is still unknown, so using a dual inhibitor gave a greater chance of CBD having an effect. 2 nM and 10 nM concentrations were chosen based on previous results (Anderson and Galileo, 2016). These concentrations were able to significantly decrease velocity in L1CAM-expressing cells, but not at the maximum effect, so I would be able to determine if CBD could potentiate its effects. From previous studies, 2 nM PF431396 had a 16.8% decrease in average velocity, while 10 nM PF431396 had a 36.9% decrease in average velocity compared to the control group (Anderson and Galileo, 2016). Similarly from the work here, the concentration 2 µM was chosen for CBD it was the highest concentration tested that showed no effect on cell velocity on its own.

The effects of CBD, PF431396, and their combination on T98G cell motility was tested by time-lapse microscopy. In the first trial, L1CAM-expressing T98G/pLKO.1 cells were incubated with DMSO only, 2 µM CBD, 2 nM FAK inhibitor, or 2 µM CBD and 2 nM FAK inhibitor. There were non-significant decreases in average velocity between DMSO only and 2 µM CBD (2.03% difference with a $P = 0.594$) and DMSO only and 2 nM FAK inhibitor (1.35% difference with a $P = 0.717$). There was a significant decrease in average velocity between the combination treatment and all other conditions; importantly, there was a significant

decrease in average velocity between 2 nM FAK inhibitor and the combination treatment (15.1% difference with a $P < 0.001$).

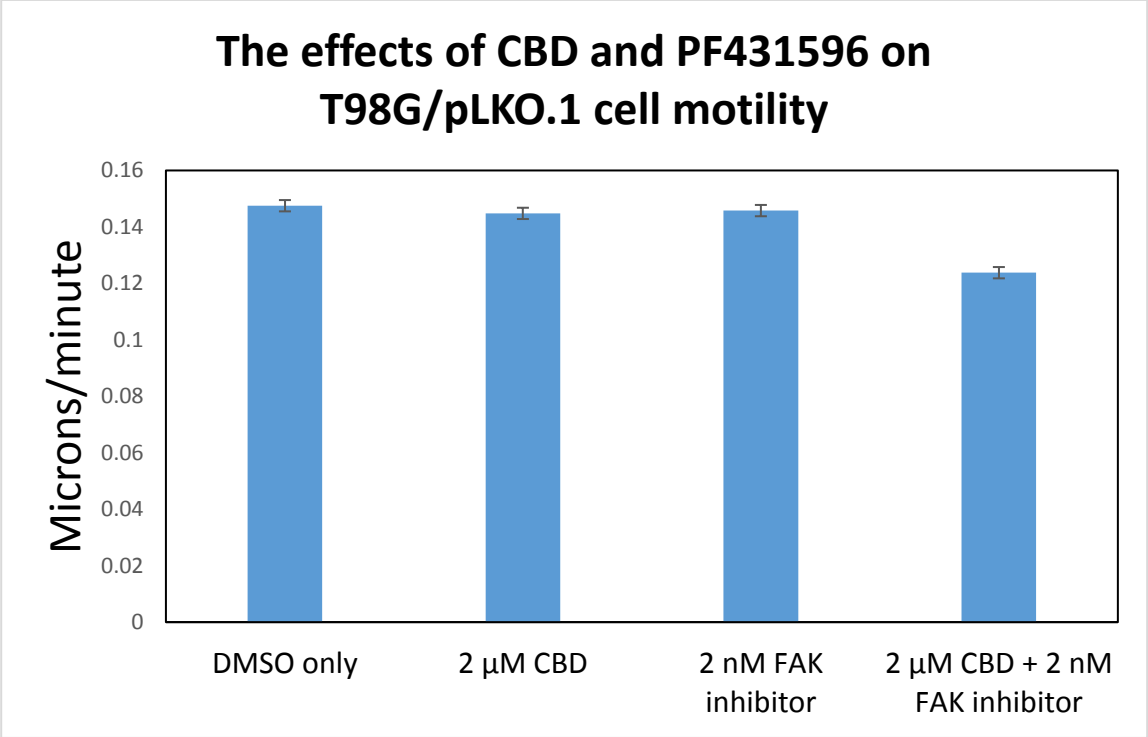


Figure 14. The effects of CBD and PF431396 on T98G/pLKO.1 cell motility. The average velocity for cells incubated with DMSO only (control) was 0.148 microns/minute, with 2 µM CBD was 0.145 microns/minute, with 2 nM PF431396 was 0.146 microns/minute, and with 2 µM CBD and 2 nM PF431396 was 0.124 microns/minute.

