PHYTOCANNABINOIDS EXERT ANTI-CANCER POTENTIAL IN PRECLINICAL IN VITRO MODELS OF BREAST CANCER

by

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Dalhousie University is located in Mi'kma'ki,
the ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

DEDICATION PAGE

To my grandparents, Cecil and Linda, and my parents, Wade and Lesley, for always supporting my academic endeavours and exemplifying that hard work pays off.

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Abstract

There are a multitude of challenges that are faced in the treatment of breast cancer, such as resistance to chemotherapeutic agents and chemotherapy-induced adverse side effects. *Cannabis sativa* is used in cancer patients under palliative care and in patients who are actively receiving chemotherapy where it functions to provide analgesic effects and mitigate some of the adverse effects induced by chemotherapy. With the use of cannabis in cancer patients comes an important question: how does cannabis or its components affect cancer or its treatment? Phytocannabinoids produced by the cannabis plant, particularly Δ-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), have been shown to exert desirable anti-cancer effects in several cancers, including breast cancer. Some of these anti-cancer effects include reducing cancer cell proliferation, inducing apoptosis and limiting metastasis. Cannabinoids other than THC and CBD are produced by the cannabis plant and have been far less studied for their potential anti-cancer properties. The objective of this study was to explore the anti-cancer effects of multiple cannabinoids in preclinical models of breast cancer. Our study revealed that cannabinoids were cytotoxic, induced apoptosis, and reduced the invasion of breast cancer cells. Cannabinoid combinations as well as cannabinoids with doxorubicin produced additive or synergistic reductions in cell viability of breast cancer cells, including a resistant model. This study provides insight that cannabinoids and combination treatments involving cannabinoids may have therapeutic benefits as novel anti-cancer agents in the treatment of breast cancer.

List of Abbreviations and Symbols Used

ABC	ATP binding cassette
AC-T	Anthracycline and cyclophosphamide followed by taxane
AEA	Anandamide
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CBC	Cannabichromene
CBCA	Cannabichromenic acid
CBD	Cannabidiol
CBDV	Cannabidivarin
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBN	Cannabinol
CBV	Cannabivarin
CIPN	Chemotherapy-induced peripheral neuropathy
CNS	Central nervous system
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid

ECM	Extracellular matrix
ECS	Endocannabinoid system
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
GBM	Glioblastoma multiforme
GPCR	G-protein-coupled receptor
GPR18	G-protein-coupled receptor 18
GPR55	G-protein-coupled receptor 55
GST	Glutathione S-transferase
HER-2	Human epidermal growth factor receptor 2
HIF-1	Hypoxia-inducible factor 1
HIF-2	Hypoxia inducible factor 2
HR	Hormone receptor
IC_{50}	Half maximal inhibitory concentration
MCD	Methyl-beta-cyclodextrin
MDR	Multidrug resistance
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MRP	Multidrug resistance protein
MRP1	Multidrug resistance associated protein 1

NBD	Non-binding domain
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OLA	Olivetolic acid
PBS	Phosphate buffered saline
PGP	P-glycoprotein
PI	Propidium iodide
PR	Paclitaxel-resistant
PgR	Progesterone receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SEM	Standard error of the mean
SNRI	Serotonin-norepinephrine reuptake inhibitor
SR144528	Selective CB2 antagonist
THC	Delta-9-tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid
THCV	Tetrahydrocannabivarin
TIMP-1	Tissue inhibitor of MMP-1
TKI	Tyrosine kinase inhibitor
TKS	Tetraketide synthase
TMD	Transmembrane domain
TMZ	Temozolomide
TNBC	Triple-negative breast cancer
Topo II	Topoisomerase II

TRP	Transient receptor potential channel
TRPM8	Transient receptor potential cation channel subfamily M member 8
TRPV1	Transient receptor potential vanilloid 1
2-AG	2-arachidonoylglycerol
2D	2-dimensional
3D	3-dimensional
β	Beta
Δ	Delta
γ	Gamma
µL	Microlitre
µM	Micromolar
~	Approximately
°C	Degrees Celsius
*****	Indicates statistical significance between specified groups
%	Percent
±	Plus minus

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First and most importantly, I want to sincerely thank my supervisor, Dr. Denis Dupré, who provided me with the opportunity to take on an MSc project in his lab. When I first came to the Dupré lab, I was very unsure of my future and lacked confidence in an academic setting. All of the technical skills I gained in the lab throughout these two years were new to me… I quite literally came into this having never passed cells before. Dr. Dupré was able to provide his expertise with so much patience and in such a supportive way that I will be forever grateful for. I will always be thankful for the encouraging, fun, cohesive lab environment that he provided as well. He allowed me to learn on my own while still providing his utmost support, which really gave me the confidence that I needed to succeed in my Masters, and also in my future endeavours. I would also like to thank my co-supervisor, Dr. Lee-Ellis, and my advisory committee members, Dr. Christopher Sinal and Dr. Christian Lehmann, for giving me feedback, guidance, constructive criticism and encouragement during these last two years. Although I always found committee meetings to be intimidating beforehand, I always left feeling supported, encouraged, and more confident, which motivated me to work hard throughout this project. I could not be more thankful for the amazing lab environment and labmates that I was so fortunate to have in the Dupré lab during this degree. Lauren and Andrea made me feel welcome from the first day I started. Andrea quickly became a special friend while she helped teach me new lab techniques that I needed for my project. I am so grateful for her friendship and support during my time in the Dupré lab. Her patience during my initial training, and her laid back, positive personality really helped me learn to keep my cool and push forward during times of stress. Thank you, Andrea, for being a great lab member and an even better friend. To my family and friends that were always there to support me during my degree, thank you. The constant encouragement and kind words never went unnoticed. Tal, although you had no idea what I was talking about most of the time, you never failed to listen and pretend to understand what was going on. To my partner, Nick, thank you for providing me with endless support and motivation to be my best during this degree. Your constant dedication to working hard is so admirable.

Chapter 1: Introduction

1.1 Breast Cancer

Breast cancer is the most commonly diagnosed form of cancer in women, and it is ranked as the second leading cause of cancer-related deaths in women as well (Ullah, 2019). Incidence rates of breast cancer increased significantly globally during the 1980s and 90s and unfortunately continue to increase with time. It is estimated that 1 in 8 women will be diagnosed with breast cancer during their lifetime (Canadian Cancer Society, 2021). Overall, breast cancer mortality has declined as a result of improved therapies and earlier diagnosis, however metastatic breast cancers continue to develop in 20-30 percent of women who experience breast cancer (Ullah, 2019). Metastatic breast cancer is most often considered to be incurable and is the primary cause of breast cancer mortality (Sledge, 2016). The American Cancer Society reported that the 5-year survival rate for women with metastatic breast cancer is 22% (Sledge, 2016). In the case of metastatic tumors, surgery is not a reliable option for removal of the tumor, as it is difficult to locate and remove any secondary tumors (Morris et al., 2009). Although non-metastatic breast cancers have more viable treatment options available, there still remain many limitations. Often, breast cancers develop resistance to chemotherapeutics over the course of treatment, or possess innate resistance, which makes treatment even more difficult in these patients. Metastasis and chemotherapeutic resistance result in poor prognosis and reduced survival rates in patients, highlighting the need for novel treatment options to improve patient outcomes in these cases.

One of the difficulties that arises with the treatment of breast cancer is that every tumor is unique. Breast cancers are heterogenous, meaning that there is variation in

morphologies and biological features of tumors between patients (intertumor heterogeneity) (Figure 1.1 A) (Tsang, 2020). Intertumor heterogeneity can also refer to differences between a primary breast tumor and a tumor in a new location as a result of metastasis (Figure 1.1 B). In addition to patient-to-patient differences, breast cancer solid tumors themselves are heterogenous and vary in morphology and biological features (intratumor heterogeneity), resulting in difficulties and uncertainties surrounding how they respond to treatment (Figure 1.1 C) (Tsang, 2020). As a result of this, breast cancer tumors are often assessed and classified on a patient-to-patient basis based on a variety of biomarkers to allow treatment to be as tailored as possible. Recently, it was recommended that all cases of breast cancer be assessed for the following to assist with prognosis and to determine therapeutic options: histological grade, estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER-2) and Ki-67 (Colomer et al., 2018).

The two most routine assessments of breast cancer are the patient's intrinsic subtype and immunophenotype (Tsang, 2020; Dai et al., 2015). There are five main intrinsic subtypes of breast cancer: luminal A, luminal B, HER-2-enchriched, triple-negative, and normal-like (Figure 1.2) (Dai et al., 2015). Immunophenotyping is defining a breast tumor by the presence or absence of the estrogen receptor, progesterone receptor, or human epidermal growth factor receptor 2 (Tsang, 2020). The intrinsic subtype and immunophenotype of a tumor can be used as predictive factors in the prognosis and treatment of breast cancer patients (Dai et al., 2015). Estrogen and progesterone receptors are sex steroid receptors that are expressed in nearly 75 percent of breast cancers and serve as indicators for the tumor's responsiveness to hormone therapy (Tsang, 2020).

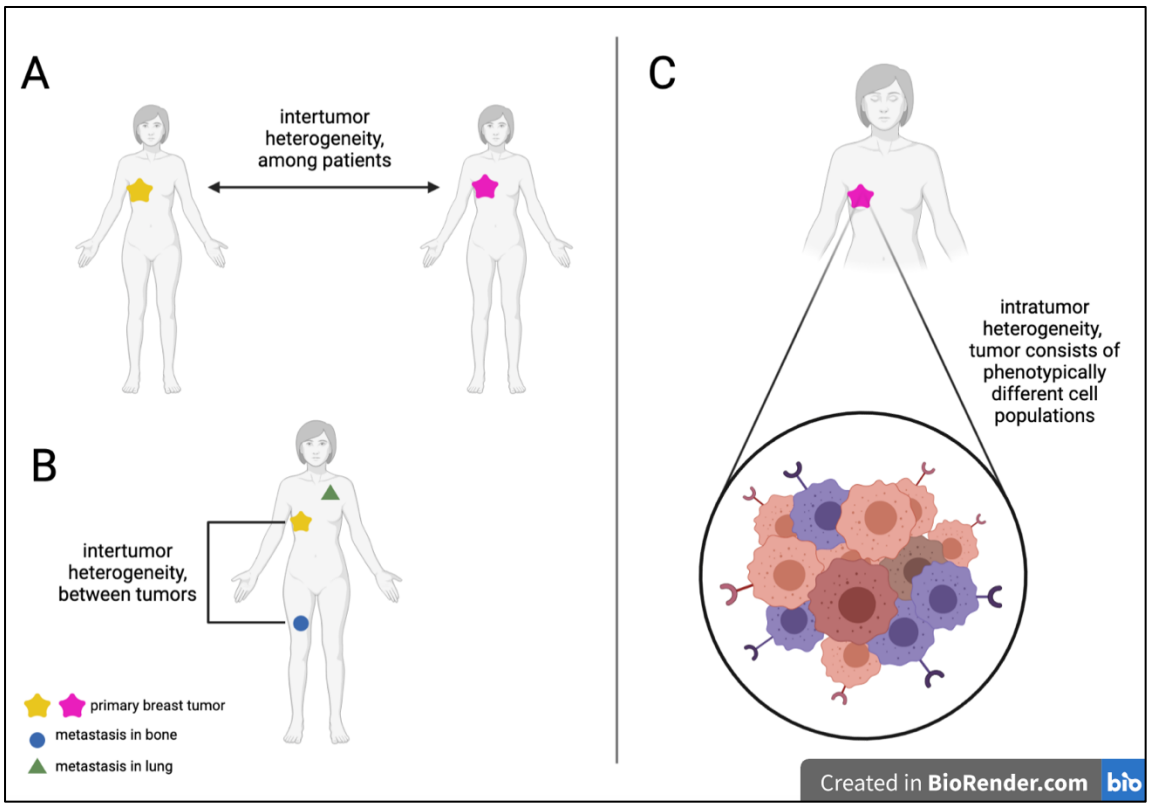


Figure 1.1. Intertumor and intratumor heterogeneity of breast cancer. (A) Tumors differ between patients, (B) tumors can metastasize to a new location and differ within a single patient, and (C) a single tumor consists of differing cell populations with varying phenotypes. Tumor heterogeneity can make it more difficult to find an effective treatment option and results in different responses to the same treatment among patients. Intratumor heterogeneity can make it difficult to target an entire tumor with a single treatment, as some cell types present may not respond to select treatments, highlighting the importance of combination treatments. Created with BioRender.com.

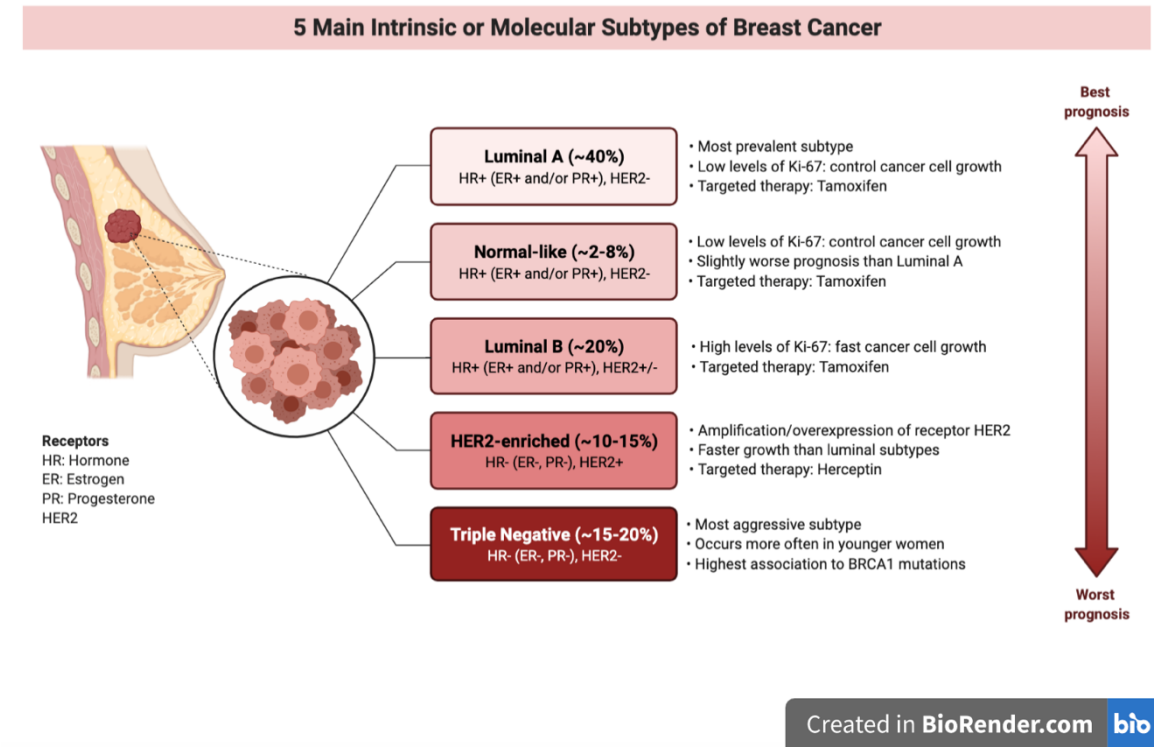


Figure 1.2. The five primary intrinsic subtypes of breast cancer and their respective immunophenotypes. The most prominent subtype is luminal A, followed by luminal B, HER-2-enriched, triple-negative, and normal-like. Luminal A has the best prognosis while triple-negative subtypes are very challenging to treat and have the worst prognosis. Reprinted from "Intrinsic and Molecular Subtypes of Cancer", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

Most often, breast cancers that are ER-positive are also PgR-positive, and these cancers tend to be less aggressive (ie. slow growing/spreading) and low-grade (ie. cancer cells are closer to "normal" phenotype) compared to ER-/PgR-negative tumors (Tsang, 2020). Breast cancers that are classified as HER-2-positive account for roughly 10 to 15 percent of all breast cancers diagnosed and are generally associated with poor prognosis and an aggressive clinical course. The benefit to breast cancers that are ER-, PgR- and HER-2-positive is that they usually respond to hormonal/immune-therapies. On the other hand, triple-negative breast cancers (TNBC) express none of the three receptor markers previously discussed. As a result, patients with TNBC tend to have poor prognosis and high-grade tumours that do not benefit from currently available therapies that target ER, PgR or HER-2 (Tsang, 2020).

1.2 Current Breast Cancer Therapy

1.2.1 Surgery, Radiation, Endocrine Therapy

Non-metastatic breast cancers have multiple effective treatment options with a main goal to eradicate the tumor from the breast tissue and prevent future recurrence (Waks et al., 2019). One of the most common methods of local therapy for non-metastatic breast tumors is surgical resection. The typical approaches for local removal are a total mastectomy, or excision of the tumor followed by a period of radiation (Waks et al., 2019). Radiation therapy is another viable option for non-metastatic breast tumors. Radiation can occur before (neoadjuvant) or following (adjuvant) surgery to remove a tumor (Waks et al., 2019). Another therapy for non-metastatic breast cancer is systemic treatment, where anti-cancer agents are delivered throughout the body to eliminate cancerous cells. Systemic therapies include hormonal (endocrine), chemotherapy and

immunotherapy (Waks et al., 2019). Endocrine therapy is the primary systemic therapy used in the treatment of hormone receptor-positive breast cancers and functions to reduce estrogen-stimulated tumor growth. Tamoxifen is one of the most well-studied endocrine therapies and functions by inhibiting the binding of estrogen to the ER and acts as a selective estrogen receptor modulator (Li et al., 2016). Tamoxifen is effective at preventing the recurrence of breast cancer, reducing the size of HR-positive breast tumors before surgical removal, and slowing or stopping the growth of advanced HR-positive breast cancer (Li et al., 2016). Aromatase inhibitors (eg. Anastrozole) are another form of endocrine therapy that function by inhibiting the conversion of androgens to estrogen, which results in reduced levels of circulating estrogen, however these are generally more effective in postmenopausal women (Waks et al., 2019). Unfortunately, endocrine therapies have adverse side effects, such as hot flashes, myalgias, increased risk of uterine cancer, and osteoporosis-related bone fracture (Waks et al., 2019).

1.2.2 Immunotherapy

The Canadian Cancer Society (2021) states that immunotherapy can be used in the treatment of local advanced or metastatic breast cancer. Cancer cells are known to escape the body's immune response, and immunotherapy is given in an attempt to restore or strengthen the ability of the immune system to detect and eliminate cancer. Three primary mechanisms have been described to contribute to the ability of cancer cells to escape immune detection and killing: reduced immune detection and immune cell activation, increased resistance to cytotoxicity, and increased suppression of immune cells due to the tumor microenvironment (Basu et al., 2019). There are currently several immunotherapy treatments that have been approved to treat breast cancer, including

immune checkpoint inhibitors and immune targeted therapies. In normal cells, immune checkpoints are surface proteins that help immune cells recognize cells in the body as "self" and therefore they avoid attack by the immune system (Garcia-Aranda & Redondo, 2019). Cancer cells often use these immune checkpoints as a method to avoid attack by the immune system, and immune checkpoint inhibitors target these proteins to help the immune system recognize and kill cancerous cells (Garcia-Aranda & Redondo, 2019). In immune targeted therapy, monoclonal antibodies are commonly used. These antibodies are designed to target certain antigens present on cancer cells (eg. HER-2) and render the target protein non-functional, leading to cancer cell death (Garcia-Aranda & Redondo, 2019). Monoclonal antibodies used in immunotherapy have response rates ranging from 3-84% against HER-2-positive breast tumors, however, acquired resistance to immunotherapy is a common issue in HER-2-positive breast cancers (Garcia-Aranda & Redondo, 2019). Finally, common adverse effects experienced by patients undergoing immunotherapy include pain, nausea, vomiting, and chills.

1.2.3 Chemotherapy

Chemotherapy is another form of systemic therapy that serves as an essential treatment for preventing recurrence of breast cancer in many patients (Wak et al., 2019). Typically, patients with HER-2 positive breast cancer are treated with a combination of HER-2 targeted therapy and chemotherapy (Luque-Bolivar et al., 2020). Breast cancer patients with triple-negative tumors receive primarily chemotherapy treatment. For early-stage or local advanced breast cancer (non-metastatic), there are a multitude of efficacious chemotherapy regimens currently used. Chemotherapy drug regimens are typically used because research has consistently supported that treatment with more than

one drug is more effective than treatment with a single agent (Canadian Cancer Society, 2021). The mono-therapy approach to cancer treatment is still a relatively common option, particularly in triple-negative metastatic breast cancers, however this approach has been routinely deemed less effective than combination therapy (Mokhtari et al., 2017). The key rationale behind multidrug therapy is that using drugs with different mechanisms of action reduces the chances of developing resistant cancer cells. Even in HR-positive breast cancers where endocrine therapy is the primary systemic therapy used, chemotherapy can be added to maximize anti-cancer benefits (Canadian Cancer Society, 2021). Clinical research has demonstrated that the AC-T (anthracycline and cyclophosphamide followed by taxane) or A-T (anthracycline followed by taxane) regimens are some of the most effective for early-stage and local advanced breast cancers (Canadian Cancer Society, 2021; Fujii et al., 2015; Rivera & Gomez, 2010; Zheng et al., 2015). Unfortunately, in cases of advanced metastatic breast cancer, combination therapy regimens are still not overly effective, and doctors may treat with a single agent in order to reduce adverse side effects (Zheng et al., 2015). In addition to their use in a sequential regimen, anthracyclines and taxanes are used alone in the treatment of advanced metastatic breast cancers as well.

Anthracyclines (eg. doxorubicin) were originally isolated from *Streptomyces peucetius* and elicit anti-tumor effects through three mechanisms. Anthracyclines intercalate into DNA by interacting with DNA gyrase and topoisomerase II to form a cleavable complex (Jasra, 2018). Following this, double-stranded DNA breaks occur which causes inhibition of transcription and DNA replication, ultimately resulting in cell cycle arrest and subsequent apoptosis. Anthracyclines can also alter signal transduction

pathways like the Fas ligand pathway (Jasra, 2018). The Fas ligand pathway plays a central role in the regulation of apoptosis. Another anti-cancer action of anthracyclines is that they promote the production of reactive oxygen species (ROS), which can result in DNA damage as well as lipid peroxidation. Unfortunately, anthracyclines like doxorubicin are well known to induce cardiotoxicity as a result of accumulation in myocardial cells, leading to the production of ROS and subsequent cell damage (Jasra, 2018). Alopecia is also a universal negative side effect experienced by breast cancer patients treated with anthracyclines.

The two main taxane drugs that are available for use are paclitaxel and docetaxel. These drugs act by inhibiting the cellular processes that are required for functional cell division. During the cell division process of mitosis, microtubules play a key role in the organization and functionality of the mitotic spindle, which ensures the integrity of the segregating DNA (Abal et al., 2003). The anti-cancer effects exerted by taxanes comes from their ability to bind microtubules during the M-phase of cell division and prevent them from depolymerizing, which inhibits the process of mitosis and ultimately results in the induction of apoptosis (Abal et al., 2003). Unfortunately, it is thought that taxanes also exert their effects on microtubule stabilization and subsequent cell cycle arrest in healthy peripheral nerves as well. A major adverse effect with the use of taxanes like paclitaxel is that they have been shown to result in chemotherapy-induced peripheral neuropathy (CIPN) in approximately 60 percent of patients (Hammond et al., 2019). The pathophysiology and mechanisms that underly CIPN are currently unknown, however it is likely a result of several mechanisms including the impairment of microtubules, reduced mitochondrial function, remodelling of ion channels and genetic predisposition

(Hammond et al., 2019). There are no agents recommended to directly prevent CPIN in patients undergoing taxane therapy, however current clinical guidelines suggest some moderate relief with the use of a serotonin-norepinephrine reuptake inhibitor (SNRI), duloxetine. Duloxetine is also often associated with negative side effects like dizziness, nausea, and insomnia, so it's use in these cancer patients is not ideal (Hammond et al., 2019). It has also been shown that cannabinoids produced by *Cannabis sativa* (eg. cannabidiol) reduce CIPN without affecting the efficacy of chemotherapy (Ward et al., 2014). There remains a clear need for the exploration of novel breast cancer treatments, or co-treatments that could allow reduced dosing of chemotherapeutic drugs to lower adverse effects experienced by patients.

1.3 Resistance in Breast Cancer

A major problem faced in the management of breast cancer is resistance to chemotherapeutic agents. Tumors can possess natural (primary) resistance to chemotherapy drugs where they do not respond to common chemotherapeutic drugs from the beginning (Choi, 2005). Additionally, tumors can develop resistance to chemotherapeutics after drug exposure, where they show initial responsiveness to treatment but develop acquired resistance later (Choi, 2005). Often when tumors develop resistance following exposure to anti-cancer drugs, they become resistant to multiple agents with differing structures and mechanisms of action at the same time, referred to as multidrug resistance (MDR) (Wind & Holen, 2011). In a clinical setting when drug resistance occurs, the method to overcome this is by selecting other anti-cancer agents for treatment in hopes that the tumor will respond. This change in treatment is usually done without any understanding of the potential molecular mechanisms of resistance in a

particular case, and better understanding of the mechanisms involved could improve treatment strategies to achieve a more beneficial response to therapy, while minimizing adverse effects (Luque-Bolivar et al., 2020).

As briefly mentioned earlier, breast cancer is a highly heterogenous disease where each patient has unique molecular and morphological features. Multiple studies have shown that even in patients with the same type of breast cancer, different responses to the same treatments are often observed, highlighting the need to gain better understanding of patients' individual resistance characteristics (Luque-Bolivar et al., 2020). A better understanding of the complex mechanisms that underly resistance to chemotherapeutics would also help to develop effective strategies to overcome MDR. Multidrug resistance is of most concern in triple-negative breast cancers since chemotherapy is the primary treatment option for this type of breast cancer. In addition to chemotherapeutic resistance, resistance to endocrine and targeted (HER-2) therapies in HR- and HER-2-positive breast cancers can also develop. The focus here is resistance to chemotherapeutic agents and the different mechanisms that lead to this resistance including: ABC transporters, enzymes, and the tumor microenvironment.

1.3.1 ABC Transporters

Transmembrane transporter proteins have been widely shown to faciliate resistance in tumors to a multitude of chemotherapeutic agents (Wind & Holen, 2011; Luque-Bolivar et al., 2020; Choi, 2005). ATP-binding cassette (ABC) transporters are a family of transmembrane transporter proteins present in the cell membrane of cancerous cells as well as healthy cells (Linton, 2007). These ABC transporters use the energy generated through adenosine triphosphate (ATP) hydrolysis to move molecules in and out

of the cell. ABC transporters move ligands across the cell membrane through active transport and their function is critical for many aspects of healthy cell physiology (Linton, 2007). ABC transporters that move molecules from the inside to the outside of the cell are referred to as efflux pumps/transporters. Most human ABC transporters are efflux pumps that function to remove cytotoxic molecules–such as dietary cytotoxins–from healthy cells. One of the other important functions of ABC transporters in healthy cells is lipid transport across the cell membrane (Linton, 2007). Cancer cells can use efflux pumps to their advantage by up-regulating their expression to pump out chemotherapeutic agents, such as doxorubicin and paclitaxel, thereby preventing their anti-cancer actions.

Doxorubicin and paclitaxel are ABC transporter substrates and resistance is commonly developed to these agents as a consequence (Wind & Holen, 2011; Luque-Bolivar et al., 2020). The description that follows provides a brief overview of how cancer cells use ABC efflux transporters to pump out anti-cancer agents like doxorubicin and paclitaxel to achieve MDR. ABC transporters have four main domains, consisting of two transmembrane domains (TMD) and two non-binding domains (NBD) (Figure 1.3). The ligand binding site is formed by the two TMDs, and the NBDs reside in the cytoplasm and are responsible for binding and hydrolyzing ATP, which drives ligand translocation across the membrane (Linton, 2007). The non-binding domains are homologous throughout the ABC family of transporters, whereas the transmembrane domains differ and provide ligand specificity (Linton, 2007). The ligand with specificity for the binding pocket (eg. doxorubicin/paclitaxel) formed by the transmembrane

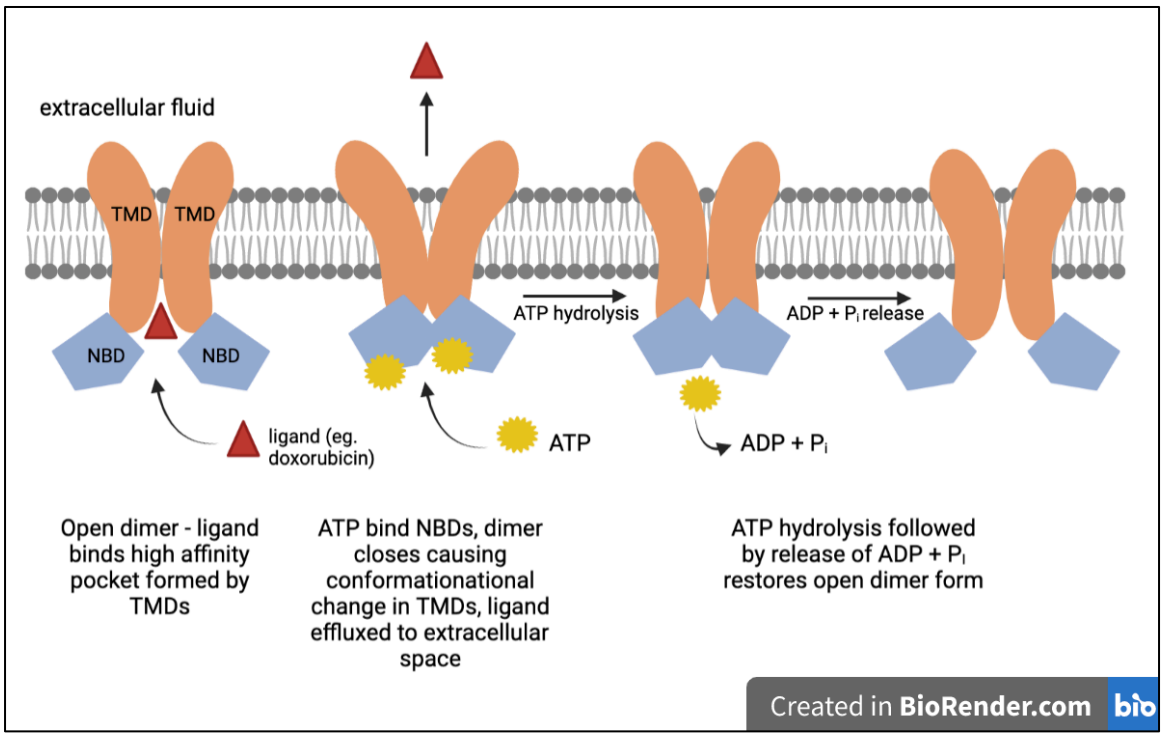


Figure 1.3. ATP-binding cassette (ABC) efflux transporter translocation of ligand to the extracellular fluid. The ligand binds the TMD binding pocket during the NBD open dimer state. ATP binding causes conformational changes in the NBDs and TMDs, resulting in efflux of the bound ligand. ATP hydrolysis and subsequent release of ADP and a phosphate from the NBDs restores the open dimer form that can accept another ligand. Cancer cells often use ABC efflux transporters to achieve MDR, rendering multiple anti-cancer agents less effective or ineffective. Created with BioRender.com.

domains binds when the NBD dimer is in its open form. This is followed by the binding of ATP to the NBDs, causing the dimer to move into a closed state and a subsequent conformational change in the TMDs (Linton, 2007). This conformational change in the TMDs causes the ligand to be effluxed out of the cell into the extracellular fluid (Figure 1.3). Following ligand efflux, the NBDs hydrolyze ATP and release ADP and phosphate, restoring the open dimer form that can accept another ligand for efflux.

Unfortunately, due to their inherent nature, ABC efflux pumps are responsible for a large portion of drug resistance by pumping drugs out of cells, rendering them significantly less effective or completely ineffective altogether (Choi, 2005). Multidrug resistance that occurs because of over-expression of ABC efflux pumps is considered classical MDR. There are three main ABC transport proteins that have been indicated in the development of MDR in breast cancer: P-glycoprotein (PGP), multidrug resistance associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) (Wind & Holen, 2011). P-glycoproteins are encoded by the human *MDR* genes, consisting of *MDR1* and *MDR2* (Kuo, 2013). Only *MDR1*-encoded PGP facilitates multidrug resistance, as *MDR2*-encoded PGP functions as a transporter of phospholipids (Kuo, 2013). P-glycoproteins are the most commonly found efflux pumps in the cell membrane and are found in many different tissues. PGP was the first ABC efflux transporter that was identified as over-expressed in breast cancers exhibiting MDR (Riordan et al., 1985). Neutral, cationic, and hydrophobic ligands are transported by PGP, including common anti-cancer agents such as vinblastine, vincristine, doxorubicin, etoposide and paclitaxel (Wind & Holen, 2011; Luque-Bolivar et al., 2020). This is a great example of how resistance to multiple anti-cancer drugs with differing mechanisms of action and

structures can arise as a result of a single resistance mechanism; in fact, many *in vitro* models to study MDR breast cancer exhibit over-expression of ABC efflux transporters that are deemed responsible for conferring resistance (Liu et al., 2005; Hall et al., 2017; Mechetner et al., 1998; Tang et al., 2015).

Multidrug resistance associated protein 1 (MRP1) is another ABC efflux transporter that is present in many different tissues and cell types, including breast cancer cells (Zaman et al., 1994). In breast cancer cells that overexpress MRP1, resistance to an array of anti-cancer drugs–such as doxorubicin–occurs, as this efflux transporter has been shown to have broad substrate specificity, similar to PGP (Wind & Holen, 2011). MRP1 transports uncharged hydrophobic molecules, anions that are lipid soluble, and hydrophilic anions (Abaan et al., 2009). Interestingly, glutathione conjugation stimulates MRP1-mediated efflux of many drugs as well (Abban et al., 2009). The third ABC transporter indicated in the development of MDR in breast cancer is the breast cancer resistance protein. The human BCRP is encoded by the *ABCG2* gene and like PGP and MRP1, it has broad substrate specificity (Mao & Unadkat, 2015). Substrates for BCRP include mitoxantrone, methotrexate, and several tyrosine kinase inhibitors (TKIs). The exact mechanism of action for drug transport mediated by BCRP is not as well characterized, however the overall mechanism is likely similar to that of PGP and MRP1 (Wind & Holen, 2011).

ABC transporters and multidrug resistance have been investigated in some clinical studies to relate levels of MDR efflux pumps present in breast tumors to clinical outcome. A 2006 study by Park et al. looked at whether the expression pattern of an ABC transporter gene panel could be used to anticipate responses to neoadjuvant

chemotherapy. They found that higher expression levels of genes for ABC transporters for specific drugs correlated with reduced pathological response to those drugs, indicating that examining the tumor ABC transporter gene expression profile of a patient could be useful to predict drug responses. In another study, high expression levels of PGP in primary invasive breast cancer were associated with shorter overall survival, a shorter progression-free period, and higher tumor grade (Surowiak et al., 2005). PGP expression at the time of breast cancer diagnosis may predict poor prognosis and clinical response to neoadjuvant chemotherapy (Chintamani et al., 2005). MRP1 expression has also been demonstrated to play a key role in resistance to chemotherapy and correlates with a shorter relapse-free survival rate (Filipits et al., 2005). Other studies have found that there is no significant correlation between PGP/MRP1 expression and 5-year disease free survival or survival overall (Moureau-Zabotto et al., 2006), however these contradictory findings are likely due to different techniques used to measure levels of MDR pumps (Wind & Holen, 2011).

Multidrug resistance inhibitors have been studied in an attempt to improve the susceptibility of MDR cancer cells to chemotherapy (Robinson & Tiriveedhi, 2020). Most of the clinical studies have specifically looked at the potential for PGP inhibitors since many solid tumors have high constitutive expression or chemotherapy-induced expression of PGP. Verapamil and cyclosporine-A were some of the PGP inhibitors that were first studied in a clinical setting. Unfortunately, most clinical trials have failed for several reasons, the most notable being the high dose of drug needed to achieve relevant PGP inhibition (Robinson & Tiriveedhi, 2020; Nanayakkara et al., 2018). The required high dosing led to serious adverse effects including unsafe cardiac and

immunosuppressive effects (Holt et al., 1992; List et al., 2001). Other PGP inhibitor candidates such as valspodar and biricodar had superior tolerability but did not have high specificity for PGP and resulted in unpredictable drug interactions and interactions with other transport proteins (Thomas & Coley, 2003; Binkhathlan & Lavasanifar, 2013). Although some argue that these complications do not dimmish the potential for PGP inhibitors (Nanayakkara et al., 2018), systemic toxicity and limited specificity are important concerns that limit their use clinically.

1.3.2 Enzymes

Cell detoxifying enzymes can also confer resistance to multiple chemotherapeutic agents in breast cancer cells. These enzymes serve the purpose of counteracting or breaking down chemotherapeutic agents in cells, rendering them much less effective or ineffective (Ji et al., 2019). Some of these detoxifying enzymes include aldehyde dehydrogenase (ALDH) and glutathione S-transferases (GST). Aldehyde dehydrogenase-1 has been shown to be a signature of breast cancer stem cells, where expression is highly correlated with chemoresistance and poor prognosis (Attia et al., 2020). Glutathione S-transferases are detoxifying enzymes that function to protect macromolecules in the cell, and they have been implicated in MDR to chemotherapeutic agents in cancer cells (Attia et al., 2020; Townsend & Tew, 2003). GST activity in cancer cells has been demonstrated to reduce the duration of effective drug concentration inside cells, ultimately leading to decreased drug efficacy (Attia et al., 2020). Many anti-cancer drugs target DNA repair enzymes, such as topoisomerase II (Topo II). While Topo II is not a cell detoxifying enzyme, cancer cells, including breast cancer, often reduce its cellular expression levels in order to diminish susceptibility to drugs that target it (Attia et al., 2020). In addition to

altering its expression, gene mutations of Topo II in cancer cells can alter its nuclear localization, which also leads to resistance to Topo II-targeting drugs (Bukowski et al., 2020).

1.3.3 Tumor Microenvironment

The microenvironment of a tumor includes elements such as the extracellular matrix, signaling molecules, stromal cells, soluble factors, mechanical signals, and hypoxia (Luque-Bolivar et al., 2020). While it facilitates favorable conditions for metastasis and tumor progression, many elements of the tumor microenvironment also confer resistance to chemotherapeutic agents. Tumor-associated macrophages have been shown to protect cancer cells from the effects of cytotoxic chemotherapy through cathepsin-dependent function (De Palma & Lewis, 2011). To reinforce this, it was found that targeting tumor associated macrophages in combination with chemotherapeutic agents can improve therapeutic drug efficacy (Mitchem et al., 2013). In breast cancer, fibroblasts are the main component of the tumor microenvironment, and they have been shown to modulate the behaviour of breast cancer cells and promote resistance to chemotherapy (Chaiwun et al., 2011). In addition to the cellular components of the tumor microenvironment, soluble factors can also play a role in resistance to chemotherapeutic agents in breast cancer. NF-κB, epidermal growth factor, transforming growth factor-β and insulin-like growth factor are all important soluble factors that have the ability to directly modulate the transcriptional regulation of BCRP and increase its expression, causing increased resistance to chemotherapy (Attia et al., 2020). Factors in the tumor microenvironment can also faciliate the transfer of cytokines, micro-RNAs and PGP from resistant to sensitive cells, resulting in increased survival (Luque-Bolivar et al., 2020).

A feature of the tumor microenvironment is a reduction in oxygen concentration, creating a hypoxic microenvironment. Under hypoxic conditions, energy is predominantly produced via glycolysis, which results in increased expression of metabolic enzymes (eg. lactate dehydrogenase) (Luque-Bolivar et al., 2020; Attia et al., 2020). This increase in metabolic enzymes can in turn render chemotherapeutic drugs less effective due to increased metabolic breakdown. Multidrug resistance proteins (MRP) are also sensitive to oxygen conditions, as their genes are hypoxia responsive (Attia et al., 2020). Hypoxia-inducible factor-1 (HIF-1) exists under hypoxic conditions in mammals and it can directly regulate the expression of MRPs, like PGP, by binding to hypoxia response elements on the genes that encode them (Comerford et al., 2002; Tsuruo et al., 2003). HIF-1 and HIF-2 have also been shown to bind hypoxia response elements in the BCRP gene, causing its increased expression (Attia et al., 2020). These up-regulations in MRPs as a result of hypoxia leads to increased resistance to chemotherapeutic agents. Finally, hypoxia in the tumor microenvironment has also been shown to reduce Topo II expression in cancer cells, which facilitates resistance to a multitude of chemotherapy drugs that target it (Attia et al., 2020).

ABC-efflux transporters, specific cellular enzymes, and the tumor microenvironment are some of the most common mechanisms through which multidrug resistance to chemotherapeutic agents is achieved in breast cancer. Chemotherapy is essential for general treatment and to prevent recurrence of breast cancer (Luque-Bolivar et al., 2020). In summary, chemotherapy is the primary form of systemic therapy with some potential efficacy against triple-negative breast cancers, so it is especially important in these patients. Chemotherapy also plays an important role as a complement to

endocrine therapy in order to prevent recurrence of hormone receptor positive breast cancers (Luque-Bolivar et al., 2020). Sadly, resistance mechanisms exist for many of the currently available anti-cancer agents used in breast cancer treatments. When patients exhibit multidrug resistance, their prognosis and clinical outcome is often poor since their tumors are unresponsive to many treatments. There is a strong need for novel agents in the treatment of breast cancer to improve prognosis and to achieve a better therapeutic response when patients are resistant to current chemotherapeutic options.

1.4 Cancer and the Endocannabinoid System

The endocannabinoid system is a complex network of cannabinoid receptors (CB1 and CB2), endocannabinoids, and the enzymes necessary for their biosynthesis and degradation of (Laezza et al., 2020; Moreno et al., 2019). The ECS and its individual components were initially explored during the characterization of the pharmacological targets of delta-9-tetrahydrocannabinol (THC) extracted from *Cannabis sativa* (Devane et al., 1992; Matsuda et al., 1990; Moreno et al., 2019). The CB1 and CB2 receptors belong to the G-protein-coupled receptor (GCPR) family and are some of the receptors known to faciliate the actions of cannabinoids. While CB1 and CB2 receptors are considered the main pathways through which cannabinoids exert their effects, additional GPCRs and several channels can faciliate actions of cannabinoids as well. Other GPCRs that cannabinoids can exert their actions through include GPR55, GPR18, GPR3, GPR6, GPR12, 5-HT, PPARα, and PPARγ (Laun et al., 2019; De Gregorio et al., 2019; O'Sullivan, 2016; Tomko et al., 2019). Cannabinoids have also been shown to affect transient receptor potential (TRP) channels such as TRPV1, TRPV2, TRPA1, and TRPM8 (Weber et al., 2016; de la Harpe et al., 2021; Watkins, 2019).

CB1 was the first discovered cannabinoid receptor and was deemed responsible for THC's psychotropic, intoxicating effects in the CNS (Matsuda et al., 1990; Fraguas-Sanchez et al., 2018). CB2 was later identified and is found primarily in non-CNS sites, particularly on immune system cells (Munro et al., 1993). Both receptors are also expressed in the peripheral nervous system and peripheral tissues (Mackie, 2005; Maccarrone et al., 2015; Moreno et al., 2019). The ECS initially attracted interest in the area of cancer due to its role in several biological processes such as appetite stimulation, energy balance, analgesia, and control of nausea and vomiting (Moreno et al., 2019; Khan et al., 2016). Cannabinoids are known to stimulate appetite in a CB1-dependent manner in the hypothalamus (Di Marzo & Matias, 2005). A number of studies have also documented cannabinoids' analgesic effects in various types of pain, including cancer pain (Hall et al., 2005). While it is likely that the majority of cannabinoid analgesic effects are mediated through CB1 in the CNS, there has also been evidence of CB2 and TRPV1 involvement (Akopian et al., 2009; Jhaveri et al., 2007). As highlighted previously, cancer patients undergoing treatment experience a multitude of adverse effects, such as weight loss, nausea and vomiting, and pain, making the ECS a fantastic target to help mediate these effects. Cannabinoids produced by the *Cannabis sativa* plant act on ECS (and other) receptors and have been used in cancer patients undergoing chemotherapy and in palliative care to provide analgesia, reduce nausea and vomiting, and promote food intake and weight gain (Guzman, 2003; Hall et al., 2005).

With the use of cannabis and cannabinoids in cancer patients under palliative care or to help mitigate the negative side effects and pain associated with anti-cancer therapy comes an important question to be addressed: how do cannabinoids affect cancer and its

treatment? The endocannabinoid system has been shown to be dysregulated in a variety of disease states, including cancer (Ramer et al., 2019). The types of dysregulation that have been observed include alterations in the concentrations of endocannabinoids and the up- or down-regulation of cannabinoid receptors (Schwarz et al., 2018). In one study, Perez-Gomez et al. (2015) found an association between high CB2 expression in HER-2 positive breast cancer and poor patient outcome. Other studies have also shown a link between the variation in cannabinoid receptor expression and patient outcomes in many different cancers (Schwarz et al., 2018). Correlation analyses have shown the relationship between high CB1 expression and poor patient prognosis in prostate, ovarian, pancreatic and colorectal cancer (Chung et al., 2009; Messalli et ak., 2014; Michalski et al., 2008; Jung et al., 2013). There also appears to be an association between high CB2 expression in head and neck squamous cell carcinoma and negative patient outcomes (Klien Nulent et al., 2013). Although there is a clear association between altered cannabinoid receptor expression and poor patient outcomes in multiple cancers, the underlying mechanisms that cause this are not well understood; the majority of research however does support that increased CB receptor expression in various cancers, including breast cancer, is associated with worse severity and outcome (Schwarz et al., 2018).

In addition to the mitigation of adverse effects caused by anti-cancer therapy and the palliative effects mediated by cannabinoids, the ECS may provide promising targets for anti-cancer treatment (Ramer et al., 2019). Over the last decade, the ECS has been explored for its role in both tumorigenesis and tumor suppression. The ECS is highly implicated in many essential processes in the body to help maintain homeostasis, which may explain its opposing roles in terms of tumorigenesis and tumor suppression (Moreno

et al., 2019). On one hand, endocannabinoids can cause changes in tissue homeostasis which results in a pro-tumorigenic environment and contributes to the subsequent development of cancer (Bissell & Hines, 2011; Kienzl et al., 2020). Cannabinoid receptor expression has also been related to pro-tumorigenic signaling. In a study that analyzed breast tumor samples, elevated CB2 expression activated HER-2 pro-oncogenic signaling (Perez-Gomez et al., 2015). In contrast to the pro-tumorigenic effects of ECS components, a multitude of studies have identified that the ECS, including its enzymes and receptors, plays an inhibitory role in tumor progression in terms of regulation of tumor growth, tumor vascularization (angiogenesis), apoptosis, and metastasis (Guindon & Hohmann, 2011).

An early study by De Petrocellis and colleagues (1998) demonstrated the anti-proliferative action of AEA through the CB1 receptor on nerve growth-factor challenged breast cancer cells. Later, AEA was shown to cause cell cycle arrest of breast cancer cells by preventing S phase exit through the loss of Cdk2 activity, up-regulating $p21^{waf}$, and reducing the formation of the active complex cyclin E-Cdk2 kinase (Laezza et al., 2006). CB2 agonists also blocked the cell cycle in breast cancer cells at the G2/M phase by downregulating Cdc2 (Caffarel et al., 2006). In glioma, CB2 agonists were able to cause cell death via the induction of apoptosis and autophagy (Salazar et al., 2009). In glioma and melanoma, the synthetic cannabinoid receptor agonist WIN-55,212,2 inhibited vascular endothelial growth factor and HIF-1, it's main transcription factor (Blazquez et al., 2003; Blazquez et al., 2004; Casanova et al., 2003). Interestingly, WIN-55,212,2 stimulation of CB receptors in non-transformed epidermal cells did not affect the viability, while in tumorigenic epidermal cells CB receptor stimulation induced cell death

via apoptosis (Casanova et al., 2003). In cervical and lung cancer, AEA has been shown to reduce metastasis by increasing the expression of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) (Ramer et al., 2008). The epithelial-mesenchymal transition (EMT) is a process that occurs during the progression of cancer cells to an invasive phenotype (Pisanti et al., 2013). Anandamide was shown to impede EMT of breast cancer cells in a CB1-dependent manner by inhibiting β-catenin in nuclear and cytoplasmic fractions (Laezza et al., 2012). The anti-cancer functions mediated by ECS components have made it an attractive target in the treatment of many cancers, including breast cancer. In addition to the anti-cancer effects of endo- and synthetic cannabinoids previously highlighted, phytocannabinoids that target ECS receptors as well as other GPCRs and channels have also been explored for their anti-cancer potential (Ramer et al., 2019). Due to the evidence that various endo- and synthetic cannabinoids provide anti-cancer effects and may preferentially target cancer cells over healthy cells, phytocannabinoids could be a promising addition to current cancer therapies.

1.5 Phytocannabinoids

Cannabis sativa L. is one of the oldest plants cultivated, dating back several thousand years (Ren et al., 2019). Today, it is one of the most widely used recreational psychoactive drugs and it is very commonly used among people experiencing various types of pain as well (Ren et al., 2019). Included in the types of pain managed by cannabis and cannabinoids is cancer pain (Noyes et al., 1975; Hall et al., 2005; Johnson et al., 2010). The cannabis plant produces hundreds of different compounds during its life cycle, many of which are phytocannabinoids (Figure 1.4) which are largely responsible

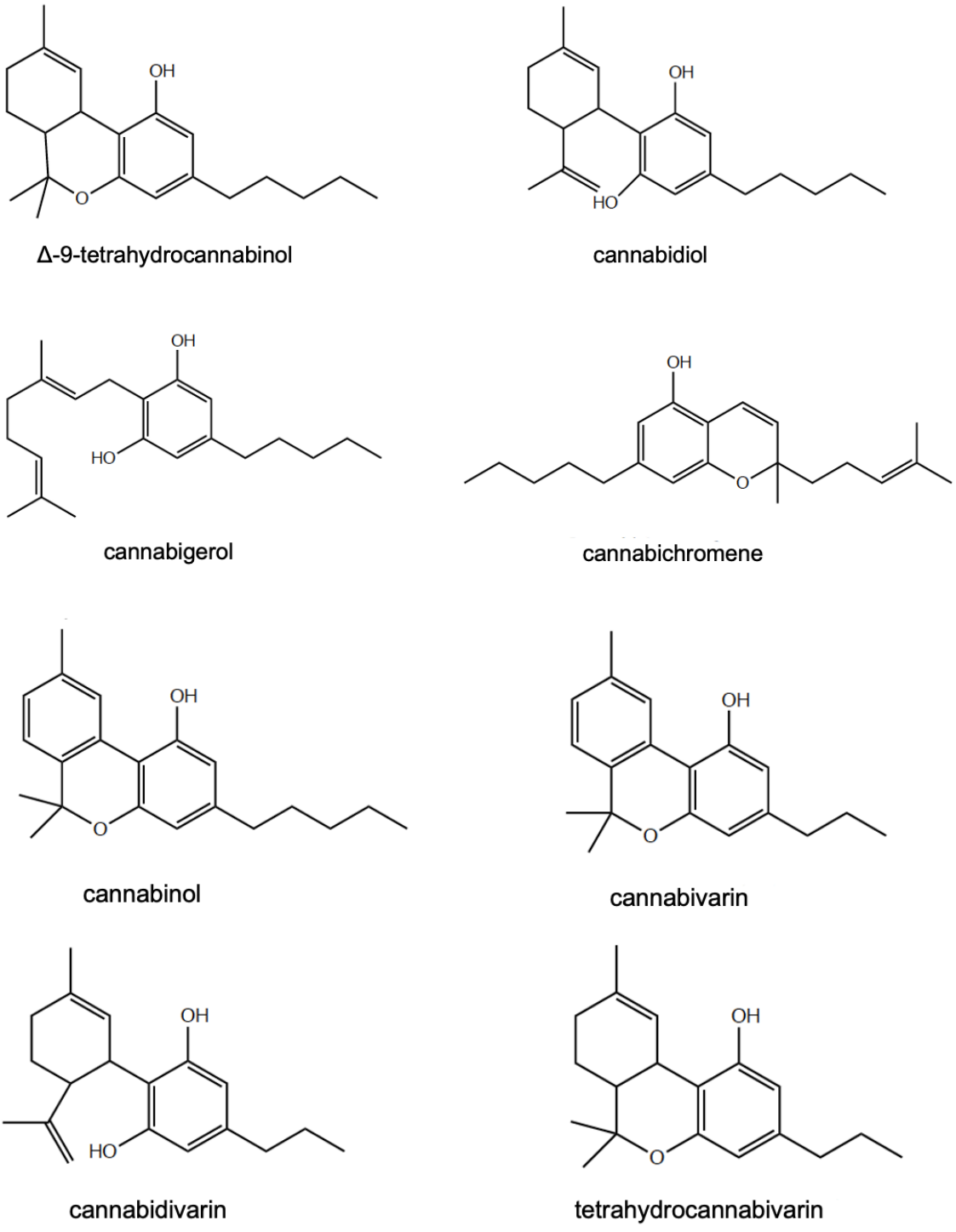


Figure 1.4. Phytocannabinoids and their structures. Delta-9-tetrahydrocannabinol and cannabidiol are major cannabinoids. Cannabichromene, cannabigerol, cannabivarin, cannabidivarin, cannabinol, and tetrahydrocannabivarin are minor cannabinoids.

for mediating various effects experienced by users. Cannabinoids can also be found in plants other than cannabis, such as *Acmella oleracea* (Woelkart et al., 2008). Cannabinoids are synthesized in glandular trichomes of female cannabis plant flowers, and very low levels are synthesized in male plants since they are trichome-poor. The biosynthesis of all cannabinoids shares an initial step where tetraketide synthase (TKS) catalyzes the production of 3,5,7-trioxododecaneoyl-coA (Tahir et al., 2021). This then undergoes cyclization and aromatization to yield olivetolic acid (OLA). Aromatic prenyltransferase then inserts a prenyl group to OLA to produce cannabigerolic acid (CBGA) (Figure 1.5). Other cannabinolic acids (eg. Δ-9-tetrahydrocannabinolic acid) are then derived from CBGA and further proceed to the finalized cannabinoid form by non-enzymatic decarboxylation of their acidic precursor (Tahir et al., 2021; Figure 1.5). Cannabigerolic acid is sometimes referred to as the mother of all phytocannabinoids because it is the precursor to all other cannabinoids produced by the cannabis plant (Tahir et al., 2021).