Following the first trial, I increased the FAK inhibitor condition to 10 nM, and used both L1CAM-expressing and L1CAM-attenuated T98G cells. For T98G/pLKO.1 cells 2 µM CBD decreased the average velocity by 7.92% ($P < 0.05$), 10 nM FAK inhibitor decreased the average velocity by 15.9% ($P < 0.001$), and the combination of

both CBD and the FAK inhibitor decreased the average velocity by 5.94% ($P = 0.107$). Interestingly, there was an increase of 10.5% ($P < 0.05$) between the 10 nM FAK inhibitor condition and the combination treatment average velocities. When compared to the DMSO-only control condition, T98G/shL1 cells had significant decreases in average velocity when incubated with 2 µM CBD and the combination treatment CBD and FAK inhibitor. The decreases were 27.9% ($P < 0.001$) and 26.3% ($P < 0.001$). There was a non-significant increase of 3.28% ($P = 0.408$) between the control and 10 nM FAK inhibitor.

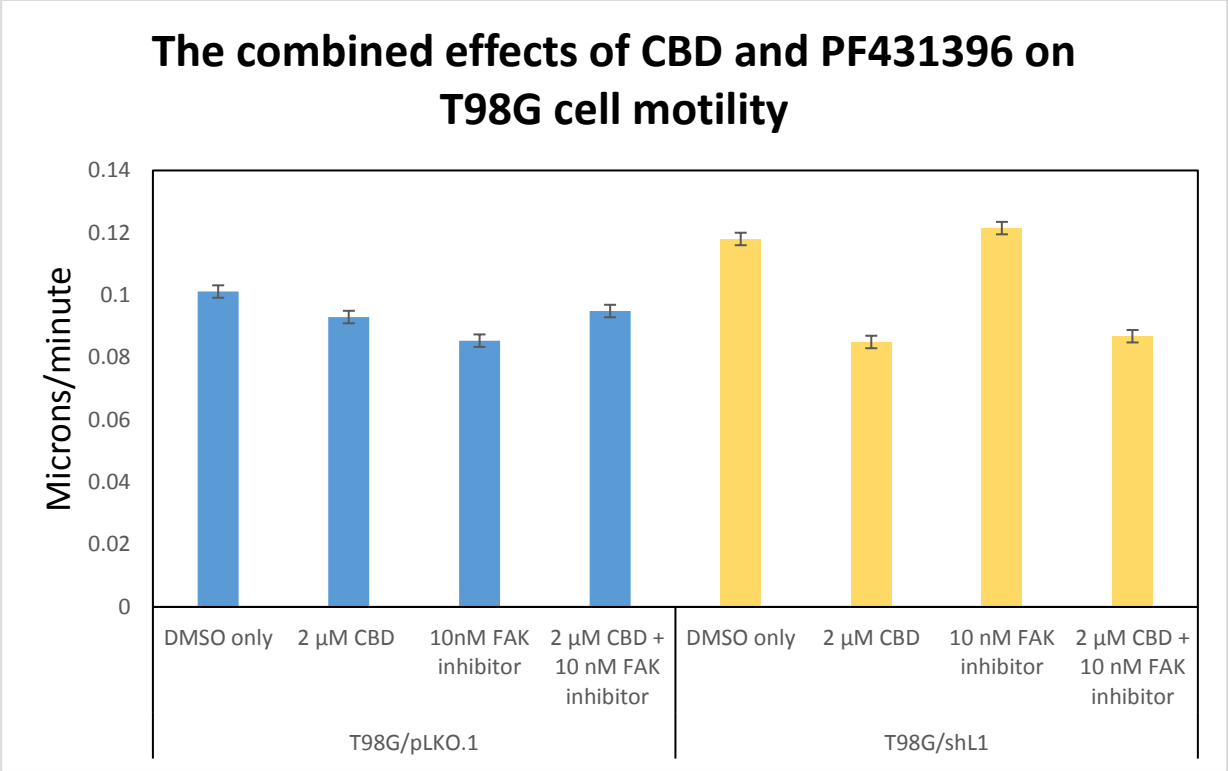


Figure 14. The combined effects of CBD and FAK inhibitor PF431396 on motility of T98G cells. The average velocity of T98G/shL1 cells incubated with DMSO only (control) was 0.118 microns/minute, with 2 µM CBD was 0.085 microns/minute, with 10 nM PF431396 was 0.122 microns/minute, and with 2 µM CBD and 10 nM PF431396 was 0.087. The average velocity of T98G/pLKO.1 cells incubated with DMSO only (control) was 0.101 microns/minute, with 2 µM CBD was 0.093 microns/minute, with 10 nM PF431396 was 0.085 microns/minute, and with 2 µM CBD and 10 nM PF431396 was 0.095 microns/minute.