1.5.1 *Δ-9-tetrahydrocannabinol and Cannabidiol*

Delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are considered the two major phytocannabinoids produced by the cannabis plant. Not all cannabinoids are found in the same quantities in every cannabis cultivar, and the most abundant cannabinoids (THC and CBD) are considered major cannabinoids while the other cannabinoids found in less abundance are minor cannabinoids (Marcu, 2016). THC was isolated from cannabis in 1964 by Gaoni and Mechoulam. THC is typically one of the most abundant cannabinoids found in cannabis and is the major psychoactive component of cannabis cultivars where it mediates its effects in the central nervous system primarily

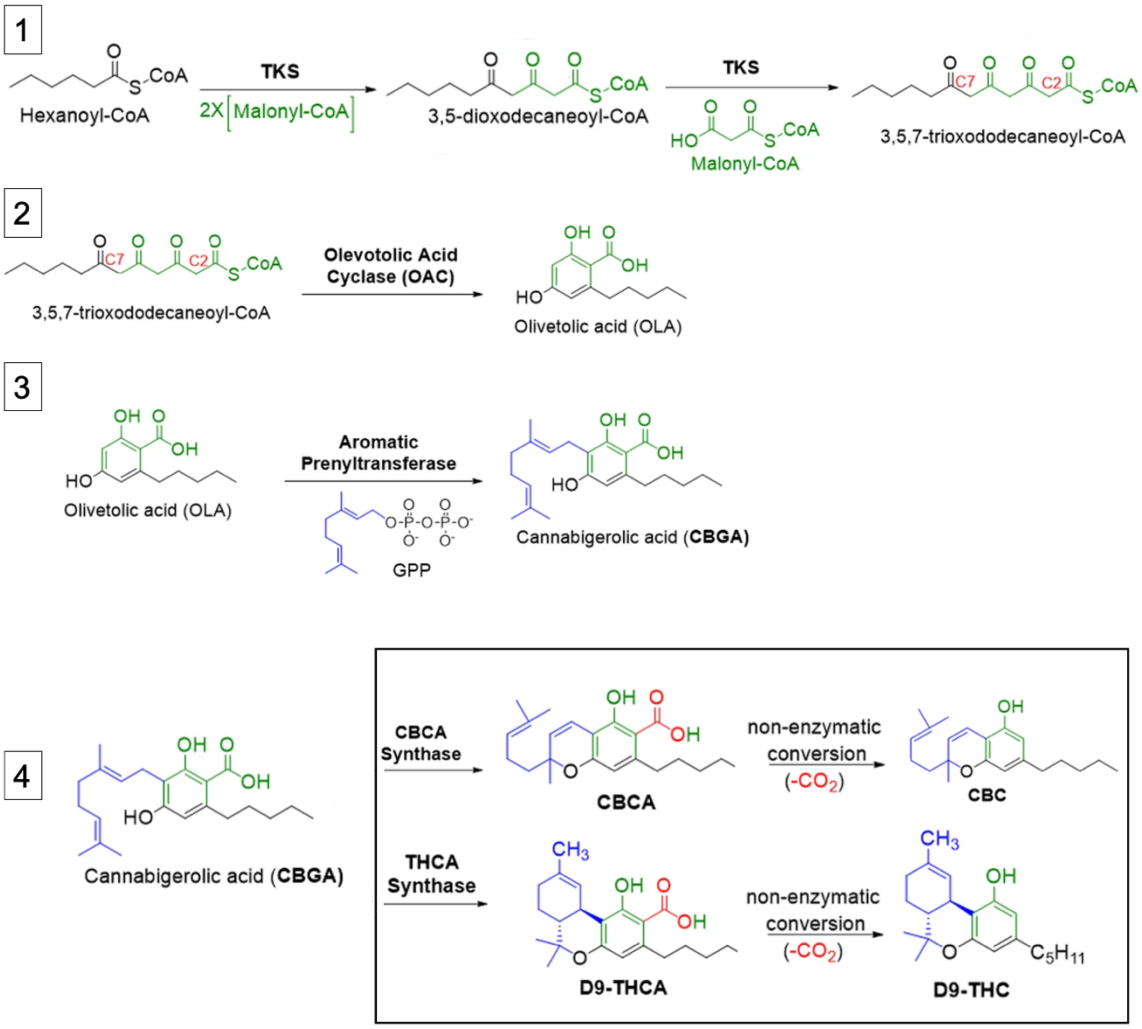


Figure 1.5. Biosynthesis of phytocannabinoids. (1) Tetraketide synthase (TKS) catalyzes the production of 3,5,7-trioxododecaneoyl-coA, (2) olivetolic acid cyclase (OAC) facilitates the production of olivetolic acid (OLA) from 3,5,7-trioxododecaneoyl-coA, (3) a prenyl group is added to OLA to form cannabigerolic acid (CBGA), and (4) CBGA is converted to other cannabinolic acids (eg. CBCA, THCA) which are converted non-enzymatically to final cannabinoid form. Adapted with permission from Springer Nature: Journal of Cannabis Research (Tahir et al. 2021), Copyright © 2021, The Authors: https://s100.copyright.com/AppDispatchServlet?title=The%20biosynthesis%20of%20the%20cannabinoids&author=M.%20Nazir%20Tahir%20et%20al&contentID=10.1186%2Fs42238-021-00062-4&copyright=The%20Author%28s%29&publication=2522-5782&publicationDate=2021-03-15&publisherName=SpringerNature&orderBeanReset=true&oa=CC%20BY.

via CB1 receptors (Bloomfield et al., 2016). When THC binds CB1 receptors in the CNS it results in the intoxicating effects described by cannabis users. THC has also been shown to allosterically modulate opioid receptors in the CNS (Chartoff & Connery, 2014). Common routes of administration for THC include inhalation, intravenously, intramuscularly, and orally. There are concerns with the use of THC medicinally due to its psychoactive effects in the CNS, as well as the potential for dependence, tolerance, and issues regarding abuse; it is however likely that the potential anti-cancer benefits outweigh those risks with THC use (Afrin et al., 2020; Bridgeman & Abazia, 2017). Cannabidiol is another major cannabinoid produced by the cannabis plant that was isolated shortly before THC (Mechoulam & Shvo, 1963). Cannabidiol has been shown to interact with a large array of targets within the endocannabinoid system and in medical settings it is most commonly prepared as an oil (Tomko et al., 2020). Unlike THC, CBD is non-intoxicating as it does not bind CB1 receptors in the CNS, therefore its use in a medical context is more appealing due to lack of intoxicating effects.

THC and CBD are by far the most extensively studied cannabinoids in the area of cancer research. As mentioned previously, cannabis and cannabinoids (THC and CBD) were first used in the field of cancer to provide some relief from the adverse effects and pain associated with anti-cancer treatment, and in cancer patients under palliative care (Guzman, 2003; Hall et al., 2005; Noyes et al., 1975; Johnson et al., 2010). The use of cannabis and cannabinoids in cancer patients lead to questions surrounding the pro- and anti-cancer effects of cannabis/cannabinoids. The anti-cancer properties of cannabinoids have been studied in a wide variety of cancers, and THC and CBD are the most well-characterized in terms of their anti-cancer potential. Both THC and CBD have been

shown to inhibit cell growth and proliferation, induce apoptosis and autophagy, and reduce migration/invasion of cancer cells, among other effects (reviewed by Tomko et al., 2020). *In vitro,* THC and CBD have been shown to induce cancer cell death via apoptosis and autophagy, inhibit migration and invasion, and sensitize cells to chemotherapeutic agents (Caffarel et al., 2006; Ligresti et al., 2006; McAllister et al., 2007; Scott et al., 2017;Garcia-Morales et al., 2020). *In vivo* models of breast cancer support the anti-cancer potential of CBD and THC as well, where they were able to reduce tumor growth, metastasis, and increase survival (Caffarel et al., 2010; Murase et al., 2014; Elbaz et al., 2015; Blasco-Benito et al., 2018). To date, there have been no clinical trials to directly examine the anti-cancer potential of CBD or THC in breast cancer patients, however, there are promising results in other cancers. In glioblastoma multiforme (GBM) patients, a two-part clinical study showed that Sativex (1:1 THC:CBD) and temozolomide (TMZ) treatment together increased the rate of 1-year survival by 39 percent (NCT01812603; NCT01812616). It was also recently shown that CBD may prolong the survival of patients suffering with GBM (Likar et al., 2021).

1.5.2 Minor Phytocannabinoids

The cannabis plant produces many more cannabinoids other than THC and CBD, including cannabigerol (CBG), cannabichromene (CBC), cannabinol (CBN), cannabivarin (CBV), cannabidivarin (CBDV), and tetrahydrocannabivarin (THCV) (Figure 1.4). These cannabinoids are considered minor phytocannabinoids since they are produced in less abundance than CBD and THC in cannabis plants. In addition, they have been much less characterized in terms of their anti-cancer potential relative to THC and CBD. Cannabigerol is derived from its acidic acid precursor, cannabigerolic acid, and it

has attracted interest in the area of cancer research recently due to its lack of intoxicating effects. More cannabis varieties are being developed with higher CBG and CBGA content than what is normally present (Navarro et al., 2018). Only a few studies have looked at the anti-cancer potential of CBG. Two early studies by Baek et al. (1996, 1998) found that CBG exhibited therapeutic benefits against mouse skin melanoma cells and oral epithelioid carcinoma cells. Other studies have also demonstrated CBG's anti-cancer effects in cancer cells, including breast and colorectal cancer cells (Ligresti et al., 2006; Schoeman et al., 2020). Cannabichromene is another minor phytocannabinoid with much left to be discovered regarding its pharmacology and anti-cancer abilities. CBC is appealing in terms of human health and medicine because, like CBD, it lacks intoxicating effects (Izzo et al., 2012). In a few small studies, CBC has been shown to exert anti-cancer effects including reduced cancer cell proliferation, cytotoxicity, and induction of apoptosis (Ligresti et al., 2006; De Petrocellis et al., 2013; Borrelli et al., 2014; Anis et al., 2021).

Cannabinol (CBN), cannabivarin (CBV), cannabidivarin (CBDV) and tetrahydrocannabivarin (THCV) are minor phytocannabinoids with a paucity of research relative to some of the other cannabinoids produced by the cannabis plant. Cannabinol was the first isolated cannabinoid from the cannabis plant, however it remains one of the most poorly studied (Wood, 1899). It is found often in aged cannabis and results from the degradation of tetrahydrocannabinolic acid (Huestis, 2005). Like THC, CBN possesses psychoactive effects by acting on CB1 receptors in the CNS, however the effects are estimated to be 10-fold lower than those produced by THC (Heustis, 2005). Some studies have shown anti-cancer effects of CBN in prostate and breast cancer cells, including

cytotoxicity and inhibition of multidrug transporters (De Petrocellis et al., 2013; McAllister et al., 2007; Holland et al., 2007). Cannabivarin, also referred to as cannabivarol, is an analog of cannabinol with a shortened side chain (ChemEurope, 2021). It is found in small amounts in some aged cannabis cultivars and is rarely found in fresh cannabis. To date, there is no published literature regarding the anti-cancer effects of CBV.

Cannabidivarin is very similar in structure to cannabidiol, but with a shorter side chain. Little has been discovered regarding the pharmacological or anti-cancer effects of CBDV, however two studies found that it had cytotoxic properties against human prostate and colon cancer cells (De Petrocellis et al., 2013; Borelli et al., 2014). Finally, tetrahydrocannabivarin is a homologue of THC with differing side chains that attribute its differing effects from that of THC. THCV is only found in trace amounts in most cannabis cultivars, however some *Cannabis sativa* strains with hybridized genetics may contain higher levels (ChemEurope, 2021). Like other minor phytocannabinoids, very little is known about THCV and its potential as an anti-cancer agent. One study did show that THCV had cytotoxic effects in prostate cancer cell lines (De Petrocellis et al., 2013), however no studies with THCV in the area of breast cancer have been published to date.

One of the key considerations to anti-cancer therapy is how they affect healthy, non-cancerous cells. An unfortunate side effect of the currently available chemotherapeutic agents and that they lack specificity and pose harm to normal cells, resulting in adverse effects. One of the potential advantages to cannabinoid use as an anti-cancer agent is that they do not appear to affect healthy cells and it has been suggested that they may preferentially target cancerous cells (Schoeman et al., 2020;

Chakravarti et al., 2014; Tomko et al., 2019). A few studies have shown that select cannabinoids and cannabinoid combinations only kill cancer cells and do not affect the viability of their healthy counterpart (Schoeman et al., 2020; Chakravarti et al., 2014; Tomko et al., 2019; University of Newcastle, 2020). Another study that used a synthetic cannabinoid, WIN-55,212,2, found that it was able to induce apoptosis of cancerous epidermal cells while leaving non-cancerous epidermal cells unaffected (Casanova et al., 2003).

1.6 Cannabinoid Synergy

In the literature, cannabinoids have been predominantly studied as individual extracts to examine their anti-cancer abilities. In addition to this, THC and CBD have been much more extensively studied in comparison to the other minor phytocannabinoids. While it is important and necessary to characterize the pharmacological and potential anti-cancer effects of individual cannabinoids, the cannabis plant produces a large array of compounds that may act together or with other agents to produce different effects. For the purposes of this thesis, cannabinoid synergy refers to the ability of combined cannabinoids or cannabinoids with other compounds (eg. terpenes, flavonoids, anti-cancer agents) to produce enhanced or synergistic effects relative to effects of pure compounds alone. The idea of synergy between cannabinoids in cannabis was first described by Mechoulam (1999), where he suggested that whole plants may provide superior effects than individual compounds isolated from them. In recent years, there has been a push for medical cannabis use because the combination of THC and CBD (and potentially other cannabinoids) has been shown to elicit enhanced therapeutic effects compared to THC alone (Sanchez-Ramos, 2015).

An interesting study by Blasco-Benito et al. (2018) investigated the potential synergistic anti-cancer effects of cannabinoids in preclinical models of breast cancer. They compared the anti-cancer effects of pure THC with those of a botanical drug preparation derived from fresh cannabis flowers. The botanical drug preparation was characterized for its cannabinoid and terpene content and contained a mixture of some cannabinoids and terpenes. They found that in both *in vitro* and *in vivo* (murine) models of breast cancer, the botanical drug preparation elicited more potent anti-cancer effects than pure THC alone. Interestingly, it was determined that the enhanced potency of the botanical preparation was not due to the most abundant terpenes present. They also highlighted that the anti-cancer actions of pure THC and the botanical extract were achieved through different targets and mechanisms of action (Blasco-Benito et al., 2018).

Combinations of cannabinoids have been explored in other cancers as well. In human glioblastoma cells, the addition of CBD to THC treatment produced synergistic anti-cancer effects compared to those observed with THC alone (Marcu et al., 2010). In a randomized controlled trial, Sativex (1:1 THC:CBD) and a THC extract were compared for their analgesic effects in patients with cancer pain that was unresponsive to opiates. Patients treated with Sativex experienced significant improvements in analgesia compared to placebo, while those who received the THC extract did not (Johnson et al., 2010). They suggested that the CBD in Sativex was necessary for significant pain relief. While this study did not examine anti-cancer effects of cannabinoids, these results do support that cannabinoid combinations may provide superior effects. These studies highlight the potential for enhanced therapeutic effects when cannabis compounds are combined rather than used as single isolated agents.

There has been some evidence that the co-treatment of cancer cells with cannabinoids and established anti-cancer agents sensitizes cancer cells to the anti-cancer drug. In leukaemia, THC was able to sensitize the cells to anti-cancer agents, and reduced PGP transport and expression (Holland et al., 2006; Liu et al., 2008; Scott et al., 2017). Similar findings were observed with CBD, where it was able to reduce PGP expression and sensitize MDR leukaemia cells to vinblastine (Holland et al., 2006). More recently in breast cancer, CBD treatment was able to increase the sensitivity of cells to anti-cancer agents cisplatin and doxorubicin (Garcia-Morales et al., 2020). In addition to sensitizing cells to anti-cancer agents, synergistic anti-cancer effects between cannabinoids and chemotherapeutic agents have been observed. It was recently shown in breast cancer that the combination of CBD with paclitaxel or doxorubicin produced synergistic anti-proliferative effects *in vitro* (Fraguas-Sanchez et al., 2020).

1.7 Rationale, Objectives, & Hypothesis

Although there have been significant advancements made throughout the years to improve breast cancer treatment and outcomes, there remains prominent issues that need to be overcome. There is a clear need for the exploration of novel breast cancer treatments, as there continue to be patients whose cancer does not respond to currently available treatment options. Of most concern is breast cancer patients where the main option for treatment is chemotherapy–as in the case of triple-negative breast cancers–yet the tumor is not overly responsive or completely unresponsive due to chemotherapeutic resistance. In addition to the issue of tumor responsiveness to treatment, chemotherapy often results in unfortunate adverse effects, which only increases the burden faced by cancer patients. These adverse effects are the result of systemically delivered

chemotherapy treatments that have off-target effects on healthy cells throughout the body. New anti-cancer agents are therefore warranted in the treatment of cancer patients to help limit or reduce chemotherapy-induced negative side effects, improve therapeutic anti-cancer benefit, and improve the quality of life in people suffering with breast cancer.

The ECS could be a promising target for new anti-cancer agents that would provide improved drug targeting and potentially fewer negative side effects. Unfortunately, when one anti-cancer drug fails, other drugs of the same class with the same target also often fail, which can limit effective treatment options available to patients. In addition to this, when multidrug resistance occurs, drugs of differing classes with different targets can be rendered ineffective due to efflux, which even further restricts treatment options. Given the abundance of literature that has shown the anti-cancer abilities of Δ-9-tetrahydrocannabinol and cannabidiol and limited evidence for the anti-cancer potential of minor phytocannabinoids, it is reasonable to explore these cannabinoids in the context of breast cancer, including MDR forms. THC and CBD have also been shown in a few studies to synergize with and sensitize cells to anti-tumor effects of currently available chemotherapeutic agents (Holland et al., 2006; Lopez-Valero et al., 2018; Fraguas-Sanchez et al., 2020). The prospective combination of cannabinoids with chemotherapeutics could increase the overall anti-cancer benefits through synergistic effects; it may also allow for a reduction in dosing of chemotherapy drugs. This would help to reduce negative side effects experienced by patients, as higher doses are typically associated with more adverse effects (Pearce et al., 2017).

The overarching objective of this study was to characterize the anti-cancer potential of phytocannabinoids produced by the cannabis plant in *in vitro* models of breast cancer. Our main objective was broken down into 3 smaller objectives as follows:

(1) Determine the anti-cancer effects of individual cannabinoids in terms of reduction in cell viability, induction of apoptosis, and inhibition of invasion.

(2) Determine the interaction (additive, antagonistic, synergistic) between cannabinoids and chemotherapeutic agents paclitaxel or doxorubicin.

(3) Determine the interaction between different cannabinoids.

It was hypothesized that phytocannabinoids would exhibit anti-proliferative and anti-invasive properties in breast cancer cells, and that the combination of cannabinoids with chemotherapeutic agents would produce synergistic reductions in cell viability. To test our hypothesis, four breast cancer cell lines were used throughout our study: two sensitive to chemotherapy and two chemotherapy-resistant forms. We performed cell viability, apoptosis and invasion assays to explore the anti-cancer abilities of cannabinoids and their synergistic potential in breast cancer. This project provides insight as to which cannabinoids and combinations may warrant further investigation in future studies.

Chapter 2: Materials and Methods

2.1 Cell Lines

Paclitaxel-resistant MDA-MB-231 cells were derived from drug sensitive MDA-MB-231 cells and were provided by Drs. Kerry Goralski, David Hoskin and Anna Greenshields (Dalhousie University). Paclitaxel-resistant MCF-7 cells were generated by serial passage of MCF-7 cells with increasing concentrations of paclitaxel and were generously provided by Drs. Robert Robey and Susan Bates (National Cancer Institute, Bethesda, MD). MCF-10A cells and respective medium were kindly provided by the lab of Dr. Yassine El Hiani (Dalhousie University). All cells were used up to 35 passages.

2.2 Anti-Cancer Agents and Cannabinoids

Paclitaxel and doxorubicin hydrochloride were obtained from Millipore-Sigma. Cannabinoids used were from a mixture of Cayman Chemical and Toronto Research Chemicals. Paclitaxel and doxorubicin were dissolved in DMSO. Cannabinoids were dissolved in methanol.

2.3 Cell Culture

Human breast adenocarcinoma MDA-MB-231 and MCF-7 cell lines were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) (Millipore-Sigma) with 10% fetal bovine serum (FBS) (Gibco, Life Technologies) and 1% penicillin-streptomycin. Paclitaxel-resistant MDA-MB-231 and MCF-7 cells were cultured under the same conditions with the addition of paclitaxel at a concentration of 470 nM. Mammary epithelial MCF-10A cells were cultured under the same atmospheric conditions in DMEM/Ham's Nutrient Mixture F-12 (Gibco, Life Technologies) with 5% horse serum, 1 ng/mL cholera toxin, 10 µg/mL human insulin, 10 ng/mL epidermal growth factor and 0.5 µg/mL hydrocortisone.

2.4 Cytotoxicity Assays

Cells were seeded at 10,000 per well in black 96-well plates (ThermoFisher Scientific) in DMEM with 10% FBS or DMEM/F-12 complete medium and allowed to grow over night prior to the addition of drug treatments. Cells were treated in DMEM containing 1% FBS or DMEM/F-12 complete medium with the following cannabinoids: cannabidiol, Δ-9-tetrahydrocannabinol, cannabigerol, cannabichromene, cannabivarin, cannabinol and tetrahydrocannabivarol. Cannabinoid concentrations were gradually increased to generate cell viability curves. Following 48-hour treatments, AlamarBlue® (Bio-Rad Laboratories) was added to total 10% of the well volume, and plates were incubated for 3-4 hours at 37°C. AlamarBlue® is an oxidation-reduction indicator that changes color upon reduction by cellular metabolism; the reduced form is highly fluorescent. Fluorescence intensity was measured with a BioTek Cytation 3 at 560 nm excitation and 590 nm emission as a measurement of cell viability. Cell viability data are expressed as the percentage ± SEM of viable cells compared to vehicle-treated control cells, normalized at 100%. Data shown was obtained from at least 3 independent experiments performed in quintuplicate.

2.5 Apoptosis Assay

MDA-MB-231 cells were grown for 24 hours in clear 96-well plates (ThermoFisher Scientific) at 3,000 cells/well in DMEM with 10% FBS. Wells were then treated with vehicle (methanol) or cannabinoids at a concentration of 2.5 μM in DMEM with 1% FBS for 24 hours. An annexin V apoptosis detection kit (Santa Cruz Biotechnology) was used to examine apoptosis. Treated cells were harvested, washed with PBS and resuspended in annexin V assay buffer. In the dark, cells were shaken

gently with propidium iodide (PI) and annexin V–fluorescein isothiocyanate–conjugated stain for a total of 20 minutes. The cells were viewed by fluorescence microscopy using an Olympus IX81 microscope with a Photometrics coolSNAP HQ2 camera and an Excite series 120Q light source. The annexin V stain was excited at 488 nm while images were taken at 525 nm. Propidium iodide was excited at 535 nm and images were taken at 617 nm. Rates of apoptosis and late apoptosis or necrosis were calculated by dividing the number of cells that stained positive for annexin V or PI by the total number of cells, respectively.

2.6 Transwell Migration

Paclitaxel-resistant MDA-MB-231 cells were trypsinized and prepared as a suspension in DMEM with no FBS containing 100,000 cells/mL. Migration chambers sat in a single well of a 24-well plate. Two hundred fifty µL of the cell suspension (2.5×10^4 cells) was added to the top portion of a Transwell migration chamber. The Transwell migration chamber had a polycarbonate membrane with a pore size of 8.0 µm (Falcon). In the well where the migration chamber rested, 700 µL of DMEM containing 10% FBS was added to activate cell migration toward the chemo-attractant (FBS). The cells were incubated in the migration chambers at 37°C for 24 hours in the presence of vehicle (methanol). Following the incubation period, the migration chamber polycarbonate membrane was rinsed with PBS and cells that did not migrate were removed with a cotton swab. Cells were fixed by immersing the migration chamber in cold methanol for 10 minutes at room temperature, followed by a 10-minute immersion in a 0.5% crystal violet solution for cell staining. The membranes were rinsed with dH_2O until the water ran clear and were left upside-down to dry for at least 24 hours. The membranes were

mounted on slides and at least 3 fields were viewed and counted with an Olympus CKX41 light microscope. The total number vehicle-treated cells that migrated served as 100% for invasion assay calculations.

2.7 Invasion Assay

Matrigel invasion chambers sat in a single well of a 24-well plate. Invasion chambers contained growth factor reduced 8.0 μm Matrigel (Corning). Invasion chambers were hydrated for 1 hour at 37°C with 250 μL of cold DMEM containing 0.2% FBS and 5 μM of cannabinoid (2X the desired final concentration). Paclitaxel-resistant MDA-MB-231 cells were trypsinized and prepared as a suspension in DMEM with no FBS containing 100,000 cells/mL. Following hydration, 250 μL of the cell suspension (2.5×10^4 cells) was added to the invasion chamber resulting in a final cannabinoid concentration of 2.5 μM. Seven hundred μL of DMEM containing 10% FBS was added to the well where the invasion chamber rested to activate invasion of cells through the Matrigel. Cells were incubated for 24 hours at 37°C. Chambers were rinsed with PBS and cells that did not invade were gently removed with a cotton swab. To fix cells, chambers were placed in cold methanol for 10 minutes, followed by a 10-minute stain in a 0.5% crystal violet solution. Chambers were rinsed thoroughly with dH_2O and allowed to dry upside down for 24 hours. Cells that invaded through the Matrigel were counted using an Olympus CKX41 light microscope. Data are expressed as percent invasion ± SEM, calculated by dividing the number of cells invaded under each condition by the number of cells that migrated under vehicle control conditions. Data was obtained from at least 3 independent experiments.

2.8 Investigation of Synergism, Additivity & Antagonism

Synergy was assessed between doxorubicin and cannabidiol, Δ-9-tetrahydrocannabinol, cannabichromene and cannabivarin. Synergy between cannabidiol or Δ-9-tetrahydrocannabinol with other cannabinoids was also assessed. The checkerboard or matrix assay was used to comprehensively assess interactions between drugs at multiple concentrations. Matrix assays were set up in 96-well plates with Drug A along the Y-axis and Drug B along the X-axis (Figure 2.1).

		Drug B			
	0μM A, 0μM B	**1μM B**	**2μM B**	**5μM B**	**10μM B**
Drug A	**1μM A**	1μM A, 1μM B	1μM A, 2μM B	1μM A, 5μM B	1μM A, 10μM B
	2μM A	2μM A, 1μM B	2μM A, 2μM B	2μM A, 5μM B	2μM A, 10μM B
	5μM A	5μM A, 1μM B	5μM A, 2μM B	5μM A, 5μM B	5μM A, 10μM B
	10μM A	10μM A, 1μM B	10μM A, 2μM B	10μM A, 5μM B	10μM A, 10μM B

Figure 2.1. Sample matrix assay set up. Drug A and B alone along X- and Y-axes. Corresponding combination of drug A + drug B present in inside wells.

Cells were plated at 10,000 cells/well in 10% FBS DMEM and allowed to incubate over night before treatment. Individual drugs were prepared at 2X the desired final concentration in DMEM containing 1% FBS. To achieve the drug combinations, 50 μL of Drug A and 50 μL of Drug B were added to the well to dilute each drug to the desired 1X concentration. Cannabinoids generally ran from 0–10 μM or 0–25 μM along the Y-axis and doxorubicin had a more varying concentration range along the X-axis depending on the cell line. For cannabinoid combinations, cannabinoids ran from 0–10 μM along the Y-axis and 0–20 μM along the X-axis. Following a 48-hour treatment, fluorescence was measured using an AlamarBlue® assay to assess cell viability, as

described previously. Cell viability in each well are expressed as the percent relative to un-treated cells, normalized as 100%. To assess potential synergy between drug combinations, SynergyFinder 2.0: visual analytics of multidrug combination synergies (SynergyFinder, 2021) was employed using the Bliss Independence drug interaction model. For each drug combination examined, 3 independent trials were used for analysis. The following parameters were selected in SynergyFinder for data analysis: readout viability, detect outliers on, curve fitting LL4, and visualize dose-response data and calculate synergy toggles were on. Three-dimensional mountain plots provided visual representations of drug combinations which had maximal or minimal effects on cell viability. For each drug combination tested in the matrix assay, SynergyFinder 2.0 provided a synergy score defining the excess response due to synergy. Scores below -10 were considered as antagonistic, between -10 and 10 additive, and greater than 10 synergistic (SynergyFinder, 2021).

2.9 Statistical Analysis & Curve Fitting

GraphPad Prism software was used to complete statistical analysis. Error bars shown are representative of the mean ± SEM. Unpaired student's T-tests were done to assess significance of two independent groups. One-way analysis of variance (ANOVA) with Tukey's post-hoc tests were performed to assess multi-group comparisons. p values are expressed as the following: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Cell viability curves were fitted using GraphPad Prism's non-linear regression function: [inhibitor] vs. normalized response (variable slope). To compare two cell viability curves and their corresponding IC_{50} values, the extra-sum-of-squares F test was used to determine if IC_{50} differences were statistically significant.

Chapter 3: Results

Four cell lines were used throughout this project, two chemotherapy (chemo)-sensitive and two paclitaxel resistant (PR). Chemo-sensitive MDA-MB-231 and MCF-7 cells are simply referred to as MDA-MB-231 or MCF-7 throughout the text, whereas paclitaxel-resistant cells are referred to as PR MDA-MB-231 or PR MCF-7. MDA-MB-231 cells are epithelial human breast adenocarcinoma cells that are triple-negative for the progesterone, estrogen and human epidermal growth factor 2 (HER-2) receptors. MCF-7 cells are another human-derived breast cancer cell line that are positive for the progesterone and estrogen receptors and negative for the HER-2 receptor. These cell lines were selected due to their differing receptor profile to investigate cannabinoid effects in two different forms of breast cancer with different immunophenotypes. First, cell viability assays were performed with THC, CBD, CBC, CBV, CBN, CBG and THCV individually to determine which cannabinoids exert inhibitory effects on cell growth and at what concentration range. The same cannabinoids were then further explored for their ability to induce apoptosis and reduce the invasion of PR MDA-MB-231 cells. Following the characterization of the anti-cancer effects produced by individual cannabinoids, we looked at the interactions between different cannabinoids, as well as between cannabinoids and chemotherapeutic agents to explore the synergistic potential of combination treatments.