The average velocities for T98G/shL1 cells were consistent across multiple trials, while the average velocities for T98G/pLKO.1 cells were inconsistent. In this trial, 2 µM CBD decreased cell velocity by 37.6% ($P < 0.001$), 10 nM FAK inhibitor decreased cell velocity by 18.8% ($P < 0.001$), and the combination of those conditions decreased cell velocity by 38.9% ($P < 0.001$). There was a non-significant decrease of

2.15% (P = 0.665) between the CBD only condition and the combination treatment, while there was a significant decrease of 24.8% (P < 0.001) between the FAK inhibitor only condition and the combination treatment.

From these experiments, I concluded that CBD could not potentiate the effects of the FAK inhibitor PF431396 on L1CAM-expressing T98G cells.

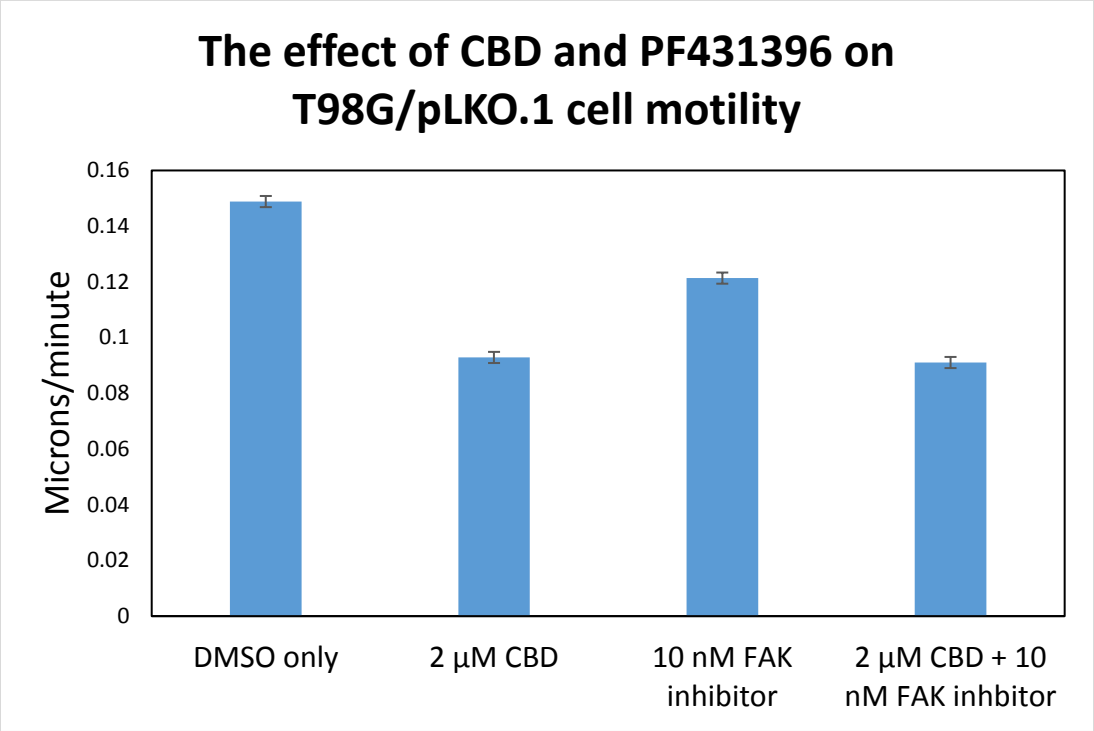


Figure 15. Shows another trial of the effects of the combination treatment on T98G/pLKO.1 cells. The average velocity for cells incubated with DMSO only (control) was 0.149 microns/minute, with 2µM CBD was 0.093 microns/minute, with 10 nM PF431396 was 0.121 microns/minute, and with a combination of 2 µM CBD and 10 nM PF431396 was 0.091 microns/minute.