3.1 Cannabinoids exerted dose-dependent cytotoxicity in MDA-MB-231 cell lines

The first part of this study was to examine the effects of individual cannabinoids on breast cancer cells. The effects of seven cannabinoids on cell viability were determined in MDA-MB-231 and PR MDA-MB-231 cells. Cells were treated with

varying concentrations of cannabinoids for 48 hours and cell viability was measured by fluorescence using an AlamarBlue assay. In both MDA-MB-231 and PR MDA-MB-231 cells, CBD, THC, CBC, CBV, CBN and THCV produced dose-dependent reductions in cell viability and IC_{50} values were calculated for each curve using GraphPad Prism's non-linear regression function (Figure 3.1). The IC_{50}s for cannabinoids in MDA-MB-231 cells were: 3.5 μM (CBD), 8.1 μM (THC), 3.1 μM (CBV), 6.3 μM (CBC), 3.7 μM (CBN), and 12.5 μM (THCV). In PR MDA-MB-231 cells, cannabinoid IC_{50}s were: 5.7 μM (CBD), 9.9 μM (THC), 4.5 μM (CBV), 6.2 μM (CBC), 5.7 μM (CBN), and 16.5 μM (THCV). For some cannabinoids–THC (Figure 3.1 B) and CBC (Figure 3.1 D)–dose-response curves had significant overlap between cell lines and IC_{50} values were very similar. On the other hand, the IC_{50} values for CBD, CBV, CBN and THCV were consistently higher in PR-MDA-MB-231 cells. CBG did not exert significant effects on cell viability in either cell line at the concentrations examined and IC_{50} values could not be calculated (Figure 3.1 G).

3.2 Cannabinoids exerted dose-dependent cytotoxicity in MCF-7 cell lines

The effects of the same seven cannabinoids previously described were assessed in MCF-7 and PR MCF-7 cells. Cells were treated with varying concentrations of cannabinoids for 48 hours and cell viability was quantified by fluorescence with an AlamarBlue assay. In both MCF-7 and PR MCF-7 cells, CBD, THC, CBC, CBV, CBN and THCV exerted dose-dependent cytotoxic effects and IC_{50} values were calculated (Figure 3.2). Most cannabinoids had higher IC_{50} values in PR MCF-7 cells, with the exception of CBD (Figure 3.2 A) and THC (Figure 3.2 B) where nearly identical IC_{50}s were observed between cell lines. IC_{50}s for cannabinoids in MCF-7 cells were as follows:

4.5 μM (CBD), 8.7 μM (THC), 4.0 μM (CBV), 6.1 μM (CBC), 5.7 μM (CBN), and 10.3 μM (THCV). In PR MCF-7 cells, IC_{50} values were: 4.3 μM (CBD), 8.9 μM (THC), 6.7 μM (CBV), 8.9 μM (CBC), 6.6 μM (CBN), and 22.5 μM (THCV). Consistent with what we found in MDA-MB-231 and PR MDA-MB-231 cells, CBG did not reduce cell viability in either MCF-7 cell line as strongly as other cannabinoids, and IC_{50}s could not be calculated (Figure 3.2 G).

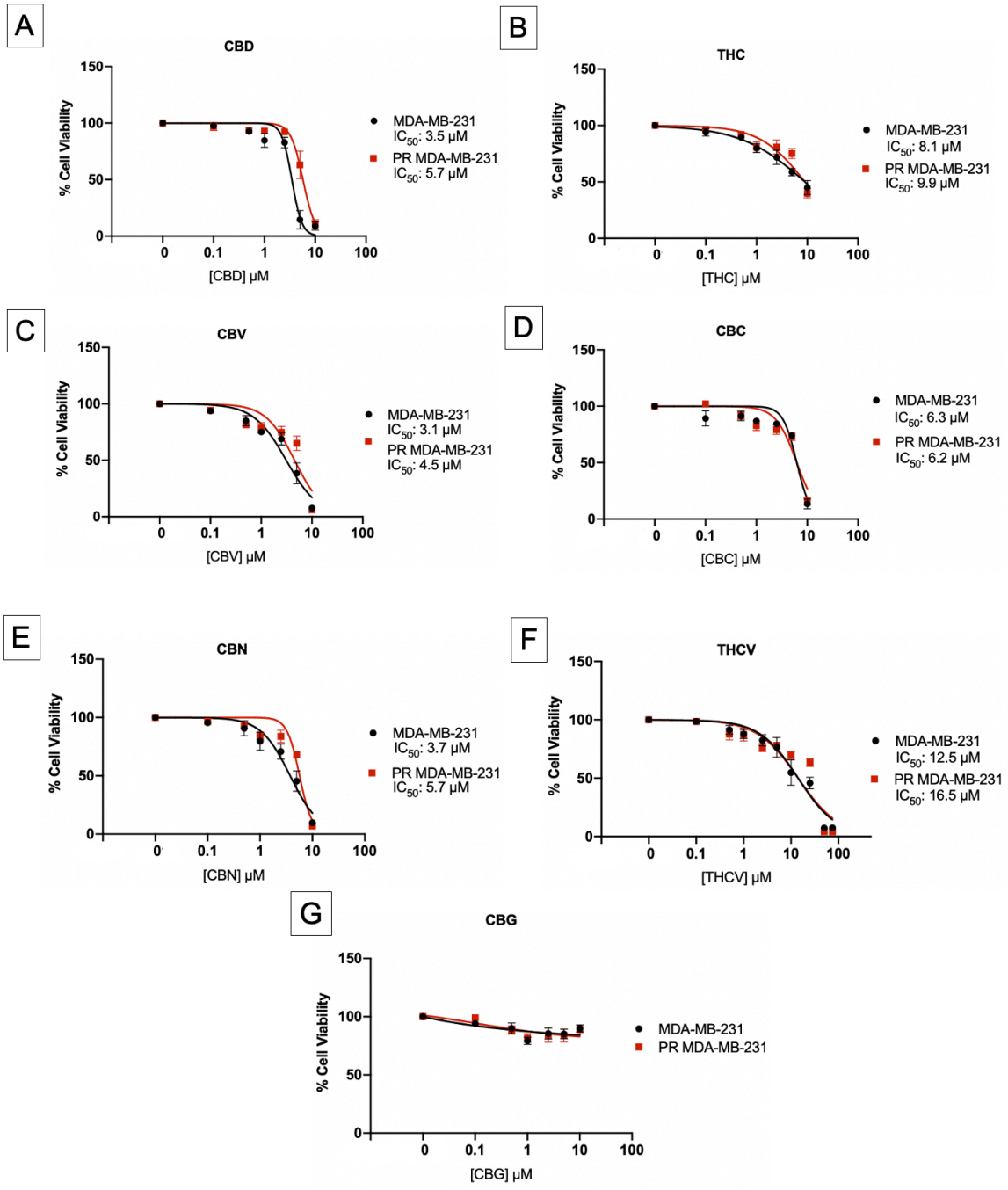


Figure 3.1. Dose-response curves generated for seven cannabinoids in MDA-MB-231 and PR MDA-MB-231 cells. Cells were treated with cannabinoids for 48 hours and cell viability was measured with an AlamarBlue assay. (A) Effects of CBD in MDA-MB-231 cell lines, (B) effects of THC in MDA-MB-231 cell lines, (C) effects of CBV in MDA-MB-231 cell lines, (D) effects of CBC in MDA-MB-231 cell lines, (E) effects of CBN in MDA-MB-231 cell lines, (F) effects of THCV in MDA-MB-231 cell lines, and (G) effects of CBG in MDA-MB-231 cell lines. Data presented as mean ± SEM of at least 3 independent experiments.

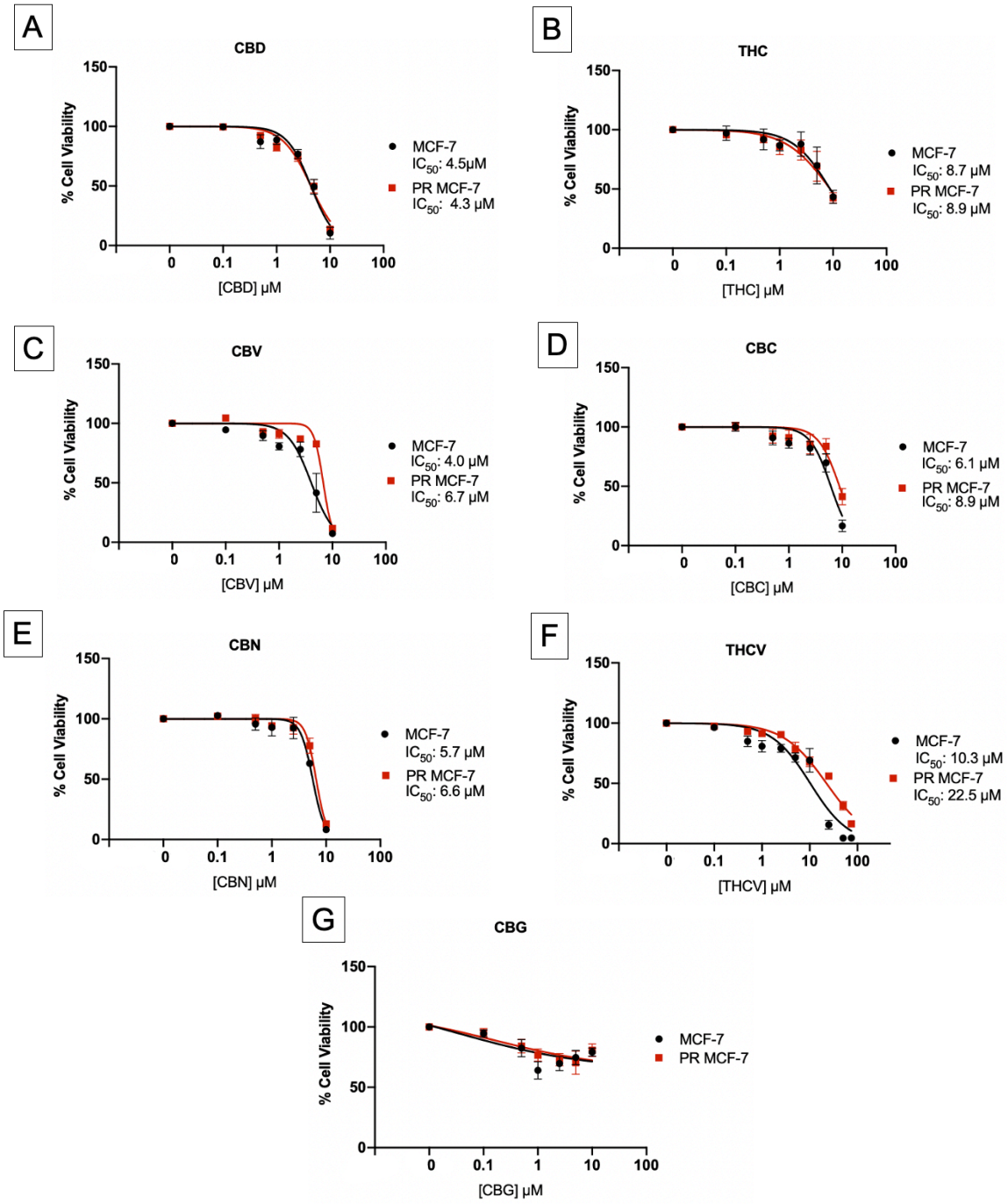


Figure 3.2. Dose-response curves generated for seven cannabinoids in MCF-7 and PR MCF-7 cells. Cells were treated with cannabinoids for 48 hours and cell viability was measured with an AlamarBlue assay. (A) Effects of CBD in MCF-7 cell lines, (B) effects of THC in MCF-7 cell lines, (C) effects of CBV in MCF-7 cell lines, (D) effects of CBC in MCF-7 cell lines, (E) effects of CBN in MCF-7 cell lines, (F) effects of THCV in MCF-7 cell lines, and (G) effects of CBG in MCF-7 cell lines. Data presented as mean ± SEM of at least 3 independent experiments.

3.3 Cannabinoids did not exert cytotoxicity in non-tumorigenic MCF-10A cells

A key consideration in the treatment of cancer is the potential harm to non-cancerous cells. Unfortunately, commonly used chemotherapeutic agents cause off-target effects by killing healthy cells in the body, ultimately resulting in the negative side effects associated with chemotherapy treatment. In breast cancer research, MCF-10A cells are frequently used as the *in vitro* standard to measure effects of drugs in healthy breast cells. They are non-tumorigenic breast epithelial cells that have been shown to express normal breast cell growth patterns and protein expression when cultured under 2-dimensional conditions (Qu et al., 2015), as they were in this study. Previous cancer research, including breast cancer, has demonstrated that some cannabinoids may favourably kill cancerous cells and leave non-tumorigenic cells unaffected, however this has only been shown with a mixture of cannabinoids, CBD, and abnormal CBD (Schoeman et al., 2020; Chakravarti et al., 2014; University of Newcastle, 2020; Tomko et al., 2019). We wanted to determine the effects of seven individual cannabinoids on the cell viability of MCF-10A cells. Cells were treated for 48 hours with CBD, THC, CBV, CBC, CBN, THCV, or CBG at or above the concentrations that produced maximal effects on breast cancer cell viability, ranging from 10 to 75 μM. None of the cannabinoids tested exhibited cytotoxic effects in MCF-10A cells following treatment (Figure 3.3). Percent cell viability measured under cannabinoid treatment were as follows: 95.21 ± 1.74 (CBD), 100.5 ± 5.64 (THC), 96.53 ± 5.15 (CBC), 104 ± 6.24 (CBV), 94.31 ± 2.61 (CBN), 98.9 ± 8.29 (THCV), and 89.93 ± 3.98 (CBG). Cell viabilities measured in each treatment group did not significantly differ from vehicle control-treated cells, reinforcing that cannabinoids may preferentially kill cancerous cells.

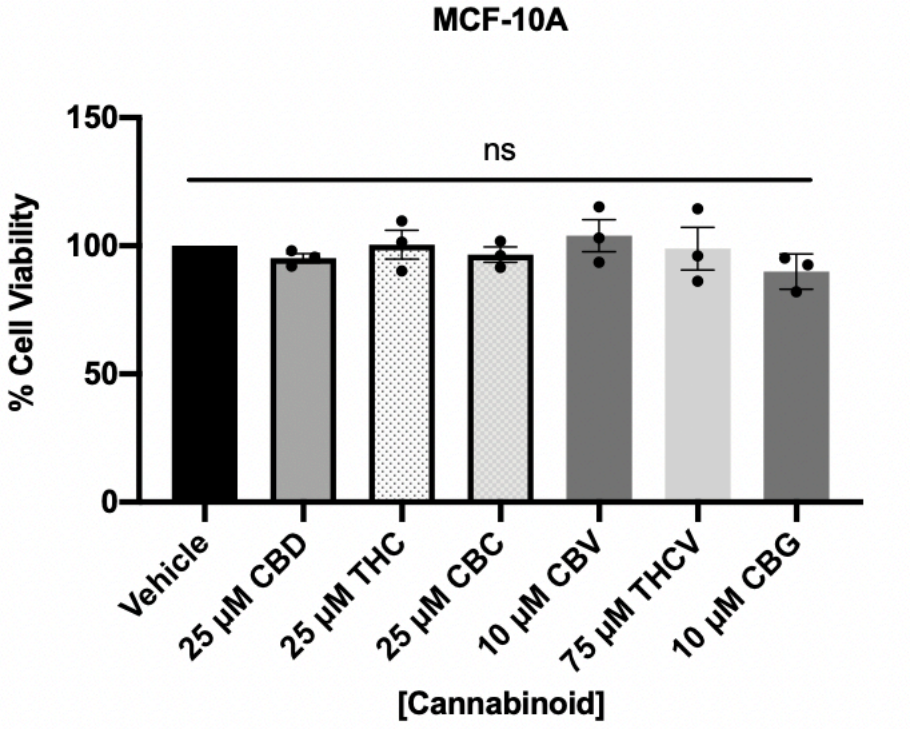


Figure 3.3. Effects of cannabinoids on cell viability in non-tumorigenic MCF-10A cells. Cells were treated with maximum concentrations of cannabinoids used during experiments in this study for 48 hours. Cell viability was measured with AlamarBlue. No significant differences were observed between treatment groups compared with cells treated with vehicle control. Data presented as mean ± SEM of at least 3 independent experiments. Unpaired student's T-tests were used to assess differences between treatments and vehicle control.

3.4 Cannabinoids induced apoptosis in MDA-MB-231 cells

Once we determined that cannabinoids were exerting cytotoxic effects on breast cancer cells, we wanted to further characterize this by looking at induction of apoptosis. To investigate if cannabinoids were inducing apoptosis, an Annexin V assay was used. Normally, phosphatidylserine residues line the inner cell membrane leaflet, however its inversion to the outer leaflet is an early event during apoptosis, making it useful in the detection of early apoptosis. Fluorescent Annexin V has a strong affinity for phosphatidylserine allowing for cells expressing it to be fluorescently labeled. In addition to this, propidium iodide (PI) staining was used detect cells whose membrane had broken down, indicating late apoptosis or necrosis. Cells that stained positive for Annexin V were considered apoptotic and cells that were PI-positive were considered late apoptotic or necrotic. Cells were treated with cannabinoids at a concentration of 2.5 μM for 24 hours and then stained for Annexin V/PI. This concentration was selected because it was not causing significant cell death and would allow us to observe rates of apoptosis without killing too many cells. All seven cannabinoids tested induced apoptosis in MDA-MB-231 cells (Figure 3.4 A). In cells treated with vehicle, 13.3 ± 1.78 percent stained Annexin V-positive. Under cannabinoid treatment conditions, on average 40 percent of cells were Annexin V-positive, the highest being 62 ± 2.26 and 48 ± 1.51 percent in CBD and CBV treatments, respectively. Following cannabinoid treatment, late apoptotic or necrotic cells MDA-MB-231 were also observed (Figure 3.4 B). In vehicle-treated cells, 6 ± 1.18 percent stained positive for PI, and on average, 15.7 percent of cells stained positive for PI in cannabinoid treatment groups. The only cannabinoids that did not cause significant elevations in PI staining were CBC and CBG.

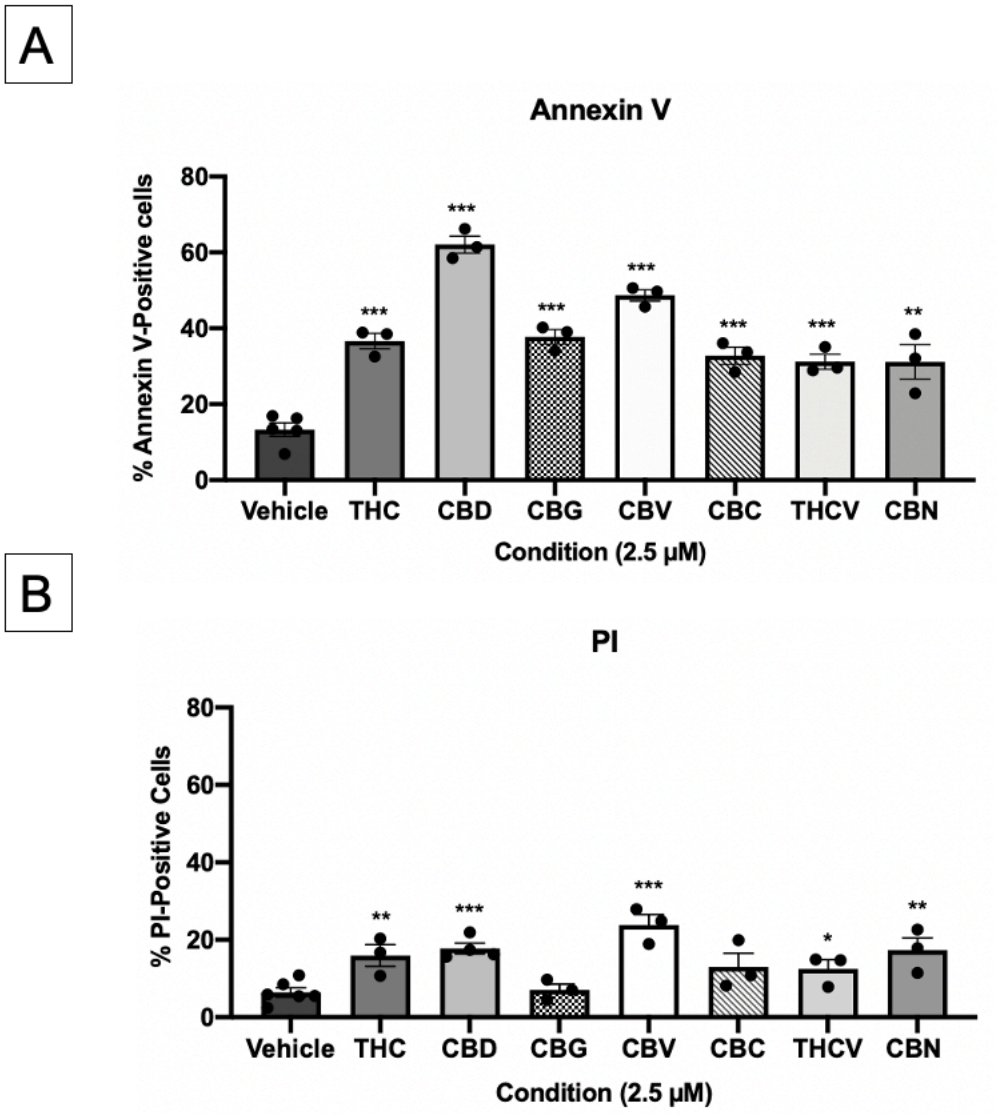


Figure 3.4. Detection of apoptosis induction by seven cannabinoids in MDA-MB-231 cells. Cells were treated with 2.5 µM of each cannabinoid separately for 24 hours. (A) Annexin V staining was used to detect rates of apoptosis and (B) PI staining for detection of late apoptosis or necrosis. THC, CBD, CBG, CBV, CBC, THCV and CBN caused significant increases in apoptosis. THC, CBD, CBV, THCV and CBN also increased levels of late apoptotic or necrotic cells. Data presented as mean ± SEM of at least 3 independent experiments. Unpaired student's T-tests were used to compare differences between treatment groups and vehicle. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5 Cannabinoids reduced the invasive ability of PR MDA-MB-231 cells

Certain forms of breast cancer cells are highly invasive, and the active invasion of these cells results in metastasis and the development of secondary tumors in breast cancer patients. Migration is defined as the passive movement of cells in response to chemical or mechanical stimuli, while invasion described the ability of cells to become actively motile and move through the extracellular matrix and disseminate from the primary tumor site into neighbouring tissues as well as distant organs (Pijuan et al., 2019). Although migration and invasion both play roles in metastasis, the focus in this study was invasion because it is the first key step in the metastatic cascade (Novikov et al., 2020; Fares et al., 2020). Triple-negative MDA-MB-231 cells are highly aggressive and invasive, and we wanted to explore cannabinoids' potential to alter their ability to invade.

We used a Matrigel invasion assay to measure the invasive capabilities of PR MDA-MB-231 cells. Matrigel functions to mimic the basement membrane *in vitro* that cells move through in order to invade surrounding areas (Pijuan et al., 2019). First, we confirmed that PR MDA-MB-231 cells were able to invade the Matrigel under vehicle conditions (Figure 3.5), as some cells have difficulty invading through a pore membrane (Pijuan et al., 2019). We then compared the percentage of cells that invaded under treatment conditions relative to control conditions (Figure 3.5). Cells were incubated under respective conditions at 2.5 μM in the Matrigel chambers and allowed to invade for 24 hours. The concentration of 2.5 μM was selected because it was not causing a substantial reduction in cell viability, allowing us to observe potential differences in invasion without significant cell death. Cells that invaded the Matrigel were stained and counted. The percent of cells that invaded through the Matrigel in each condition were

calculated relative to the number of cells that migrated through a cell culture insert under vehicle control conditions. Under control conditions, 38.32 ± 1.79 percent of cells were able to invade the Matrigel. Each cannabinoid tested was able to significantly reduce the invasive capability of PR MDA-MB-231 cells relative to control conditions. The percent of cells able to invade under cannabinoid conditions were reduced to the following: 12.07% ± 1.38 (CBD), 9.97% ± 1.08 (THC), 9.80% ± 1.39 (CBC), 8.27% ± 0.20 (CBV), 6.53% ± 0.67 (CBN), and 10.02% ± 0.82 (THCV).

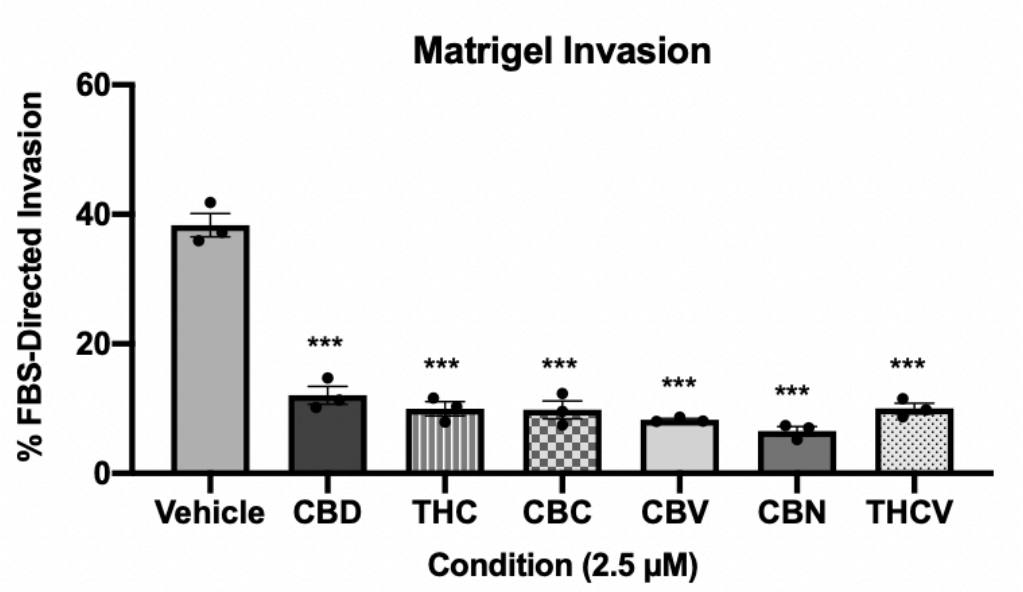


Figure 3.5. Matrigel invasion to assess the effects of cannabinoids on invasion of PR MDA-MB-231 cells. Cells were treated with vehicle or cannabinoids for 24 hours and allowed to invade through a Matrigel well. The percentage of cells that were able to invade the Matrigel under each condition was calculated relative to the number of cells that migrated through a cell culture insert (normalized as 100%). At least 3 fields of view or the entire membrane (for <1000 cells) was counted. CBD, THC, CBC, CBV, CBN and THCV were able to significantly reduce invasion of MDA-MB-231 cells. Data presented as mean ± SEM from 3 independent experiments. Unpaired student's T-tests were performed to compare reductions in invasion under cannabinoid conditions relative to vehicle treated cells. *** $p < 0.001$.

3.6 Paclitaxel did not reduce the IC_{50} for cannabinoid curves in PR MDA-MB-231 cells

Following the investigation of the effects of individual cannabinoids, we wanted to explore the addition of a constant concentration of paclitaxel (470 nM) to cannabinoid dose-response curves in PR cell lines to see if IC_{50} values could be reduced. This concentration of paclitaxel was selected because the PR cells were resistant at this concentration (Hall et al., 2017; Tomko et al., 2019), and we wanted to see if the addition of paclitaxel could enhance the effects of cannabinoids in these cells. It has been previously demonstrated that cannabinoids with anti-cancer agents produced enhanced overall anti-cancer activity, and that THC and/or CBD was able to sensitize cancer cells to chemotherapies (Lopez-Valero et al., 2018; Scott et al., 2017; Liu et al., 2008; Holland et al., 2006). As a preliminary study of cannabinoids with anti-cancer agents, we combined cannabinoid dose-response curves with 470 nM paclitaxel in the PR cell lines. Cells were treated for 48 hours with varying concentrations of cannabinoids with the addition of paclitaxel. Cell viability was measured using AlamarBlue reagent and fluorescence. Curves were fitted with Prism GraphPad and IC_{50} values were compared using the extra-sum-of-squares F-test.

We found that the addition of paclitaxel to cannabinoid dose-response curves did not significantly reduce IC_{50} values in PR MDA-MB-231 cells (Figure 3.6 A-F). The IC_{50} for CBD alone was 5.7 μM and with paclitaxel the IC_{50} was 5.9 μM. For THC alone, the IC_{50} was 9.9 μM and with paclitaxel the IC_{50} was 8.5 μM. The addition of paclitaxel to the CBV curve caused an IC_{50} shift from 4.5 μM to 3.8 μM. CBC alone and with the addition of paclitaxel had an IC_{50} of 6.1 μM. The IC_{50} for CBN did not shift

when paclitaxel was added and remained at 5.7 μM. When THCV was combined with paclitaxel, the IC_{50} increased to 20.3 μM compared to 16.5 μM for THCV alone. In the case of CBG, IC_{50} values could not be calculated, and it does not appear that the addition of paclitaxel was able to increase inhibitory effects, as shown by significant overlap between curves (Figure 3.6 G).

3.7 Paclitaxel did not reduce the IC_{50} for cannabinoid curves in PR MCF-7 cells

Cannabinoid dose-response curves were combined with 470 nM paclitaxel in PR MCF-7 cells, as described previously for PR MDA-MB-231 cells. Similar to what we found in the PR MDA-MB-231 cells, the addition of paclitaxel did not shift curves to the left or significantly reduce IC_{50} values, with the exception of THCV (Figure 3.7). CBD alone had an IC_{50} of 4.3 μM and with paclitaxel it was 4.1 μM. THC had an IC_{50} or 8.9 μM and with paclitaxel added it was 9.1 μM. CBV and CBV + paclitaxel curves had nearly identical IC_{50}s of 6.7 and 6.8 μM, respectively. The IC_{50} for CBC alone was 8.9 μM and with paclitaxel it was 9.5 μM. CBN alone had an IC_{50} of 6.6 μM and when combined with paclitaxel the IC_{50} was 6.9 μM. When the THCV curve was combined with paclitaxel, the curve marginally shifted to the right and the IC_{50} value increased from 22.5 μM to 30.4 μM. For CBG, IC_{50} values could not be calculated, and the addition of paclitaxel did not increase inhibitory effects as demonstrated by significant curve overlap (Figure 3.7 G).

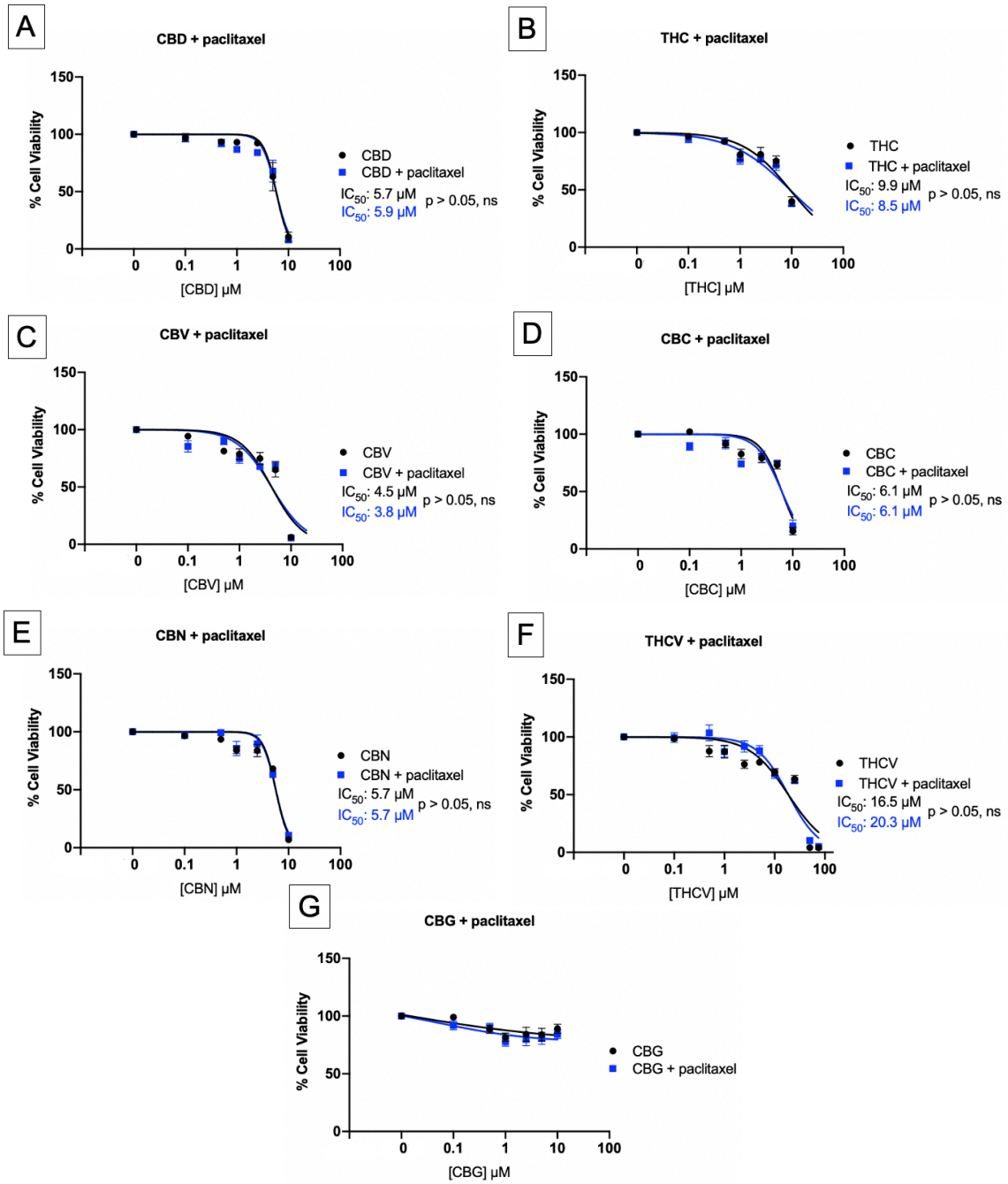


Figure 3.6. Combination of cannabinoid dose-response curves with 470 nM paclitaxel in PR MDA-MB-231. Cells were treated for 48 hours, and cell viability measured using an AlamarBlue assay. (A) Effects of CBD + paclitaxel, (B) effects of THC + paclitaxel, (C) effects of CBV + paclitaxel, (D) effects of CBC + paclitaxel, (E) effects of CBN + paclitaxel, (F) effects of THCV + paclitaxel, and (G) effects of CBG + paclitaxel. No significant differences between IC_{50}s were observed. Data presented as mean ± SEM of 3 independent experiments. Comparison of IC_{50} values performed using the extra-sum-of-squares F-test.

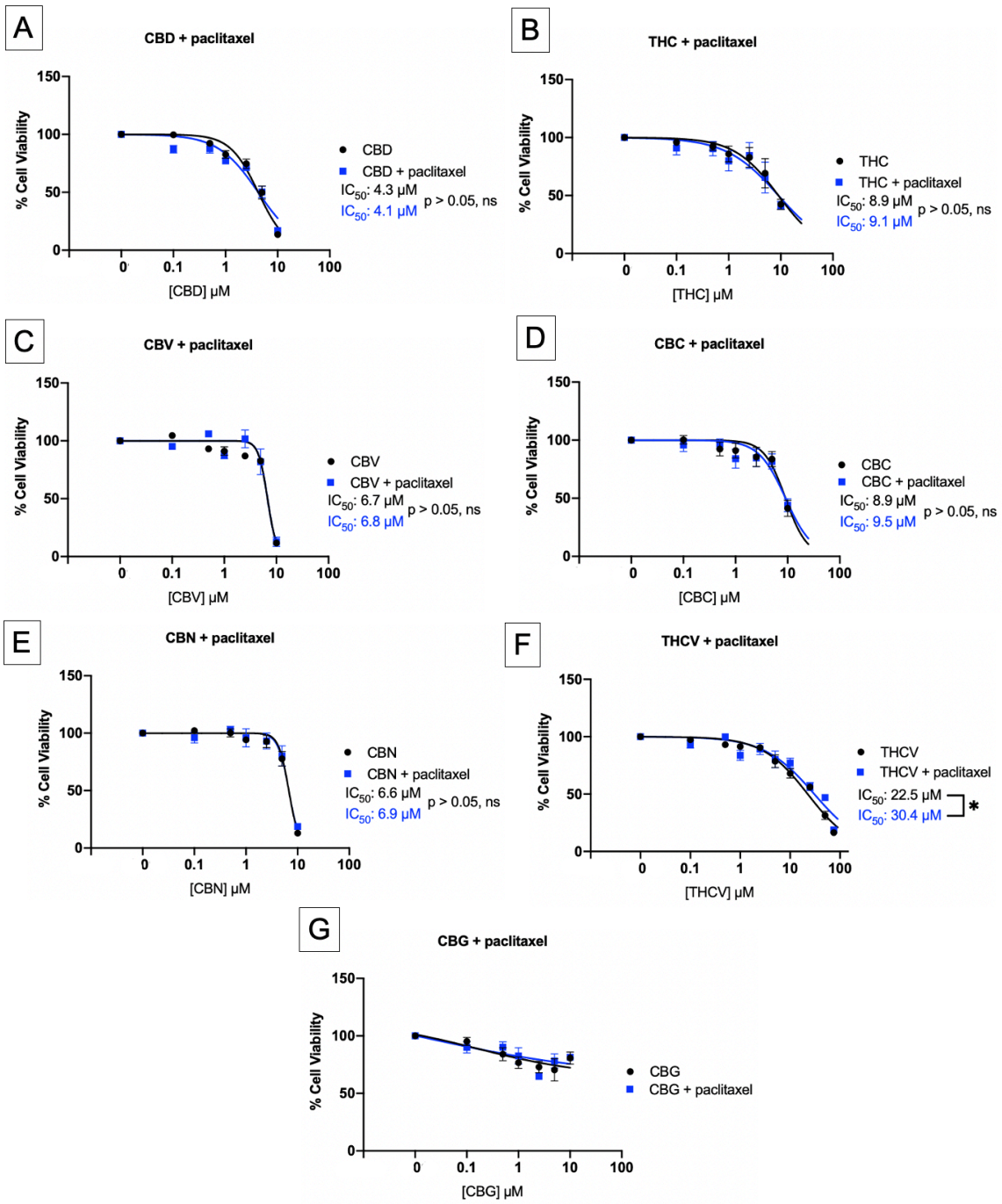


Figure 3.7. Combination of cannabinoid dose-response curves with 470 nM paclitaxel in PR MCF-7. Cells were treated for 48 hours, and cell viability was measured using an AlamarBlue assay. (A) Effects of CBD + paclitaxel, (B) effects of THC + paclitaxel, (C) effects of CBV + paclitaxel, (D) effects of CBC + paclitaxel, (E) effects of CBN + paclitaxel, (F) effects of THCV + paclitaxel, and (G) effects of CBG + paclitaxel. No significant differences between IC_{50}s were observed with the exception of THCV. Data presented as mean ± SEM of 3 independent experiments. Comparison of IC_{50} values was performed with the extra-sum-of-squares F-test. * $p < 0.05$.

3.8 CBD reduced the IC_{50} of cannabinoid dose-response curves in MDA-MB-231 cell lines

It has been documented that whole botanical extracts from cannabis elicit superior anti-cancer effects, and that the combination of specific cannabinoids (CBD and THC) show increased therapeutic effects compared to individual cannabinoids alone (Blasco-Benito et al., 2018; Johnson et al., 2010; Schoeman et al., 2020). As a preliminary study for the potential of cannabinoid combinations, we combined cannabinoid dose-response curves in all four breast cancer cell lines with a single concentration of cannabidiol at 2.5 μM. We wanted to determine if adding CBD to dose-response curves had the potential to shift curves and reduce IC_{50} values. We chose to add CBD to cannabinoid dose-response curves because it has been shown that complementing THC treatment with CBD increased the anti-cancer efficacy (Marcu et al., 2010), it was more potent than THC in our studies, and it lacks the intoxicating effects that THC possesses.

We combined CBC, CBV, CBN and THCV curves with 2.5 μM CBD in MDA-MB-231 and PR MDA-MB-231 cell lines. Cells were treated with varying concentrations of cannabinoids with the addition of CBD for 48 hours, and cell viability was measured by fluorescence using an AlamarBlue assay. Dose-response curves were fitted using GraphPad Prism and IC_{50} values were compared using the extra-sum-of-squares F-test. In MDA-MB-231 cells, we found that the addition of CBD was able to significantly reduce IC_{50} values for CBC, CBV, CBN and THCV (Figure 3.8.1.). The effect of 2.5 μM CBD alone on cell viability was also plotted on each curve in red. The addition of CBD was able to reduce the IC_{50} for CBC from 6.3 μM to 2.7 μM. CBV alone had an IC_{50} of 3.1 μM, while with the addition of CBD it was reduced to 1.5 μM. The IC_{50} for CBN was

reduced from 3.7 μM to 2.4 μM with the addition of CBD. THCV alone had an IC_{50} of 12.5 μM and CBD was able to reduce it to 6.5 μM. We also found that in PR MDA-MB-231 cells, adding CBD to CBC, CBV and CBN curves significantly reduced IC_{50}s (Figure 3.8.2). The IC_{50} for CBC shifted from 6.2 μM to 2.8 μM with the addition of CBD. CBV alone had an IC_{50} of 4.5 and with CBD it was reduced to 1.8 μM. The IC_{50} for CBN was reduced to 2.6 from 5.7 μM when CBD was added. The IC_{50} for THCV in PR MDA-MB-231 cells was reduced to 10.4 from 16.5 μM with the addition of CBD, however this shift was not significant.

3.9 CBD reduced the IC_{50} of cannabinoid dose-response curves in MCF-7 cell lines

We combined the same cannabinoid dose-response curves previously discussed with 2.5 μM CBD in both MCF-7 cell lines as well. The addition of CBD was able to significantly reduce IC_{50} values for CBV, CBC, and CBN curves in MCF-7 cells (Figure 3.9.1). CBC had an IC_{50} of 6.1 μM alone and when combined with CBD the IC_{50} was reduced to 4.0 μM. CBV's IC_{50} was reduced to 2.8 μM from 4.0 μM following the addition of CBD. The IC_{50} for CBN alone was 5.7 μM and adding CBD reduced the IC_{50} to 4.2 μM. While the addition of CBD to the THCV curve was able to reduce the IC_{50} to 9.4 μM from 10.3 μM, this decrease was not significant (Figure 3.9.1 D). In PR MCF-7 cells, CBD was able to reduce IC_{50} values of CBC, CBV, CBN and THCV dose-response curves (Figure 3.9.2). The IC_{50} for CBC was reduced from 8.9 to 6.2 μM. CBV had an IC_{50} of 6.7 μM and when CBD was added it fell to 5.1 μM. CBN's IC_{50} shifted from 6.6 to 4.0 μM following the addition of CBD. The IC_{50} for THCV was reduced from 22.5 μM to 13.2 μM.

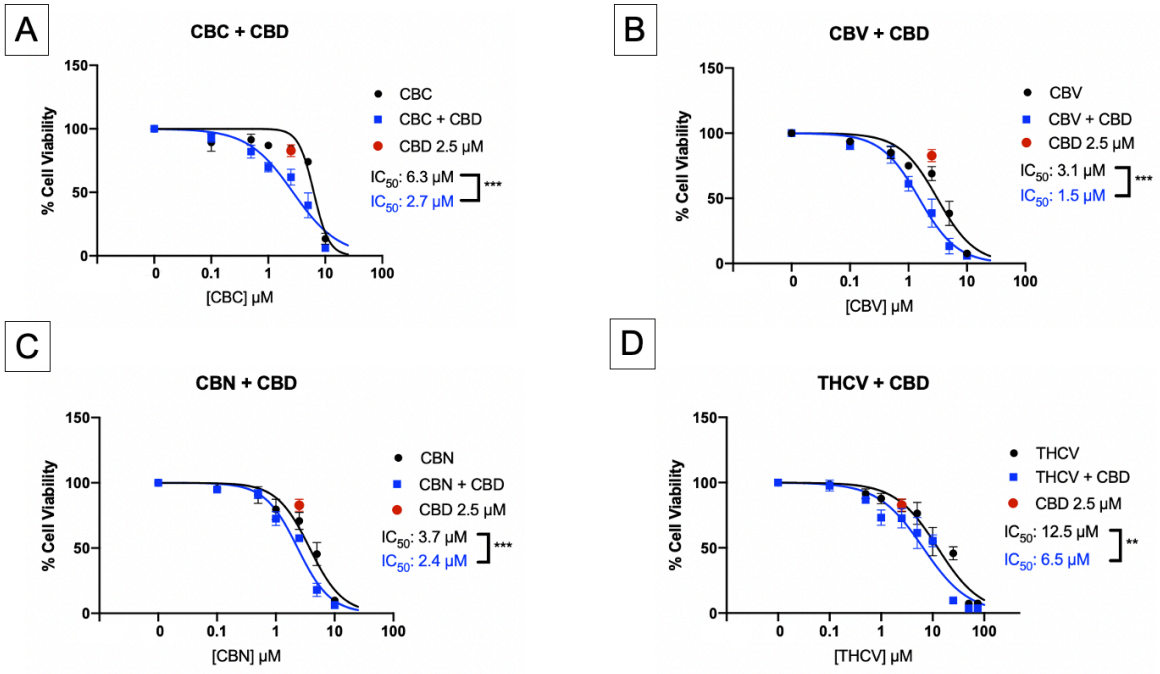


Figure 3.8.1. Combination of cannabinoid dose-response curves with 2.5 µM cannabidiol in MDA-MB-231 cells. CBC, CBV, CBN and THCV dose response curves were combined with 2.5 µM CBD to explore potential for cannabinoid combinations. Cells were treated for 48 hours, and cell viability was measured using AlamarBlue. (A) Effects of CBC + CBD, (B) effects of CBV + CBD, (C) effects of CBN + CBD, and (D) effects of THCV + CBD. Effect of CBD alone at 2.5 µM also shown on each plot in red. The addition of CBD to each cannabinoid dose-response curve was able to significantly reduce IC_{50}s, calculated using the extra-sum-of-squares F-test. Data presented as mean ± SEM of at least 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$.

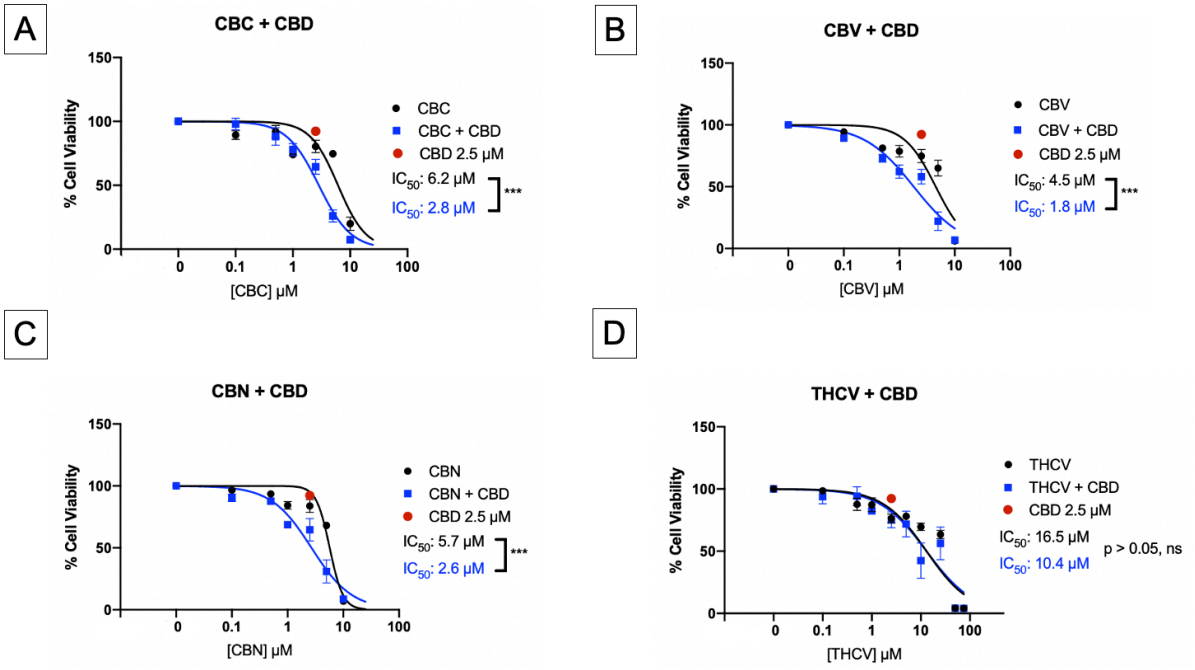


Figure 3.8.2. Combination of cannabinoid dose-response curves with 2.5 µM cannabidiol in PR MDA-MB-231 cells. CBC, CBV, CBN and THCV dose response curves were combined with 2.5 µM CBD to explore potential for cannabinoid combinations. Cells were treated for 48 hours, and cell viability was measured by an AlamarBlue assay. (A) Effects of CBC + CBD, (B) effects of CBV + CBD, (C) effects of CBN + CBD, and (D) effects of THCV + CBD. Effect of CBD alone at 2.5 µM also shown on each plot in red. The addition of CBD to CBC, CBV, and CBN dose-response curves was able to significantly reduce IC_{50}s, calculated using the extra-sum-of-squares F-test. The shift of the THCV curve with the addition of CBD was not statistically significant. Data presented as mean ± SEM of at least 3 independent experiments. *** $p < 0.001$.

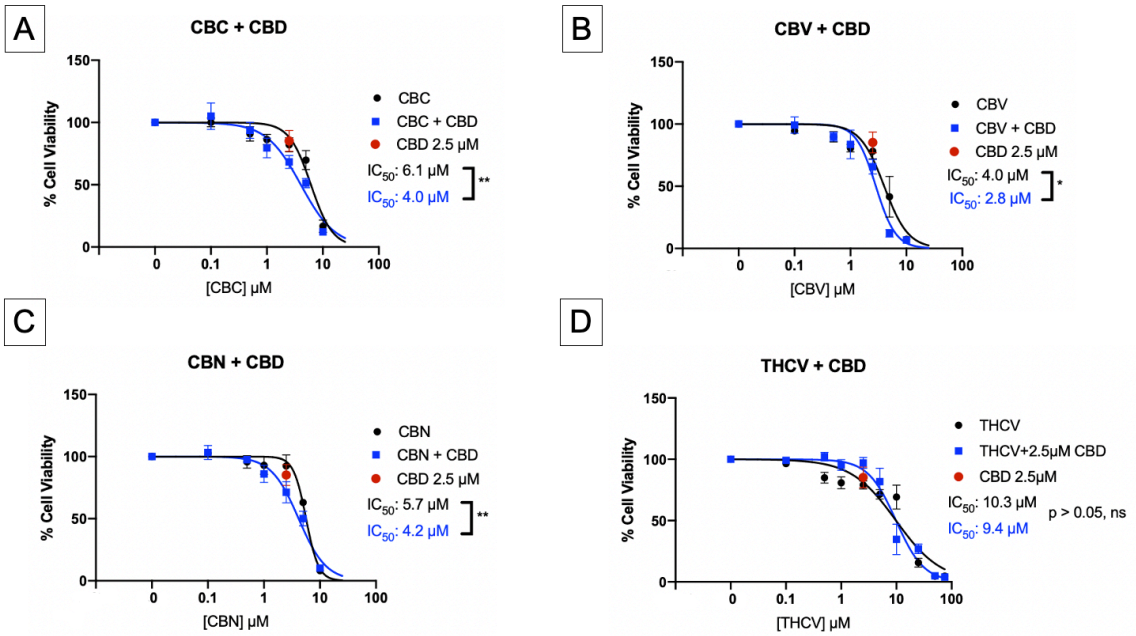


Figure 3.9.1. Combination of cannabinoid dose-response curves with 2.5 µM cannabidiol in MCF-7 cells. CBC, CBV, CBN and THCV dose response curves were combined with 2.5 µM CBD to explore the potential of cannabinoid combinations. Cells were treated for 48 hours, and cell viability was measured with an AlamarBlue assay. (A) Effects of CBC + CBD, (B) effects of CBV + CBD, (C) effects of CBN + CBD, and (D) effects of THCV + CBD. Effect of CBD alone at 2.5 µM also shown on each plot in red. The addition of CBD to CBC, CBV and CBN dose-response curves was able to significantly reduce IC_{50}s, calculated using the extra-sum-of-squares F-test. The shift of the THCV curve with the addition of CBD was not statistically significant. Data presented as mean ± SEM of at least 3 independent experiments. * $p < 0.05$, ** $p < 0.01$.

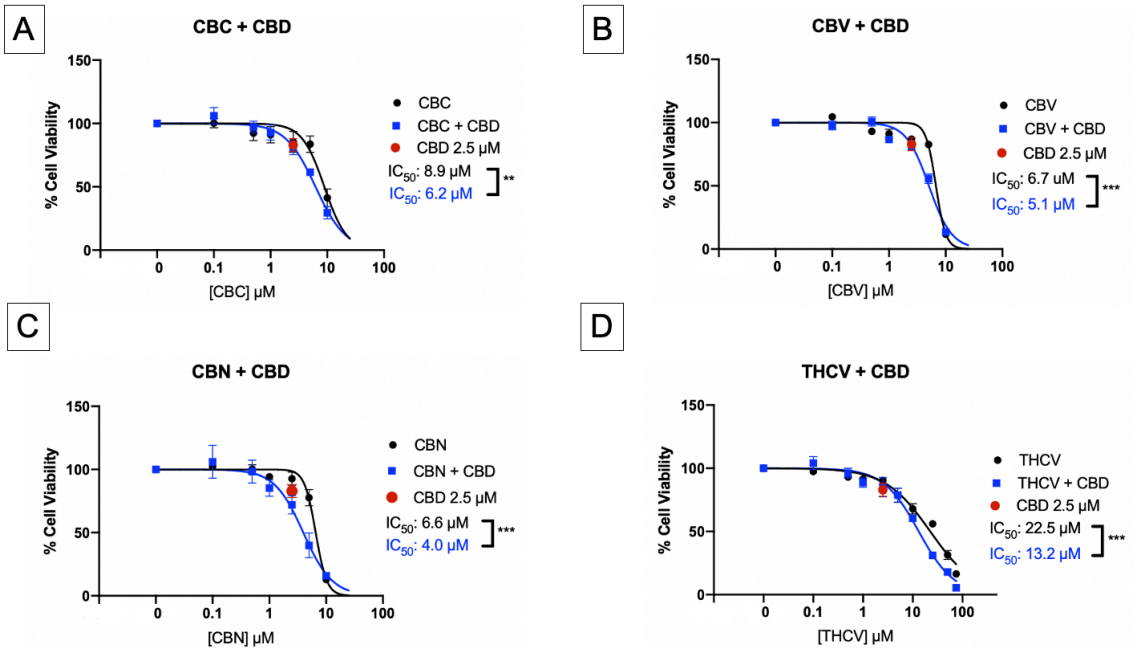


Figure 3.9.2. Combination of cannabinoid dose-response curves with 2.5 µM cannabidiol in PR MCF-7 cells. CBC, CBV, CBN and THCV dose response curves were combined with 2.5 µM CBD to explore potential for cannabinoid combinations. Cells were treated for 48 hours, and cell viability was measured with AlamarBlue. (A) Effects of CBC + CBD, (B) effects of CBV + CBD, (C) effects of CBN + CBD, and (D) effects of THCV + CBD. Effect of CBD alone at 2.5 µM also shown on each plot in red. The addition of CBD to each cannabinoid dose-response curve was able to significantly reduce IC_{50}s, calculated using the extra-sum-of-squares F-test. Data presented as mean ± SEM of at least 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$.

3.10 Doxorubicin with CBD exerted additive inhibitory effects on cell viability

Previous studies have shown that the combination of THC with CBD, and whole botanical extracts, produce enhanced therapeutic benefits (eg. pain relief) and anti-cancer effects, (Johnson et al., 2010; Blasco-Benito et al., 2018). In addition to this, it has been demonstrated *in vivo* that the combination of cannabinoids with temozolomide (TMZ) synergistically reduced the growth of xenograft glioma tumors (Lopez-Valero et al., 2018). As an extension of our preliminary studies that combined dose-response curves with single concentrations of paclitaxel or cannabidiol, we performed combination studies using the dose-response matrix assay. This comprehensive experimental method allows researchers to screen the effects of combined drugs with a large array of combination concentrations. We explored the combination of a commonly used chemotherapeutic agent, doxorubicin, with several cannabinoids using the matrix assay in MDA-MB-231, PR MDA-MB-231 and MCF-7 cells. The first step in this process was to generate dose-response curves for doxorubicin in our cell lines to determine the range of concentrations to be used in the matrix assays (Figure 3.10.1). Cells were treated for 48 hours with varying concentrations of doxorubicin and cell viability was measured by fluorescence. We found that the PR MDA-MB-231 cells were also resistant to doxorubicin with a much higher IC_{50} at 1.8 μM (Figure 3.10.1 C), relative to susceptible MDA-MB-231 cells with an IC_{50} of 20.5 nM (Figure 3.10.1 A). We concluded that the PR-MDA-MB-231 cells were resistant to doxorubicin due to efflux by the PGP, as it is a substrate for this transporter and these cells have significantly up-regulated PGP expression (Hall et al., 2017).