Chapter 4

DISCUSSION

In this study, I have shown that (1) CBD at micromolar concentrations increased GSC motility, (2) there was a differential effect of CBD on cells that have either L1CAM-expression or have had their expression of L1CAM attenuated, and (3) CBD was unable to potentiate the effects of FAK inhibitor PF431396 on L1CAM-expressing T98G cells.

4.1 GSC motility

The results in this study indicate that CBD has a stimulatory effect on both GSC # 2015-2 and #2016-4 lines, but further research should be done to better understand the reasons behind this result. The difference in the magnitude of the effect could be due to the fact that each GSC line came from a different human GBM tumor. Because of this each line has slightly different characteristics and expression of different proteins including L1CAM (Plusch, 2018). Continuing to characterize these deadly cells is an important objective of the Galileo lab, and will be beneficial in future trials with CBD. Once the GSC lines have been characterized, each can be treated with CBD to determine if there are trends depending on expression, or if the effect that CBD has on motility is different for each GSC line. Because of CBD's reported success in combination with radiotherapy, THC, and chemotherapy drugs (Marcu et al., 2010; Scott et al., 2014; Torres et al., 2011), it would be important to revisit it in the future as one component in a mixture of treatments as more treatment options become known to fight GSCs. CBD could be used to lower toxicity of the treatment by lowering the effective dose.

Instead of trying to inhibit GSCs by slowing down their invasion, one could try a different approach with CBD in the future. Higher concentrations of CBD should be analyzed for their ability to combat GSCs by inducing cell death. There has been success with decreasing cell viability in GBM-derived cell lines (Vaccini et al., 2005; Marcu et al., 2010; Scott et al., 2014; Torres et al., 2011), while only Nabissi and colleagues have had success with decreasing cell viability in GSCs.

Although the majority of research shows the anti-cancer effects of CBD and the endocannabinoid system, there is also evidence that cannabinoids can have a stimulatory effect in cancer. Cannabinoid receptors and their endogenous ligands are generally upregulated in tumor tissue compared with non-tumor tissue (Guzman, 2003; Malfitano et al., 2011; Caffarel et al. 2006; Sanchez et al., 2001). Studies have associated the expression levels of cannabinoid receptors, endocannabinoids and endocannabinoid-metabolizing enzymes with tumor aggressiveness (Malfitano et al., 2011; Nomura et al., 2010; Thors et al., 2010). This suggests that endocannabinoid overexpression may be pro-tumorigenic (Nomura et al., 2010). It is important to determine the precise signaling mechanism that cannabinoids, including CBD, use to regulate cell viability because it could clarify what circumstances cause the endocannabinoid system to act in favor of tumorigenesis.

4.2 L1CAM-positive versus L1CAM-negative cell motility

CBD is more effective against L1CAM-negative cells in comparison to L1CAM-expressing glioblastoma cells at decreasing cell motility. This brings forward the possibility of L1CAM being a protective factor in glioblastoma cells. Unfortunately, because there is a high expression of L1CAM in GBM (Moos et al., 1988; Yang et al., 2009, 2011), CBD's efficacy against GBM may be limited. It is

important to repeat these trials first before dismissing CBD as a treatment option because of the inconsistencies with the average velocities of the T98G/pLKO.1 cells across multiple trials. Because we have already shown that T98G/shL1 cells are, on average, slower than the T98G/pLKO.1 cells (Yang et al., 2011), it is suspicious to see that this was not always the case in the above results. It is possible that the T98G/pLKO.1 cells could have been too confluent in that trials #1 and #3 causing them to be contact inhibited, therefore decreasing their motility. Repeated trials hopefully also would clarify why in some trials a given concentration of CBD caused a significant decrease in average velocity in T98G/pLKO.1 cells, while in other trials, it seemed to have no effect at all. I currently have no explanation for this discrepancy.