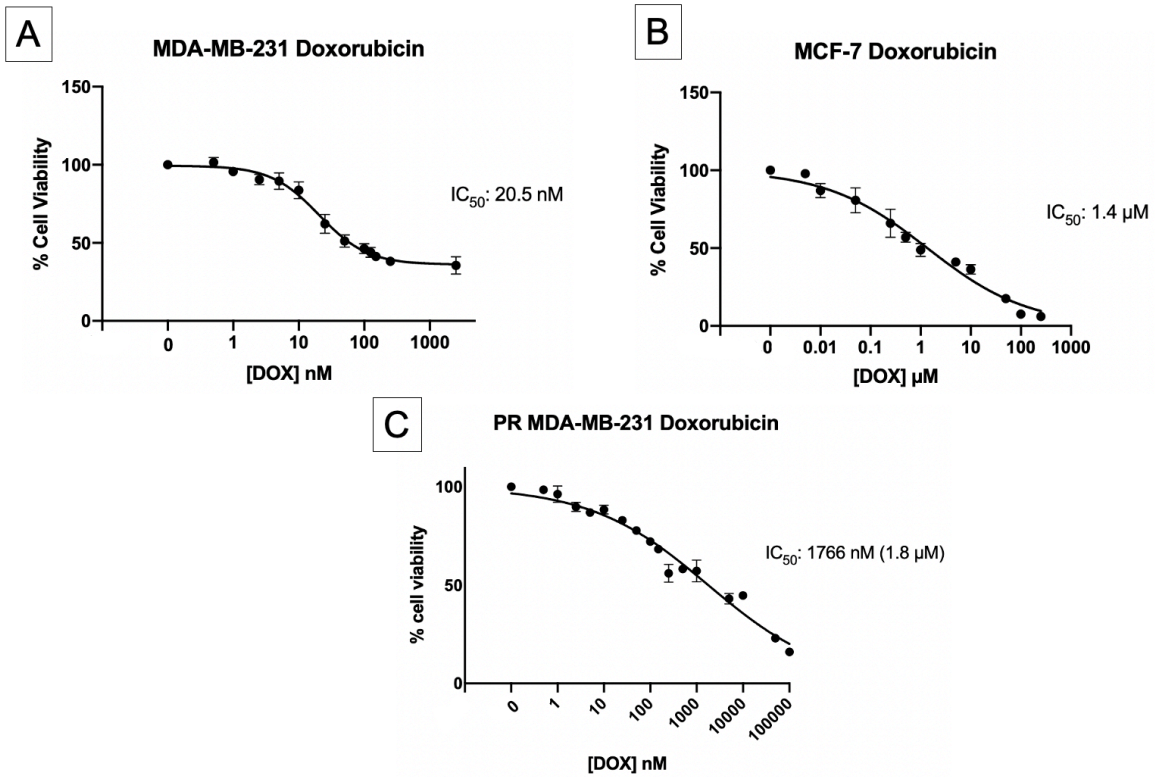


Figure 3.10.1. Dose-response curves for chemotherapeutic agent doxorubicin. Cells were treated for 48 hours, and cell viability was measured with an AlamarBlue assay. (A) Effects of doxorubicin in MDA-MB-231 cells, (B) effects of doxorubicin in MCF-7 cells, and (C) effects of doxorubicin in PR MDA-MB-231 cells. Data presented as mean ± SEM of 3 independent experiments.

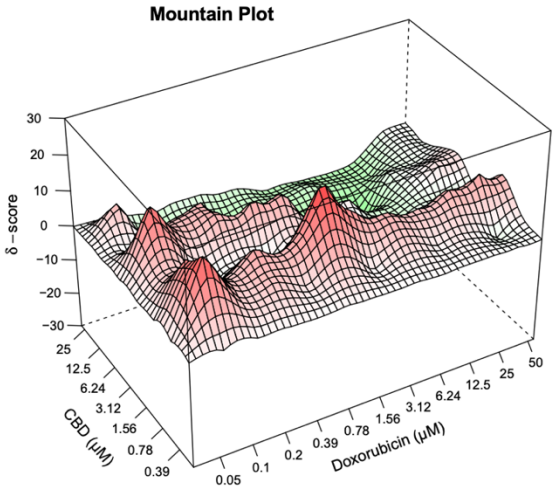
After we determined appropriate dose ranges for doxorubicin in our cell lines, matrix assays were performed for combinations of doxorubicin with CBD, THC, CBC or CBV. Matrix assays were set up as grids in 96-well plates and cells were treated with compounds for 48 hours. Cell viability was measured by fluorescence using an AlamarBlue assay. Following data collection, Synergy Finder (2.0) was used to assess potential synergistic, additive, or antagonistic dose combinations. Synergy Finder can process a large amount of data and analyze it to determine interactions between two drugs and provides an excellent, user-friendly tool for preclinical drug screening. The web-based program uses a selected reference model to compare expected responses with observed responses and assigns each dose combination a corresponding synergy score. We used the Bliss Independence reference model for our experiments.

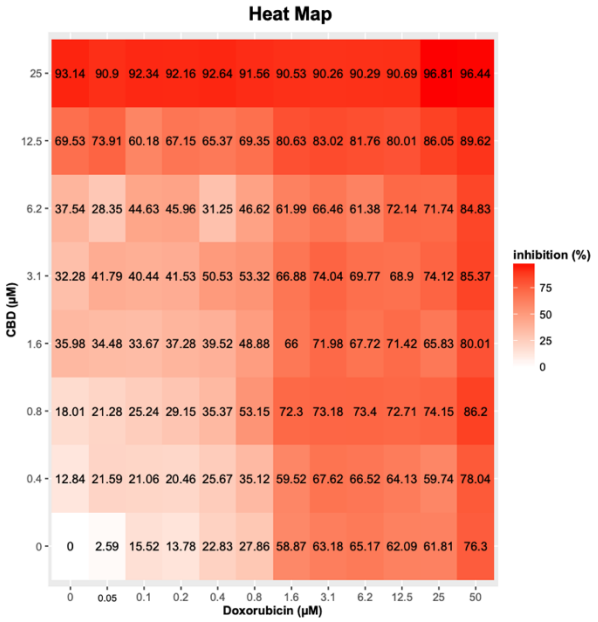
In the 3-dimensional mountain plots, X- and Y-axes are doxorubicin and cannabinoid, respectively, and the Z-axis represents the assigned synergy scores. Darker red regions on the 3D map correspond to the higher synergy scores produced for a given combination. Light red, white, and light green areas correspond to scores that indicate additivity, and the darker green areas represent antagonistic scores. Each mountain plot has a corresponding 2-dimensional heat map that shows the percent inhibition associated with each dose combination, as well as for each drug alone; this data was used to calculate the synergy scores. The Bliss Independence reference model uses the following equation to calculate expected response: $Y_{ab} = y_a + y_b - y_a y_b$, where Y_{ab} is the Bliss predicted response and y_a and y_b are the observed inhibition with drug A at dose a and drug B at dose b, respectively (Liu et al., 2018). If the observed response (inhibition) is higher than the predicted response, the interaction is considered to be synergistic

(synergy score >10). If the response is less than expected, the interaction is considered antagonistic (synergy score <-10). If the response is equal to the expected response, the synergy score would be 0 and the interaction is considered additive. For synergy scores that fall between -10 and 10, the interaction is also considered to be additive.

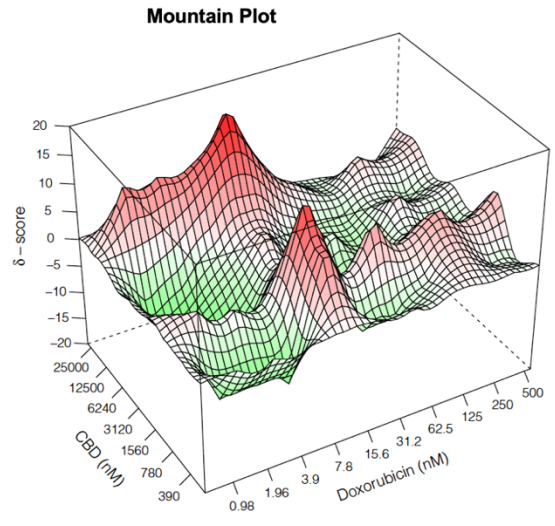
First, looking at the combination of doxorubicin with cannabidiol, in all three cell lines we found that the overall inhibitory effects on cell viability were additive, with some specific dose combinations yielding synergistic and antagonistic scores (Figure 3.10.2, Table 3.1). In MCF-7 cells, the majority of synergy scores were between -10 and 10, with the highest synergy score reaching 23.12 at 0.78 μM CBD and 0.78 μM doxorubicin (Figure 3.10.2 A). We found similar results in MDA-MB-231 cells, where the majority of scores fell between -10 and 10 and the highest synergy score was 19.83 at 12.5 μM CBD and 7.8 nM doxorubicin (Figure 3.10.2 B). It is important to point out that depending on the mountain plot, the Z-axis range varies and is automatically set by Synergy Finder. While the mountain plot peaks produced in the MDA-MB-231 cells may appear higher and darker red compared to those in the MCF-7s, the Z-axes have differing ranges. In PR MDA-MB-231 cells, doxorubicin and CBD exerted predominantly additive inhibitory effects (Figure 3.10.2 C). The majority of synergy scores fell in the -5 to 10 range, likely indicative of additivity. The highest synergy score reached 27.27 at 0.78 μM CBD and 1.23 μM doxorubicin. Table 3.1 summarizes the synergy scores produced for the combination of doxorubicin and CBD by providing the three highest and lowest synergy scores and their corresponding dose combination.

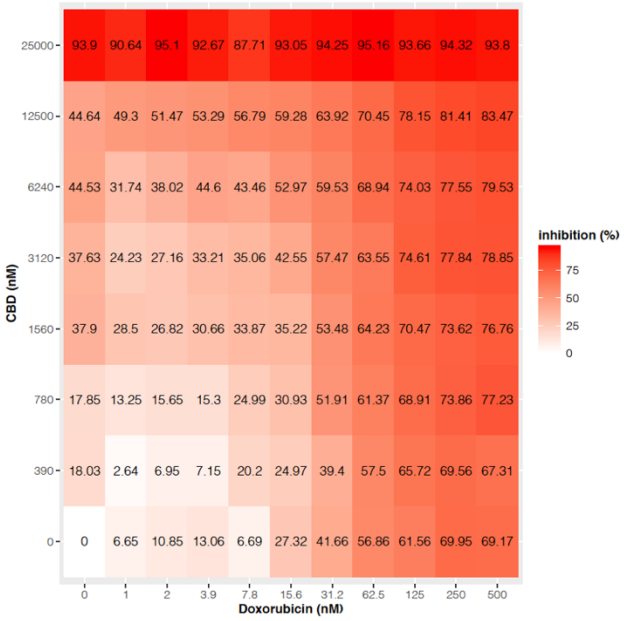
A **MCF-7: Doxorubicin + CBD**





B **MDA-MB-231: Doxorubicin + CBD**





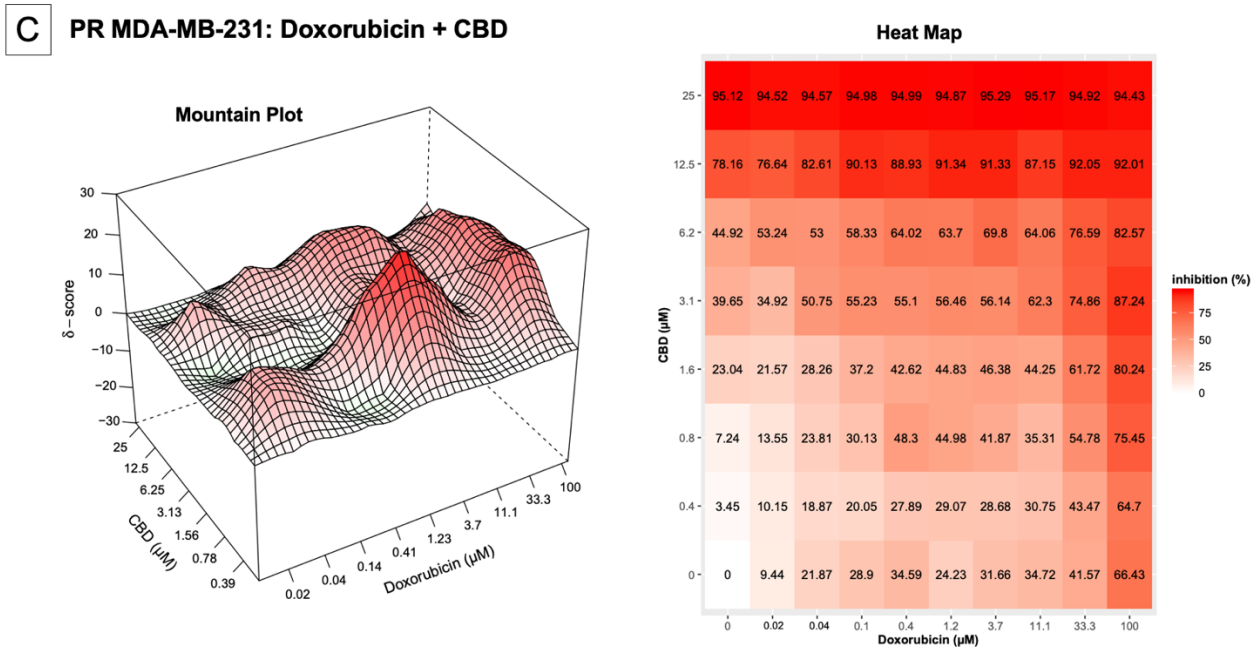


Figure 3.10.2. Assessment of synergy between doxorubicin and cannabidiol. Cells were treated for 48 hours, and cell viability was measured with an AlamarBlue assay. Data was input into Synergy Finder (2.0) to generate 3D mountain plots outlining synergy scores across the dose range, and corresponding 2D heat maps showing percent inhibition data used to calculate synergy scores. Mountain plots and heat maps were captured for the combination of doxorubicin with CBD in (A) MCF-7 cells, (B) MDA-MB-231 cells, and (C) PR MDA-MB-231 cells. Some doses on heat maps rounded to the nearest tenth or hundredth. Data presented as mean of 3 independent experiments.

Table 3.1. Summary of synergy scores for the combination of doxorubicin with cannabidiol.

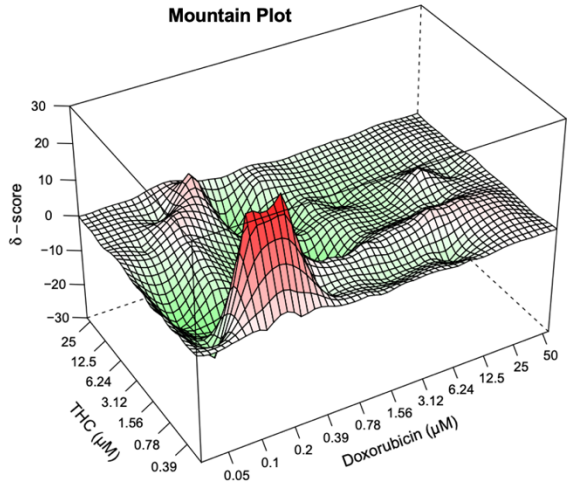
Synergy Score	[CBD]	[Doxorubicin]
	MCF-7	
23.12	0.78 μM	0.78 μM
20.61	0.39 μM	0.05 μM
19.10	3.12 μM	0.05 μM
-10.01	6.24 μM	1.56 μM
-12.00	12.5 μM	0.1 μM
-15.86	6.24 μM	0.39 μM
	MDA-MB-231	
19.83	12.5 μM	7.8 nM
16.73	0.78 μM	7.8 nM
11.13	12.5 μM	0.98 nM
-10.00	1.56 μM	1.96 nM
-11.39	0.390 μM	3.9 nM
-14.18	1.56 μM	15.6 nM
	PR MDA-MB-231	
27.27	0.78 μM	1.23 μM
18.80	0.78 μM	0.41 μM
17.88	0.78 μM	33.3 μM
-2.10	25 μM	33.3 μM
-3.92	3.13 μM	0.02 μM
-4.20	25 μM	100 μM

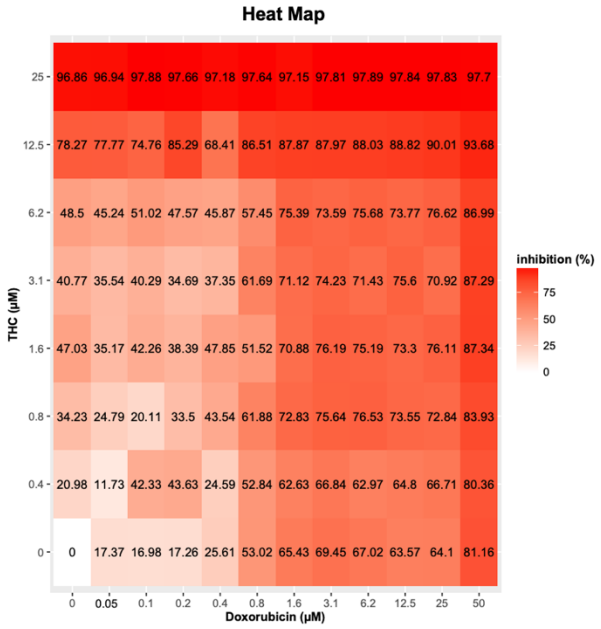
Scores in red are synergistic, black are additive, and green are antagonistic.

3.11 Doxorubicin with THC elicited additive inhibitory effects on cell viability

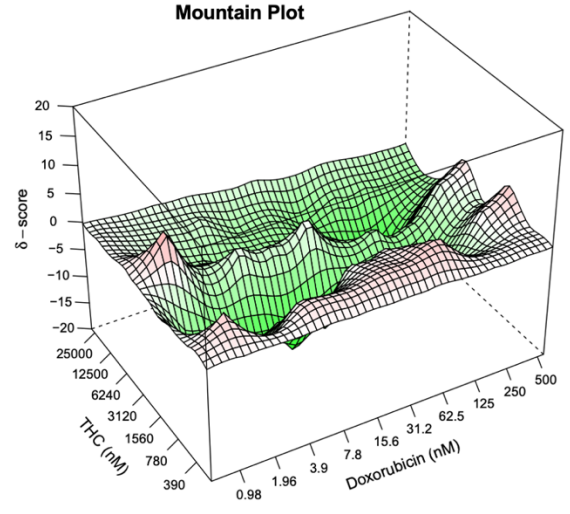
We assessed the synergistic potential of doxorubicin in combination with Δ-9-tetrahydrocannabinol using the matrix assay. Cells were treated for 48 hours with an array of combinations of doxorubicin and THC and cell viability was measured by fluorescence, as previously described. We found that the combination of doxorubicin with THC yielded mostly additive inhibitory effects on cell viability in the three cell lines used, however, depending on the concentrations, synergistic and antagonistic interactions were also observed. In MCF-7 cells, most of the synergy scores were between -10 and 10, with a few combinations producing both synergistic and antagonistic scores (Figure 3.11 A, Table 3.2). The highest synergy score reached was 28.8 at 0.39 μM THC and 0.2 μM doxorubicin. In MDA-MB-231 cells, overall synergy scores were much lower than observed in the other two cell lines, with the majority of scores falling between 0 and -10 (Figure 3.11 B, Table 3.2). There were no scores within the synergistic range, the highest being only 7.4 at 3.12 μM THC and 0.98 nM doxorubicin. In PR MDA-MB-231 cells we found that the majority of scores fell between -10 and 10, indicating overall additive effects between doxorubicin and THC in these cells (Figure 3.11 C, Table 3.2). The highest score was in the lower synergistic score range at 15.7 and fell at high concentrations of both drugs, at 12.5 μM THC and 11.1 μM doxorubicin. Table 3.2 summarizes the synergy scores produced for the combination of THC and doxorubicin, presenting the three highest and lowest scores and their corresponding dose combinations.

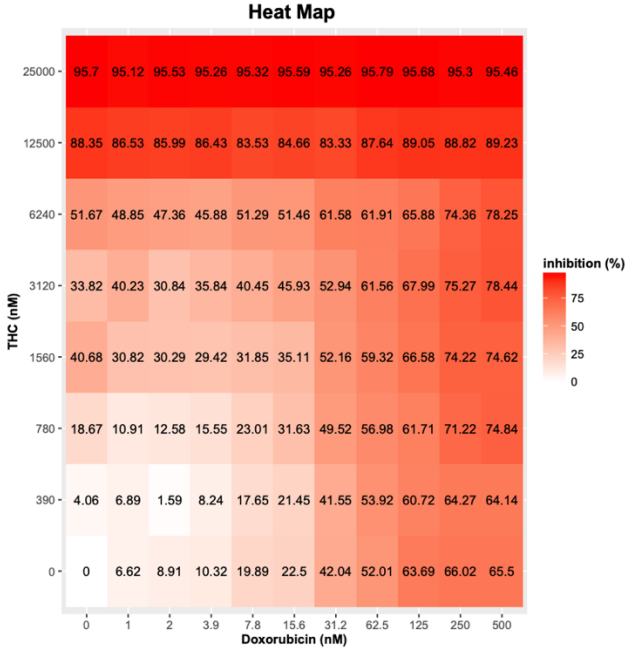
A **MCF-7: Doxorubicin + THC**





B **MDA-MB-231: Doxorubicin + THC**





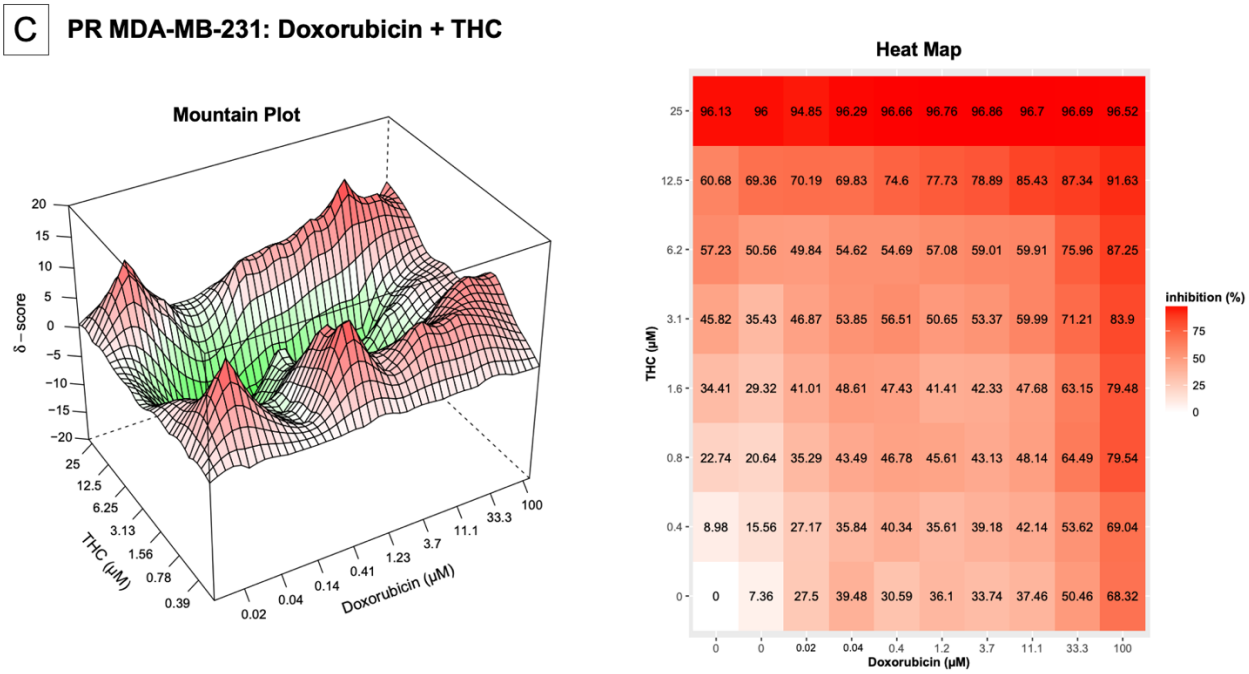


Figure 3.11. Assessment of synergy between doxorubicin and Δ-9-tetrahydrocannabinol. Cells were treated for 48 hours, and cell viability was measured with an AlamarBlue assay. Data was input into Synergy Finder (2.0) to generate 3D mountain plots outlining synergy scores across the dose range, and corresponding 2D heat maps showing percent inhibition data used to calculate synergy scores. Mountain plots and heat maps were captured for the combination of doxorubicin with THC in (A) MCF-7 cells, (B) MDA-MB-231 cells, and (C) PR MDA-MB-231 cells. Some doses on heat maps rounded to the nearest integer, tenth or hundredth. Data presented as mean of 3 independent experiments.

Table 3.2. Summary of synergy scores for the combination of doxorubicin with Δ-9-tetrahydrocannabinol.

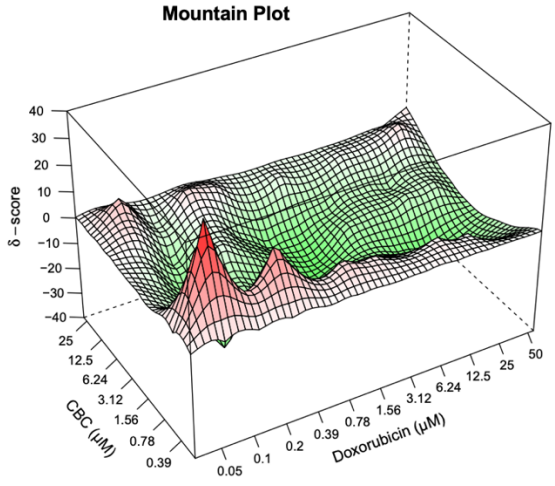
Synergy Score	[THC]	[Doxorubicin]
	MCF-7	
28.82	0.39 μM	0.2 μM
27.56	0.39 μM	0.1 μM
8.89	12.5 μM	0.2 μM
-14.46	12.5 μM	0.39 μM
-15.85	6.25 μM	0.78 μM
-16.05	0.78 μM	0.1 μM
	MDA-MB-231	
7.38	3.12 μM	0.98 nM
3.83	0.39 μM	62.5 nM
3.74	0.39 μM	0.98 nM
-14.27	1.56 μM	3.9 nM
-16.34	6.25 μM	125 nM
-18.24	1.56 μM	7.8 nM
	PR MDA-MB-231	
15.72	12.5 μM	11.1 μM
14.85	0.39 μM	0.41 μM
14.28	0.39 μM	0.02 μM
-13.24	6.25 μM	1.23 μM
-16.70	6.25 μM	0.04 μM
-17.97	6.25 μM	0.14 μM

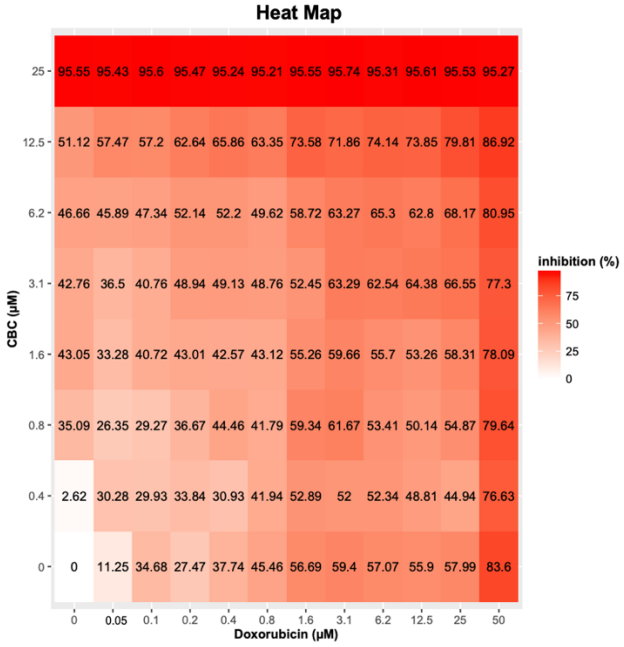
Scores in red are synergistic, black are additive, and green are antagonistic.