If repeated trials proved to be consistent with there being a differential effect of CBD depending on L1CAM expression, it would be important to investigate the mechanism that could be responsible for this difference. Given previous success of limiting cell migration of GBM-derived cell line U87 (Vaccini et al., 2005), it may be true that CBD has a cell type specific effect on cell motility.

Because of the recurring problem with having the T98G cells being too confluent to effectively run a time-lapse and compare velocities, I think it would be beneficial to standardize the *Scratch assay* protocol in any follow up trials. Instead of using a specific percentage or qualitatively determining how much of a confluent 10 centimeter plate to transfer to a 6-well plate for the time-lapse experiment, cells should be counted with a hemocytometer for a specific number of cells and allowed to grow and divide for a predetermined amount of time before beginning the trial.

As stated above with the GSCs, further research should look into how CBD can influence cell viability and proliferation of glioblastoma cells. If L1CAM does in

fact have a protective effect for cancer cell motility, then this could also be true for preventing cell death. Supporting this, effects of inhibitors of cell motility in GBM cell lines were mirrored by effect in cell proliferation (Anderson and Galileo, 2016). It would be interesting to determine if a greater concentration of CBD is needed to kill or decrease cell proliferation of L1CAM-positive cells in comparison to L1CAM-negative cells.

4.3 Combination of CBD and a FAK inhibitor

CBD did not potentiate the effects of PF431396 on L1CAM-expressing glioblastoma cells in this study. The results for T98G/shL1 cells are consistent with the previous results that showed that the FAK inhibitor had no effect on cell motility (Anderson and Galileo, 2016).

The results in this section, specifically the cells treated with 2 μM CBD, are also consistent with L1CAM-negative cells being more susceptible to CBD treatment, and with L1CAM expression being a protective factor for GBM-derived cell lines. In Figure 15, it is important to note that incubating T98G/pLKO.1 cells with 2 μM CBD caused a 37.6% decrease in average velocity. This decrease is significantly higher than any other trial where this concentration was tested on L1CAM-expressing cells. Currently, I have no explanation as to why in this particular trial T98G/pLKO.1 cells were affected to this extent by the CBD treatment. It will be important to repeat this experiment to investigate if this trial was by chance, a mistake was made, or if CBD does actually have an effect on L1CAM-positive cell motility.

Although, there was no success here using CBD in combination with PF431396, its success with enhancing THC, radiation, and TMZ treatment (Marcu et al., 2010; Scott et al., 2014; Torres et al., 2011), gives CBD a lot of potential for GBM

treatment. Until the signaling pathway that CBD uses to prevent tumorigenesis is fully characterized, it would be difficult to determine why CBD is able to potentiate some treatments, while being unable to enhance others.

4.4 Implications for treatment of glioblastoma

Because of the small size and lipophilic structure of cannabinoids coupled with the leakiness of the blood-brain tumor barrier in malignant gliomas (Sarin et al., 2009), CBD has the potential to be a treatment for GBM. If future research is consistent with CBD having a cell type specific effect, it may be a useful addition in personalized cancer treatment. Personalized cancer care utilizes molecular profiling, which determines the genes and proteins expressed in surgical specimens (Ashfaq, 2012). This gives an indication as to what treatments will be most effective against the specific tumor at hand. Because of this, it is important to determine in what circumstances CBD would be most effective against GBM. Once these circumstances or cell types are known, CBD could be added as a component of a personalized treatment when warranted. Even though CBD was not effective against L1CAM stimulation, there are many other molecular alterations that contribute to glioma invasiveness (Dunn et al., 2012; Bleeker et al., 2012). CBD should be investigated against these additional contributors to invasiveness to help determine its place in the treatment of GBM.

Approximately 10,000 people are diagnosed with GBM each year. This, coupled with the fact that only 9.5% of patients survive 2.5 years after diagnosis, shows how great the need is to find drug treatments from all angles (Ostrom et al., 2014; Smoll et al., 2013). In this study, I investigated the possibility of CBD as a treatment both on its own and in combination with another potential treatment.

Although CBD's effect on cell motility in glioblastoma cells was limited, CBD should be investigated further for its anti-tumor properties and its specific role in the treatment of GBM.

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