3.12 Doxorubicin with CBC exerted additive inhibitory effects on cell viability

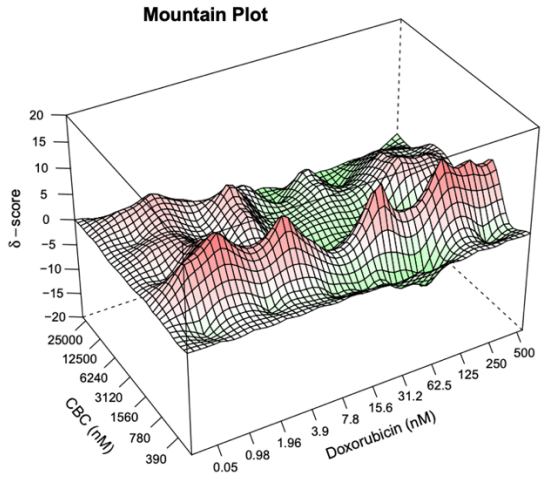
The combination potential of doxorubicin with cannabichromene was assessed in MDA-MB-231, MCF-7, and PR MDA-MB-231 cells. Cells were treated for 48 hours, and cell viability was assessed with an AlamarBlue assay. We found that doxorubicin with cannabichromene exerted predominantly additive inhibitory effects on cell viability in all three cell lines tested, while some concentrations produced synergistic and antagonistic effects (Figure 3.12). In MCF-7 cells, the majority of combinations produced scores in the low additive range between -10 and 0 (Figure 3.12 A). In addition, quite a few scores that fell below -10. The highest synergy score was 38.3 at 0.39 μM CBC and 0.05 μM doxorubicin. In MDA-MB-231 cells, most combinations of doxorubicin and CBC exerted additive inhibitory effects with synergy scores between -5 and 10 (Figure 3.12 B). There were a few synergy scores marginally above 10, the highest reached being 11.7 at 0.78 μM CBC and 31.2 nM doxorubicin. Similarly, in PR MDA-MB-231 cells most combinations of doxorubicin and CBC elicited additive effects on cell viability (Figure 3.12 C). The highest synergy score reached was 19.2 at 0.78 μM CBC and 0.14 μM doxorubicin. Some potential antagonistic effects were also observed between doxorubicin and CBC in PR MDA-MB-231 cells. Table 3.3 summarizes the three highest and lowest synergy scores produced for the combination of doxorubicin and CBC.

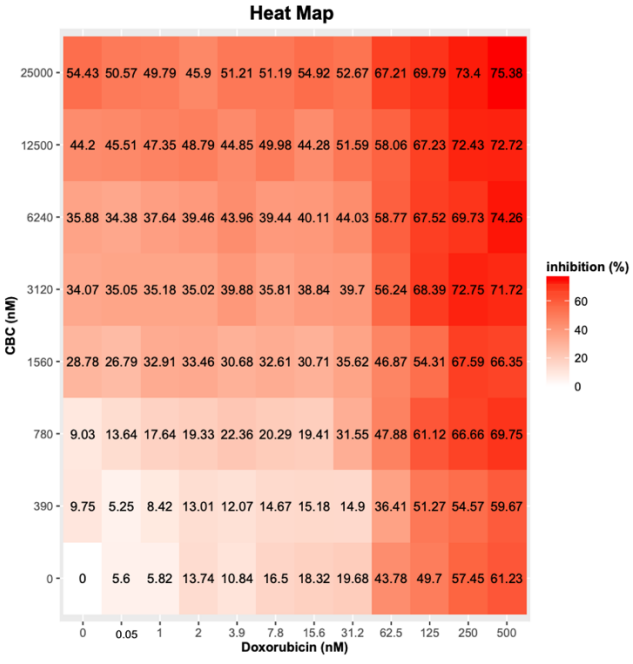
A **MCF-7: Doxorubicin + CBC**





B **MDA-MB-231: Doxorubicin + CBC**





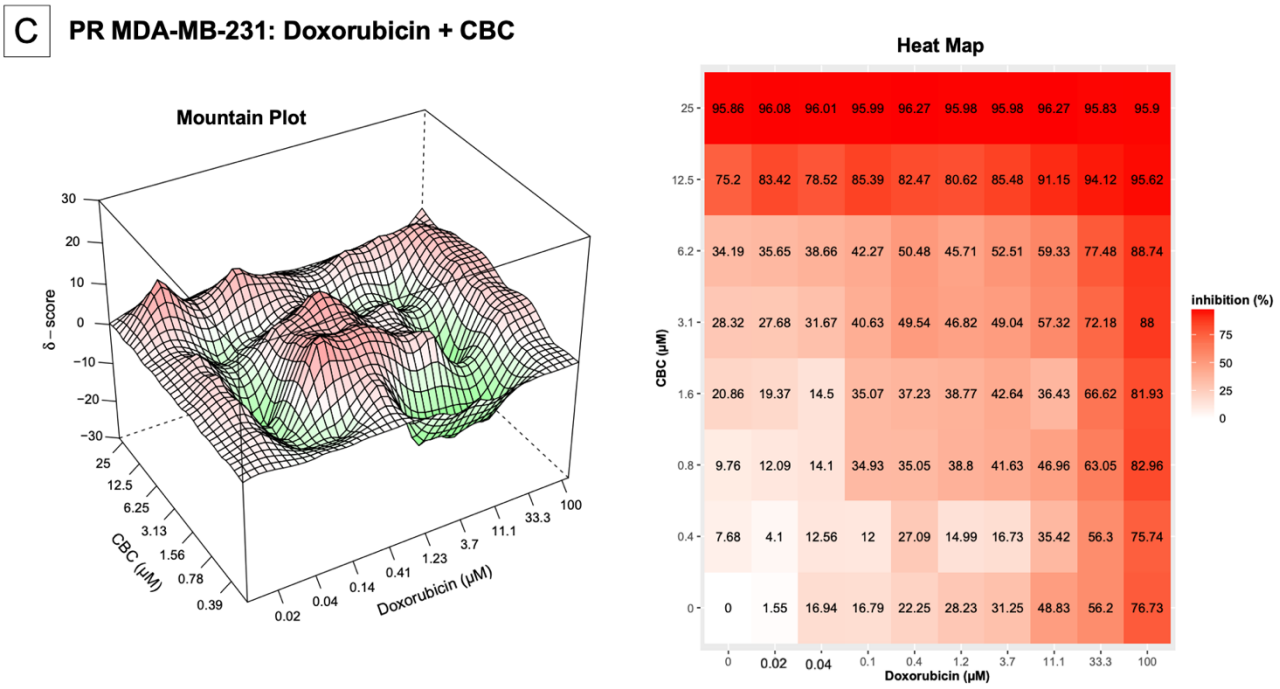


Figure 3.12. Assessment of synergy between doxorubicin and cannabichromene. Cells were treated for 48 hours, and cell viability was measured with an AlamarBlue assay. Data was input into Synergy Finder (2.0) to generate 3D mountain plots outlining synergy scores across the dose range, and corresponding 2D heat maps showing percent inhibition data used to calculate synergy scores. Mountain plots and heat maps were captured for the combination of doxorubicin with CBC in (A) MCF-7 cells, (B) MDA-MB-231 cells, and (C) PR MDA-MB-231 cells. Some doses on heat maps rounded to the nearest integer, tenth or hundredth. Data presented as mean of 3 independent experiments.

Table 3.3. Summary of synergy scores for the combination of doxorubicin with cannabichromene.

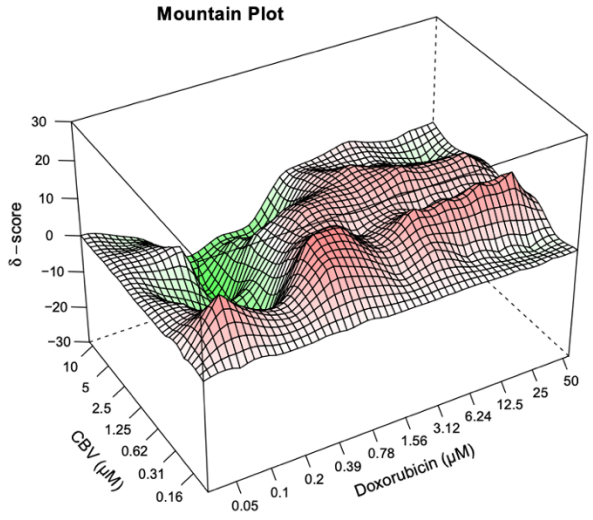
Synergy Score	[CBC]	[Doxorubicin]
	MCF-7	
38.30	0.39 μM	0.05 μM
20.45	0.39 μM	0.2 μM
10.32	12.5 μM	0.05 μM
-21.57	3.12 μM	1.56 μM
-23.84	1.56 μM	0.78 μM
-24.37	0.78 μM	0.1 μM
	MDA-MB-231	
11.66	0.78 μM	31.2 nM
11.62	0.78 μM	125 nM
11.15	0.78 μM	3.9 nM
-9.31	0.39 μM	62.5 nM
-10.54	1.56 μM	62.5 nM
-12.45	25 μM	1.96 nM
	PR MDA-MB-231	
19.19	0.78 μM	0.14 μM
13.37	0.78 μM	0.41 μM
11.27	12.5 μM	0.02 μM
-14.56	1.56 μM	0.04 μM
-14.85	0.39 μM	3.7 μM
-20.95	1.56 μM	11.1 μM

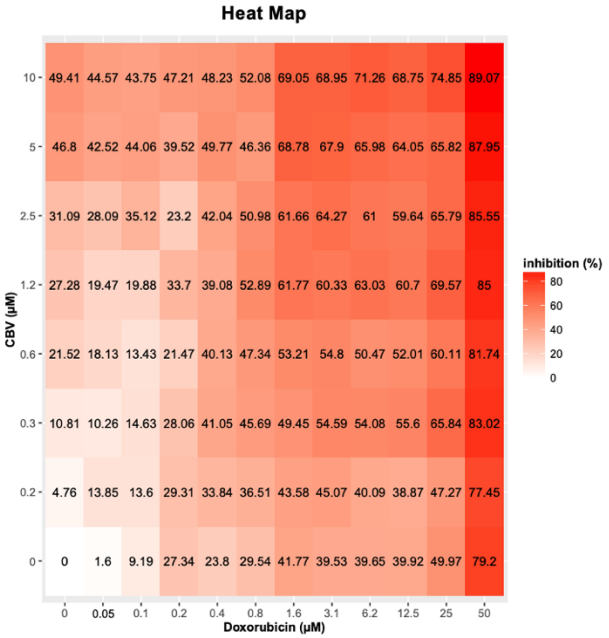
Scores in red are synergistic, black are additive, and green are antagonistic.

3.13 Doxorubicin with CBV elicited additive inhibitory effects on cell viability

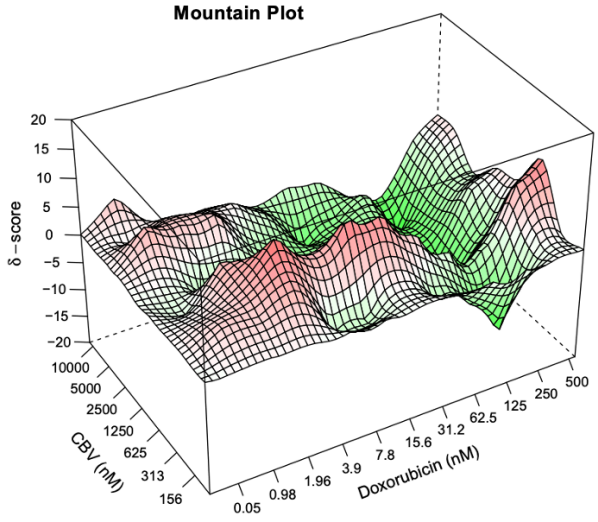
The final cannabinoid we explored in combination with doxorubicin was cannabivarin. Cells were treated with compounds for 48 hours, and cell viability was quantified by fluorescence using AlamarBlue reagent. In MCF-7, MDA-MB-231, and PR MDA-MB-231 cells we found that the combination of doxorubicin with cannabivarin produced primarily additive inhibitory effects on cell viability, however, depending on the concentrations, some synergistic and antagonistic effects were also observed. In MCF-7 cells, most synergy scores for doxorubicin with CBV fell between -10 and 10, however some higher and lower scores also occurred (Figure 3.13 A). The highest score was 13.84 at 0.31 μM CBV and 25 μM doxorubicin. Some low scores were also observed in MCF-7 cells. In MDA-MB-231 cells, doxorubicin and CBV exerted mostly additive effects with the majority of synergy scores falling between -10 and 10 (Figure 3.13 B). Almost no synergistic scores were observed for doxorubicin with CBV in MDA-MB-231 cells and the scores that did fall in the synergistic category were only marginally above 10. The highest score reached in MDA-MB-231 cells was only 11.04 at 0.313 μM CBV and 1.96 nM doxorubicin. Finally, in PR MDA-MB-231 cells, doxorubicin and CBV exerted generally additive effects as well (Figure 3.13 C). Similar to what we found in chemo-sensitive MDA-MB-231 cells, almost no synergistic scores occurred, and those that did were only slightly above 10. The highest score was 10.40 at 0.31 μM CBV and 33.3 μM doxorubicin. Table 3.4 summarizes the synergy scores for the combination of doxorubicin and CBV, presenting the three highest and lowest scores and their associated doses.

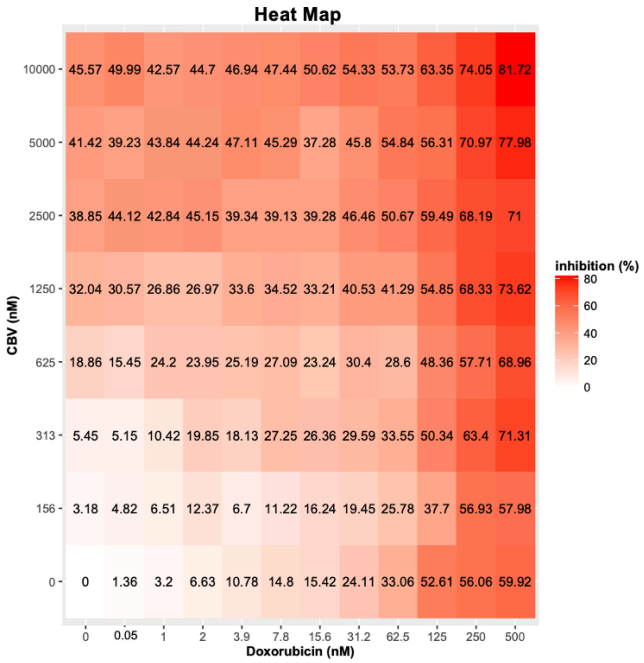
A **MCF-7: Doxorubicin + CBV**





B **MDA-MB-231: Doxorubicin + CBV**





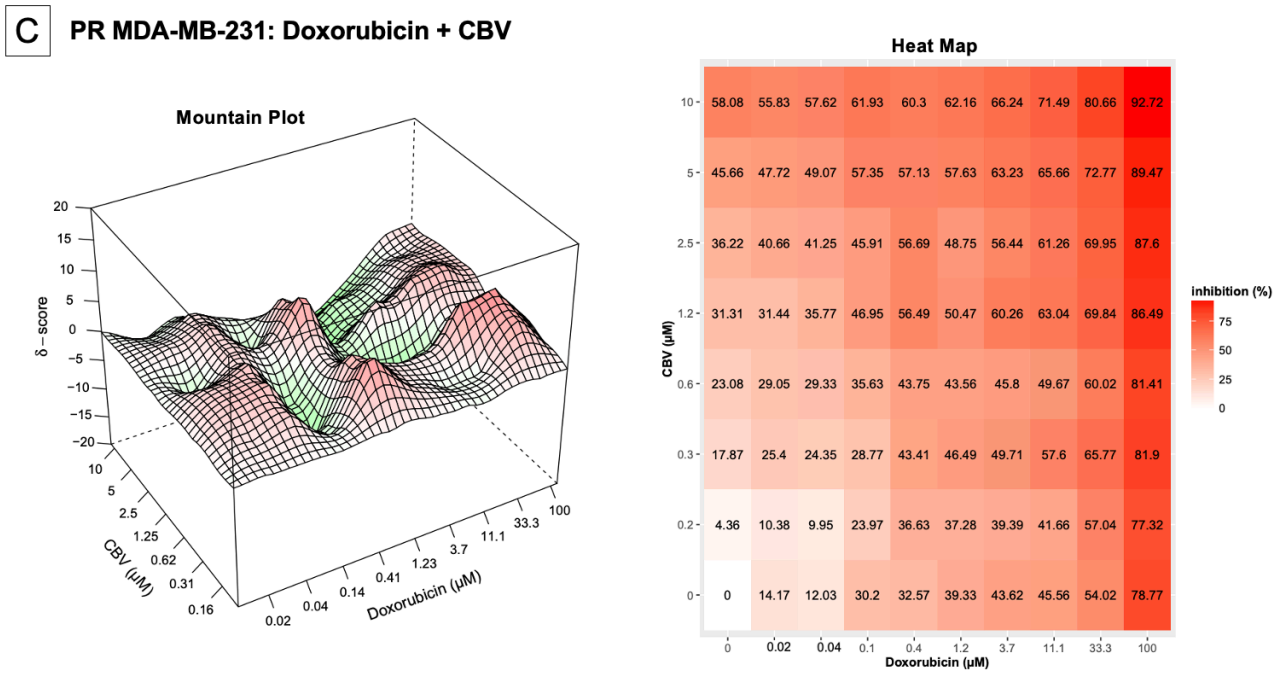


Figure 3.13. Assessment of synergy between doxorubicin and cannabivarin. Cells were treated for 48 hours, and cell viability was measured with an AlamarBlue assay. Data was input into Synergy Finder (2.0) to generate 3D mountain plots outlining synergy scores across the dose range, and corresponding 2D heat maps showing percent inhibition data used to calculate synergy scores. Mountain plots and heat maps were captured for the combination of doxorubicin with CBV in (A) MCF-7 cells, (B) MDA-MB-231 cells, and (C) PR MDA-MB-231 cells. Some doses on heat maps rounded to the nearest tenth. Data presented as mean of 3 independent experiments.

Table 3.4. Summary of synergy scores for the combination of doxorubicin with cannabivarin.

Synergy Score	[CBV]	[Doxorubicin]
	MCF-7	
13.84	0.31 μM	25 μM
13.82	0.16 μM	0.05 μM
13.75	0.31 μM	0.39 μM
-19.23	0.62 μM	0.2 μM
-20.71	5 μM	0.2 μM
-25.19	2.5 μM	0.2 μM
	MDA-MB-231	
11.04	0.313 μM	1.96 nM
10.62	0.313 μM	500 nM
10.47	0.313 μM	7.8 nM
-15.52	0.156 μM	125 nM
-15.56	5 μM	125 nM
-15.94	0.625 μM	62.5 nM
	PR MDA-MB-231	
10.40	0.31 μM	33.3 μM
10.24	0.31 μM	11.1 μM
8.99	1.25 μM	0.41 μM
-9.22	2.5 μM	1.23 μM
-9.93	0.62 μM	0.14 μM
-10.47	0.62 μM	3.7 μM

Scores in red are synergistic, black are additive, and green are antagonistic.

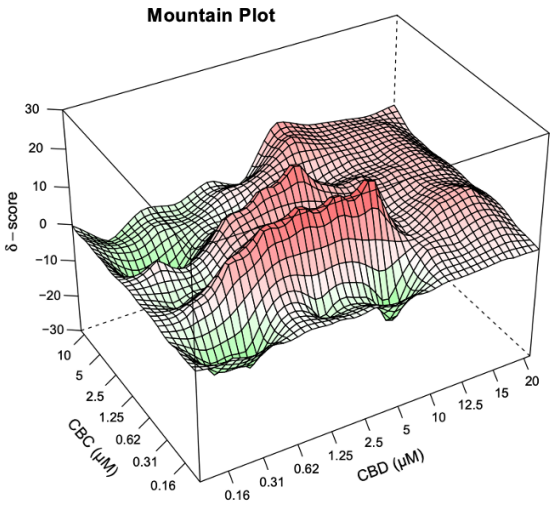
3.14 Cannabinoids in combination with CBD produced additive and synergistic inhibitory effects on cell viability

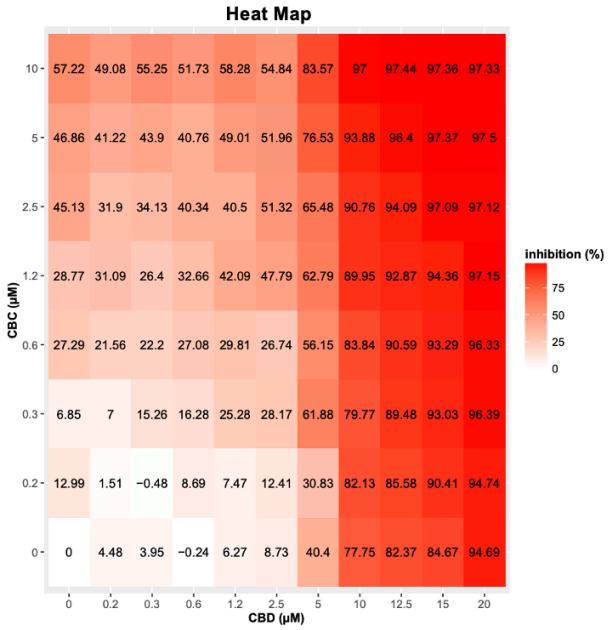
Multiple cannabinoids affect different targets and can modulate the effects of other cannabinoids at the same target (Blasco-Benito et al., 2018; Chung et al., 2019). For example, CBD has been shown to modulate the effects of THC at CB1 (Chung et al., 2019). In addition, whole botanical extracts and mixtures that contain more than one cannabinoid exert different, enhanced anti-cancer effects compared to cannabinoids alone (Blasco-Benito et al., 2018; Schoeman et al., 2020; Anis et al., 2021). As an extension of our preliminary studies that combined cannabinoid dose-response curves with a single concentration of CBD, we performed matrix assays to explore the combination of CBD with other cannabinoids. In MDA-MB-231 cells, we combined CBD with CBC, CBN or CBV in 8x11 grids. In each grid, the concentration of CBD ranged from 0 μM – 20 μM along the X-axis and the concentration of CBC, CBN, or CBV ranged from 0 μM – 10 μM along the Y-axis. Cells were treated for 48 hours, and cell viability was assessed using an AlamarBlue assay. We used Synergy Finder (2.0) to analyze cell viability data to assess the synergistic potential of cannabinoid combinations. Figure 3.14 presents the 3D mountain plots depicting the synergy scores over the dose range and the associated 2D heat map showing the percent inhibition achieved at each point within the matrix.

The first combination we explored was cannabidiol with cannabichromene. In MDA-MB-231 cells, we found that CBD with CBC exerted primarily additive effects, however, depending on the concentrations, possible synergistic and antagonistic interactions also occurred (Figure 3.14 A). Most of the synergy scores calculated fell between 0 and 10. The highest score for CBD with CBC reached 22.15 at 0.31 μM CBC

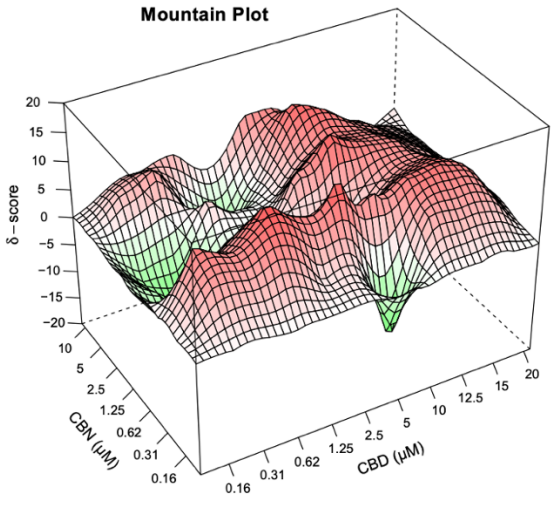
and 5 μM CBD. While a few low antagonistic scores were observed, most of the scores were in the higher additivity range. Next, we looked at the combination of cannabidiol with cannabinol. The combination of CBD with CBN produced primarily additive effects in MDA-MB-231 cells, however some synergistic and antagonistic scores occurred as well (Figure 3.14 B). The majority of scores fell between 0 and 10 and the highest score was 14.51 and 0.31 μM CBN and 2.5 μM CBD. There were very few scores that fell into the antagonistic range and almost all synergy scores were greater than 0 for the combination of CBD and CBN. Finally, we looked at the combination of cannabidiol with cannabivarin. CBD and CBV exerted mainly additive effects, however, many synergistic dose combinations occurred at higher concentrations of CBD (Figure 3.14 C). While the majority of scores fell between 0 and 15, the highest score was 41.52 at 0.31 μM CBV and 12.5 μM CBD. Only a single antagonistic score of -14.60 occurred for the combination of CBD and CBV at 0.31 μM of both drugs. Table. 3.5 summarizes synergy scores and associated doses for the combination of CBD with CBC, CBN or CBV.

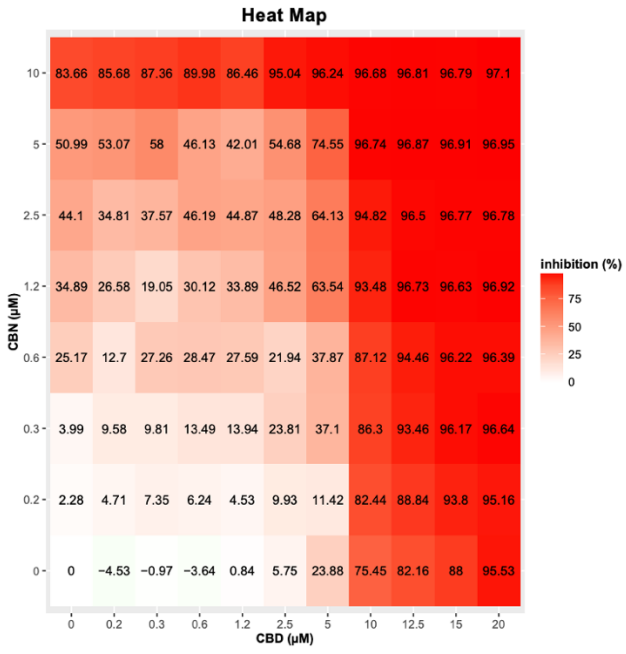
A **MDA-MB-231: CBD + CBC**





B **MDA-MB-231: CBD + CBN**





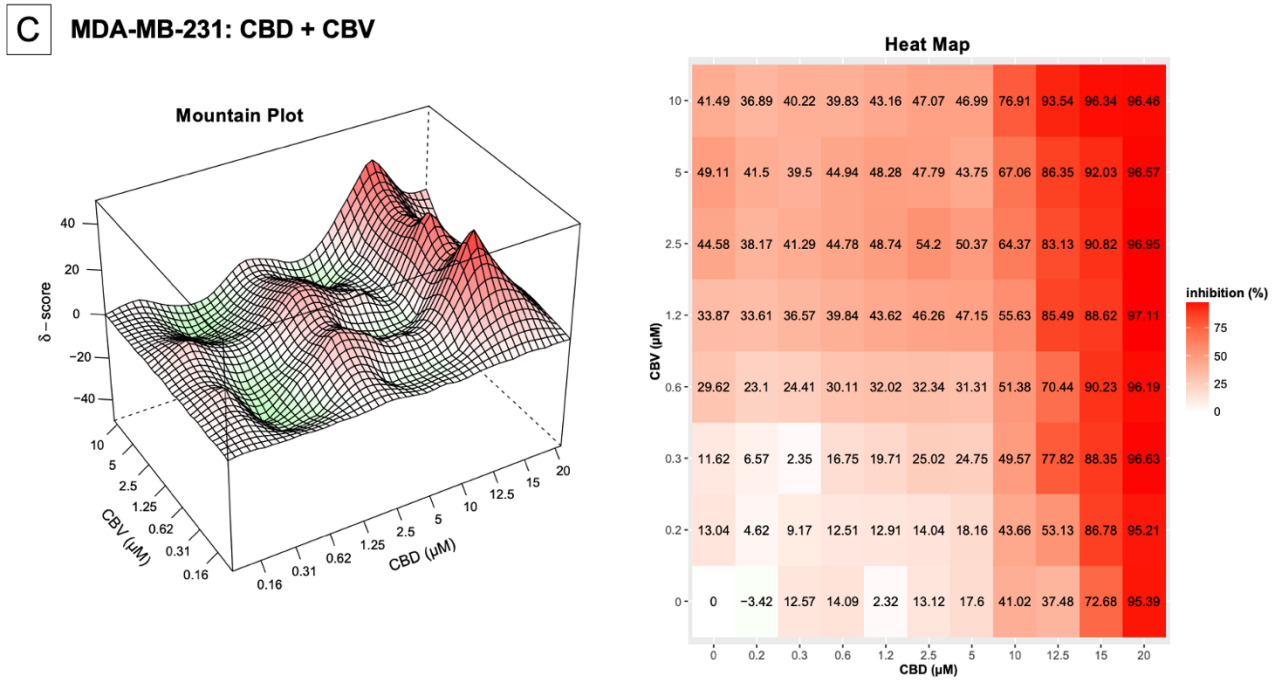


Figure 3.14. Assessment of synergy between cannabidiol and cannabichromene, cannabinol, or cannabivarin. Cells were treated for 48 hours, and cell viability was quantified using an AlamarBlue assay. Data was input into Synergy Finder (2.0) to generate 3D mountain plots outlining synergy scores across the dose range, and corresponding 2D heat maps showing percent inhibition data used to calculate synergy scores. Mountain plots and heat maps were captured for the combination of CBD with (A) cannabichromene, (B) cannabinol, and (C) cannabivarin in MDA-MB-231 cells. Some doses on heat maps rounded to the nearest tenth. Data presented as mean of 3 independent experiments.

Table. 3.5. Summary of synergy scores for the combination of cannabidiol with cannabichromene, cannabinol, or cannabivarin.

CBD + CBC			CBD + CBN			CBD + CBV		
Synergy Score	[CBD] µM	[CBC] µM	Synergy Score	[CBD] µM	[CBN] µM	Synergy Score	[CBD] µM	[CBV] µM
22.15	5	0.31	14.51	2.5	0.31	41.52	12.5	0.31
19.63	2.5	0.31	13.23	5	1.25	36.46	12.5	10
17.90	2.5	1.25	13.22	0.62	0.31	33.35	12.5	1.25
-10.84	0.16	0.16	-9.31	1.25	5	-8.63	0.31	0.16
-13.20	0.16	2.5	-14.07	5	0.16	-9.25	0.31	0.62
-18.45	5	0.16	-15.11	0.31	1.25	-14.60	0.31	0.31

Scores in red are synergistic, black are additive, and green are antagonistic.

3.15 Cannabinoids in combination with THC exerted predominantly additive inhibitory effects on cell viability

In addition to cannabinoids in combination with CBD, the final set of combination studies we performed were with THC and other cannabinoids. In MDA-MB-231 cells, THC was combined in matrix assays with cannabichromene or cannabivarin. Cells were treated for 48 hours with an array of cannabinoid combinations, and cell viability was assessed with AlamarBlue. We used Synergy Finder (2.0) to analyze data and calculate synergy scores for each dose combination tested. Looking at the combination of THC with CBC, the majority of dose combinations resulted in scores between 0 and 13, indicative of mostly additivity (Figure 3.15 A). In addition to this, some higher scores in the synergistic range occurred at high concentrations of both drugs. The highest score was 26.07 at 10 μM CBC and 15 μM THC. Only two scores fell into the antagonistic range for the combination of THC and CBC, the lowest being -13.91 at 0.62 μM CBC and 15 μM THC. The combination of THC with cannabivarin also produced primarily additive effects, with most of the synergy scores falling between -10 and 5 (Figure 3.15 B). Depending on the concentrations, a few synergistic and antagonistic scores also occurred. At high concentrations of both THC and CBV, some higher synergy scores were observed, the highest reaching 22.18 at 12.5 μM THC and 10 μM CBV. Table 3.6 summarizes synergy scores for the combination of THC with CBC or CBV and their associated doses.

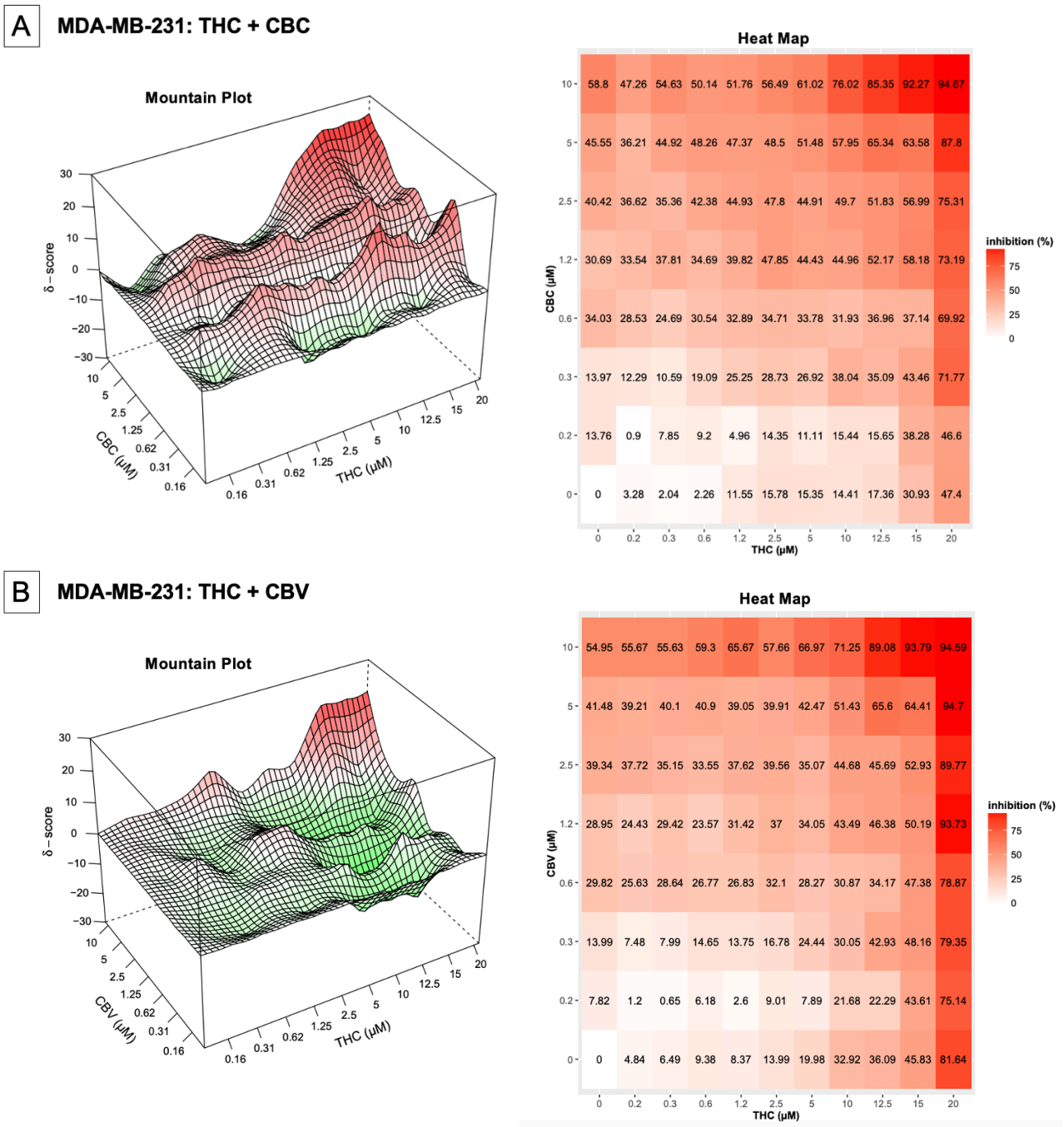


Figure 3.18. Assessment of synergy between Δ-9-tetrahydrocannabinol and cannabichromene or cannabivarin. Cells were treated for 48 hours, and cell viability was quantified using an AlamarBlue assay. Data was input into Synergy Finder (2.0) to generate 3D mountain plots outlining synergy scores across the dose range, and corresponding 2D heat maps showing percent inhibition data used to calculate synergy scores. Mountain plots and heat maps were captured for the combination of THC with (A) cannabichromene, and (B) cannabivarin in MDA-MB-231 cells. Some doses on heat maps rounded to the nearest tenth. Data presented as mean of 3 independent experiments.

Table 3.6. Summary of synergy scores for the combination of Δ-9-tetrahydrocannabinol with cannabichromene or cannabivarin.

THC + CBC			THC + CBV		
Synergy Score	[THC] μM	[CBC] μM	Synergy Score	[THC] μM	[CBV] μM
26.07	15	10	22.18	12.5	10
25.24	12.5	10	22.11	15	10
23.89	20	0.31	11.49	1.25	10
-9.71	0.16	10	-14.83	12.5	0.16
-12.06	1.25	0.16	-18.54	12.5	0.62
-13.91	15	0.62	-19.50	10	0.62

Scores in red are synergistic, black are additive, and green are antagonistic.

Chapter 4: Discussion

4.1 General Overview

In Canada, breast cancer is the most commonly identified form of cancer in women, and it is estimated that 27,400 Canadian women were diagnosed with it in 2020 alone (Canadian Cancer Society, 2021). Fortunately, over the last few decades, breast cancer mortality has steadily declined due to earlier diagnosis and improved anti-cancer therapies, however, metastatic breast cancers still develop in 20-30 percent of women who are diagnosed with breast cancer (Ullah, 2019). Metastatic breast cancer remains difficult to treat, is often considered incurable, and is the primary cause of breast cancer-related mortality (Sledge, 2016). In the case of non-metastatic breast cancers, there are more treatment options available compared to those for metastatic cases, however there are still challenges that can arise during the treatment process, such as chemotherapeutic resistance. One of the first-line chemotherapeutic regimens recommended for the treatment of metastatic breast cancer is anthracyclines and taxanes (eg. doxorubicin and paclitaxel) (Rivera & Gomez, 2010; Zheng et al., 2015). This regimen can also be used in the treatment of non-metastatic advanced breast cancers (Canadian Cancer Society, 2021; Fujii et al., 2015). Unfortunately, in cases of advanced metastatic breast cancer, combination therapy regimens are still not overly effective, and single agents can be prescribed in order to limit adverse side effects (Zheng et al., 2015).

The development of resistance to chemotherapeutic anti-cancer agents remains a prominent challenge to overcome in the treatment of cancer patients (Rivera & Gomez, 2010). Breast cancer tumors can be either inherently resistant to chemotherapy drugs, or they can develop resistance following exposure (Choi, 2005). Tumors can develop

resistance to a multitude of chemotherapy agents with differing structures and mechanisms of action, making successful treatment even more challenging (Wind & Holen, 2011). Multidrug resistance is one of the key considerations to combination therapy regimens, where the objective is to use anti-cancer drugs with differing mechanisms of action to reduce the chances of developing resistance. Monotherapy cancer treatments tend to be more susceptible to resistance as a result of consistent exposure to a single compound, which can induce cancer cells to initiate changes to prevent cell death, such as up-regulating the expression of ABC efflux transporters (Mokhtari et al., 2017). Another important consideration in the treatment of breast cancer patients (and cancer patients in general) are the negative side effects as a result of systemic administration of chemotherapy. The previously described anthracycline-taxane based treatment regimen displays several adverse effects, such as cardiotoxicity produced by anthracyclines, chemotherapy-induced neuropathy by taxanes, and other physical effects such as alopecia, nausea, and vomiting (Jasra, 2018; Hammond et al., 2019; Sibaud et al., 2016).

With the overarching goal to help mitigate negative side effects, many cancer patients and caregivers inquire about the use of medical cannabis during cancer treatment (Birdsall et al., 2016). Phytocannabinoids produced by the cannabis plant act on CB1 and CB2 receptors, as well as other targets within the ECS such as GPR55 and TRPV1. The ECS functions to modulate mood, appetite, and pain sensation, which are all aspects that cancer patients likely struggle with while undergoing chemotherapy (Birdsall et al., 2016). A review by Kramer (2015) highlighted clinical studies that looked at the potential for oral cannabinoids or smoked cannabis to improve some commonly experienced

negative side effects experienced by patients treated with chemotherapy. As an example, it has been shown that cancer patients undergoing chemotherapy who were given oral cannabinoids or consumed cannabis via inhalation experienced reduced chemotherapy-induced nausea and vomiting (Chang et al. 1979; Chang et al. 1981; Sallan et al. 1975; Tramer et al. 2001). *In vivo* studies have also shown that CBD palliates the cardiotoxicity related to doxorubicin and taxane-induced peripheral neuropathy associated with paclitaxel (Ward et al., 2014; King et al., 2017; Hao et al., 2015).

Interestingly, in addition to their positive effects on the quality of life of patients undergoing chemotherapy, preclinical studies have shown that cannabinoids exert a multitude of anti-cancer effects in breast and other cancers. Many G-protein coupled receptors have been linked with tumor progression and subsequent metastasis, and cannabinoid receptors have been shown to be over-expressed in cancerous cells relative to their non-malignant phenotype (Pyszniak et al., 2016; Pisanti et al., 2013). GPCRs, such as CB1 and CB2, that can be activated by cannabinoids to promote cellular anti-tumor responses are an emerging therapeutic target in cancer research (Moreno et al., 2019). To date, most of the research surrounding the anti-cancer potential of cannabinoids in breast and other cancers has been performed with pure Δ-9-tehtrahydrocannabinol or cannabidiol in preclinical studies. In addition to the preclinical work, a few clinical studies have shown the beneficial effects of cannabinoids. THC and CBD have been shown to increase the survival of glioblastoma multiforme patients and studies support them as co-treatments (NCT01812603; NCT01812616; Likar et al., 2021). The cannabis plant also produces multiple cannabinoids other than THC and CBD that have been far less extensively explored for their anti-cancer effects. Cannabinoids

may offer a novel, targeted treatment possibility to breast cancer patients at earlier stages to slow tumor progression, and to advanced patients that are limited in other effective treatment options (Kiskova et al., 2019).

Given the issues that are faced during the treatment of breast cancer, such as chemotherapeutic resistance, general drug unresponsiveness, and the adverse effects associated with treatment, there is a clear need for the investigation of novel agents in the management of breast cancer. The purpose of the present study was to investigate the anti-cancer potential of a variety of cannabinoids in *in vitro* models of breast cancer. We sought to reinforce the well-described anti-cancer effects of Δ-9-tetrahydrocannabinol and cannabidiol, as well as a few lesser characterized cannabinoids–cannabichromene, cannabivarin, cannabinol, cannabigerol, and tetrahydrocannabivarin–in breast cancer cells. We looked at the effects of individual cannabinoids in terms of their cytotoxicity and ability to induce apoptosis and reduce invasion of breast cancer cell lines, including multidrug resistant forms. Following the characterization of individual cannabinoids, we investigated the synergistic cytotoxic potential of cannabinoid combinations as well as cannabinoids in combination with chemotherapy, since previous literature has shown that, relative to monotherapy, enhanced anti-cancer effects occur when cannabinoids are combined with other cannabinoids or existing chemotherapeutic agents (Blasco-Benito et al., 2018, Marcu et al., 2010; Lopez-Valero et al., 2018; Garcia-Morales et al., 2020).

4.2 Cannabinoids are cytotoxic to breast cancer cells *in vitro*

Cell viability assays are one of the first essential steps in drug screening, development, and discovery. AlamarBlue is a redox indicator that is often used in bioassays to evaluate cellular health and explore the cytotoxicity of compounds, and it is

commonly used in cancer research and drug screening (Rampersad, 2012). In the first part of this project, we used AlamarBlue cell viability assays to assess the cytotoxicity of individual cannabinoids in breast cancer cells to select which cannabinoids are effective and at what concentration ranges. We used four breast cancer cell lines in this study: chemo-sensitive MDA-MB-231 and MCF-7 cells, as well as a chemo-resistant version of each. The chemo-resistant cells were originally developed to be resistant to paclitaxel, hence why they are referred to as paclitaxel-resistant (PR) cells (Hall et al., 2017). We began our investigation by generating dose response curves for cannabinoids ranging between 0 and 10 μM in all four breast cancer cell lines. This range of concentrations was selected based on the previous work of others and our lab that reported cannabinoid effects in this range (Borrelli et al., 2014; Tomko et al., 2019; Scott et al., 2017).

We verified the well-documented dose-dependent cytotoxicity of THC and CBD in all of our breast cancer cell lines (Figures 3.1–3.2). As previously mentioned, THC and CBD are by far the most extensively studied cannabinoids in cancer research, so we expected to see cytotoxicity of these compounds. Although the cytotoxic effects of THC and CBD have been previously reported in breast cancer cells, specific IC_{50}s had not yet been documented in the literature at the time of our study; this has since changed and one other study recently published IC_{50}s for THC and CBD in breast cancer cells (Schoeman et al., 2020) . In addition, to our knowledge, no other studies have reported the effects of THC or CBD in chemotherapy-resistant breast cancer models. In MDA-MB-231 cells, IC_{50}s for CBD and THC were 3.5 and 8.1 μM, respectively. In MCF-7 cells, IC_{50}s for CBD and THC were slightly higher at 4.5 and 8.7 μM, respectively. In PR MDA-MB-231 and PR MCF-7 cells, IC_{50}s for CBD and THC were similar to what was found in

their chemo-sensitive counterparts. A few other studies have reported IC_{50} values for THC and CBD, both in breast and other cancer cell lines, such as prostate, colon, glioma, and leukaemia (Schoeman et al., 2020; Goncharov et al., 2005; Scott et al., 2017; Ligresti et al., 2006). A recent study by Schoeman et al. (2020) looked at the cytotoxicity of a few cannabinoids, including CBD and THC, in both MDA-MB-231 and MCF-7 cells. They reported higher IC_{50}s than what we found in our study, however consistent with our results, THC had a significantly higher IC_{50} value than CBD in both cell lines. In our initial cell viability assays to generate dose response curves, cells were treated for a total of 48 hours, however, cannabinoid treatments were replaced at approximately 24 hours. This could explain why our IC_{50}s were lower than what Schoeman et al. (2020) found, as they did not replenish treatments while assessing cell viability and the other experimental parameters (eg. number of cells seeded per well) were nearly identical to ours. Although there is no published data regarding the half-life of cannabinoids *in vitro*, the half-life of CBD is estimated to be between 18 and 32 hours in humans, so we replenished treatments at 24 hours (Devinsky et al., 2014). Other *in vitro* studies performed with different cancer cells (eg. leukaemia) have reported IC_{50}s for THC and CBD, where similar to what we found, THC appears less potent at reducing cell viability than CBD (Scott et al., 2017). It has also been shown that MDA-MB-231 cells seem to be generally more susceptible to cannabinoid treatments compared to MCF-7 cells (Schoeman et al., 2020), which we confirmed in our results.

Cannabichromene, cannabivarin, cannabinol, and tetrahydrocannabivarin produced dose-dependent inhibition of cell viability in all four breast cancer cell lines tested (Figures 3.1–3.2), as we expected. To date, there is very limited research

surrounding the pharmacological characteristics and anti-cancer potential of these cannabinoids, however, a few studies have shown some anti-cancer effects *in vitro* (Baek et al., 1996; Baek et al., 1998; Ligresti et al., 2006; De Petrocellis et al., 2013; Borrelli et al., 2014; McAllister et al., 2007; Holland et al., 2007; Schoeman et al., 2020; Anis et al., 2021). In chemo-sensitive MDA-MB-231 and MCF-7 cells, we found that CBV was the most potent inhibitor of cell viability, producing the lowest IC_{50}s (3.1 and 4.0 μM, respectively) observed across all cannabinoids tested. CBV also produced the strongest effects on cell viability in PR MDA-MB-231 cells compared with the other cannabinoids. There has been no published research surrounding the anti-cancer effects of CBV, however the results of this study indicate its potential as a novel potent inhibitor of cell viability in breast cancer cells. In PR MCF-7 cells, CBV and CBN were found to have nearly identical potency, with IC_{50}s falling at 6.7 and 6.6 μM, respectively. The Schoeman study (2020) reported higher IC_{50} values for CBN in MDA-MB-231 and MCF-7 cells than what we found, but as previously highlighted, this could be because of differences in treatment protocols. Only two other studies have been published that very briefly looked at the effects of cannabinol on cell proliferation, and the results supported that CBN exerts anti-proliferative effects on aggressive breast cancer and prostate cancer cells (McAllister et al., 2007; De Petrocellis et al., 2013). In all four breast cancer cell lines, THCV had the highest IC_{50}s, ranging between 10.3 and 22.5 μM depending on the cell line. THCV has been shown to exert cytotoxic effects in prostate cancer cell lines, with IC_{50} values above 17.5 μM, which is similar to what we found in our PR breast cancer cell lines (De Petrocellis et al., 2013). Only one study has briefly looked at the effects of CBC in breast cancer cells, where it inhibited cell viability and demonstrated

similar potency to THC (Ligresti et al., 2006). In our study, we found that, depending on the cell line, CBC had slightly higher or very similar potency to THC in terms of cell viability reduction. In prostate carcinoma cells, CBC was found to have similar potency to CBD as an inhibitor of cell viability (De Petrocellis et al., 2013). Another study found that CBC was able to inhibit the growth of colorectal cancer cells, but only at a concentration of 30 μM (Borrelli et al., 2014). We found that CBC was able to inhibit cell viability of breast cancer cells at much lower concentrations than 30 μM, however, our treatment time was 48 hours compared to only 24 hours in the Borrelli (2014) study.

Cannabigerol was the only cannabinoid that did not demonstrate significant inhibitory effects on cell viability in any of the breast cancer cells used (Figure 3.1 G –3.2 G). While only a handful of studies have been performed that explored CBG's anti-cancer potential, promising results were observed. Two early studies by Baek et al. (1996, 1998) found that CBG exerted significant inhibitory effects on the proliferation of mouse skin melanoma cells with an IC_{50} of 9.5 μM, and that CBG was the most effective cannabinoid tested at reducing the cell viability of oral epithelioid carcinoma cells. More recently, CBG showed cytotoxic effects and IC_{50} values of 28.4 and 31.45 μM in MDA-MB-231 and MCF-7 cells, respectively (Schoeman et al., 2020). In the present study, we were unable to observe CBG's documented effects on cell viability at the concentrations tested (0–10 μM). The experimental methods that we used in this study were very consistent with those used by Schoeman et al. (2020), in fact, most of the cannabinoids that we tested were more potent at reducing cell viability than what they reported, likely because we replenished treatments at ~24 hours. To confirm that our results were not due to a defective drug batch or problems with the cells, we purchased another batch of CBG

from the same manufacturer and grew new cells. The CBG was readily dissolved in methanol at a concentration of 1 mg/mL and should not have been coming out of solution at that concentration, as it is soluble in methanol up to 25 mg/mL (Cayman Chemical). We observed the same lack of effects even with the new cells and CBG. Thinking that we simply were not reaching a high enough concentration of CBG to generate effects, we tried increased concentrations up to 50 μM and still did not observe significant inhibitory effects (data not shown), therefore we decided to move forward, as we had six other cannabinoids that successfully reduced cell viability. It is still not clear why CBG did not cause significant reductions in cell viability as anticipated based on previous studies.

4.3 Cannabinoids induce apoptosis and reduce invasion of aggressive breast cancer cells

Controlled, regulated cell death, otherwise referred to as apoptosis, is a key outcome for anti-cancer agents. Cannabinoids have been shown to induce apoptosis of cancer cells *in vitro*, however, most of this research has focused on THC and CBD (Reviewed by Tomko et al., 2020). In the present study, we wanted to determine if cannabinoids were reducing cell viability of breast cancer cells by inducing apoptosis. We used an Annexin V apoptosis assay to see if cannabinoids at a concentration of 2.5 μM were able to increase rates of apoptosis relative to cells treated with vehicle alone. The concentration of 2.5 μM was selected for the apoptosis assay because based on our dose-response curves, cannabinoids at this dose were not drastically reducing cell viability to a degree that would make it difficult to observe rates of early apoptosis. If we chose a higher concentration, many cells may have been dead, which would have made it difficult to detect apoptosis efficiently. In addition, compared to the cell viability assays,

the apoptosis assays required a lower cell seeding density to facilitate counting. This could allow cannabinoids at 2.5 μM to have increased effects compared to those observed at a higher seeding density, as it has been shown that rapidly dividing cells tend to have increased drug sensitivity when seeded at lower density (Haverty et al., 2016). Cells that stained positive for Annexin V were considered to be undergoing apoptosis, whereas cells that stained positive for propidium iodide (PI+) were late apoptotic or necrotic. Given that we observed higher rates of Annexin V-positive cells compared to PI-positive cells, we can conclude that early apoptosis was occurring more than late apoptosis or necrosis.

We confirmed that THC and CBD were able to induce apoptosis in MDA-MB-231 cells, and in addition, we found that the lesser characterized cannabinoids CBC, CBV, CBN, CBG and THCV induced apoptosis as well (Figure 3.4). Under vehicle control conditions, only 13.3 percent of cells were Annexin V+ compared to an average of 40 percent of cells under cannabinoid treatment conditions. Another study by our lab reported very similar basal rates of early apoptotic breast cancer cells under vehicle control conditions (Tomko et al., 2019). Cannabivarin and cannabidiol caused the largest increases in Annexin V staining, reaching 62 and 48 percent of cells, respectively. The other cannabinoids tested induced slightly lower and comparable levels of apoptosis, with on average 40 percent of cells staining Annexin V+. Select cannabinoids have been shown to induce apoptosis of cancer cells in other studies as well, including breast cancer. One study found that THC induced apoptosis in breast cancer cells through the activation of CB2 receptors by co-treating with THC and a selective CB2 antagonist, SR144528 (Caffarel et al., 2006). They found that THC lost its ability to induce apoptosis

in the presence of SR144528, indicating the role of CB2. Another study with glioma cells also found that THC treatment was able to induce apoptosis via a CB1-independent mechanism, suggesting the possible involvement of CB2 and sphingolipids (Sanchez et al., 1998). It has also been found that THC treatment significantly increases caspase-3 activity in breast cancer cells and that THC-mediated apoptosis may occur as a result of ceramide accumulation (Caffarel et al., 2006; Caffarel et al., 2010; Galve-Roperh et al., 2000). A few studies have shown the ability of cannabidiol to induce apoptosis of cancer cells as well. Two studies have suggested that in breast cancer and glioblastoma cells, CBD induces endoplasmic reticulum stress and results in the production of reactive oxygen species (ROS) and subsequent apoptosis (Shrivastava et al., 2011; Singer et al., 2014). To date, only a single study has shown the ability of phytocannabinoids other than THC or CBD to induce apoptosis in cancer cells. Cannabigerol stimulated apoptosis in colorectal cancer cells by increasing ROS production, which is in agreement with other studies that suggest the role of ROS in cannabinoid-mediated apoptosis (Borrelli et al., 2014). To our knowledge, the findings that CBC, CBV, CBN and THCV induce apoptosis of breast cancer cells is novel, and the complex molecular mechanisms behind cannabinoid-induced apoptosis are not well characterized.

The development of secondary tumors in a different location of the body relative to the primary tumor is termed metastasis. Metastases occur in 20-30 percent of women diagnosed with breast cancer and are the primary cause of treatment failure and mortality (Ullah, 2019; Fares et al. 2020). Despite this, tumor metastasis continues to remain poorly understood, however the activation of invasion is a hallmark of cancer metastasis (Hanahan & Weinberg, 2011; Fares et al., 2020). The five main contributing steps to

tumor metastasis are: activation of invasion, intravasation, circulation, extravasation, and final colonization in a new tissue (Fares et al., 2020; Figure 4.1). Tumor cell invasion is the first key step in the metastasis cascade and if it can be avoided through treatment, metastases may be prevented. Cannabinoids (THC and CBD) have been shown to reduce the invasion of cancer cells in a few studies, which prompted us to investigate the anti-invasive potential of other cannabinoids. THC and/or CBD reduced the invasion of breast cancer, glioma, non-small cell lung cancer, melanoma, hepatocellular carcinoma, and endometrial cancer cells (Elbaz et al., 2015; Alharris et al., 2019; Blazquez et al., 2008; Preet et al., 2008; Milian et al., 2020; Verykiou et al., 2019; Leelawat et al., 2010; Ramer et al., 2008).

We sought to corroborate the anti-invasive properties of THC and CBD and explore other cannabinoids for their anti-invasive potential in breast cancer cells. We used a Matrigel invasion assay and cannabinoids at a concentration of 2.5 μM to characterize the invasion of PR MDA-MB-231 cells *in vitro*. Similar to the apoptosis assay, this dose of cannabinoids was selected because it was not drastically reducing cell viability (viability was ~80% at 2.5 μM), and in order to detect differences in invasion we needed to ensure we had viable cells capable of invading. We reinforced the anti-invasive properties of THC and CBD in PR MDA-MB-231 cells, and we determined that CBC, CBV, CBN, and THCV had anti-invasive properties *in vitro* as well. Under vehicle control conditions, ~40% of PR MDA- MB-231 cells invaded the Matrigel, while under cannabinoid treatment conditions invasion was significantly reduced to between 12 and 6.5 percent (Figure 3.5). Out of the cannabinoids tested, CBN produced the largest reduction in invasion to only 6.5 percent of cells. The lesser characterized cannabinoids

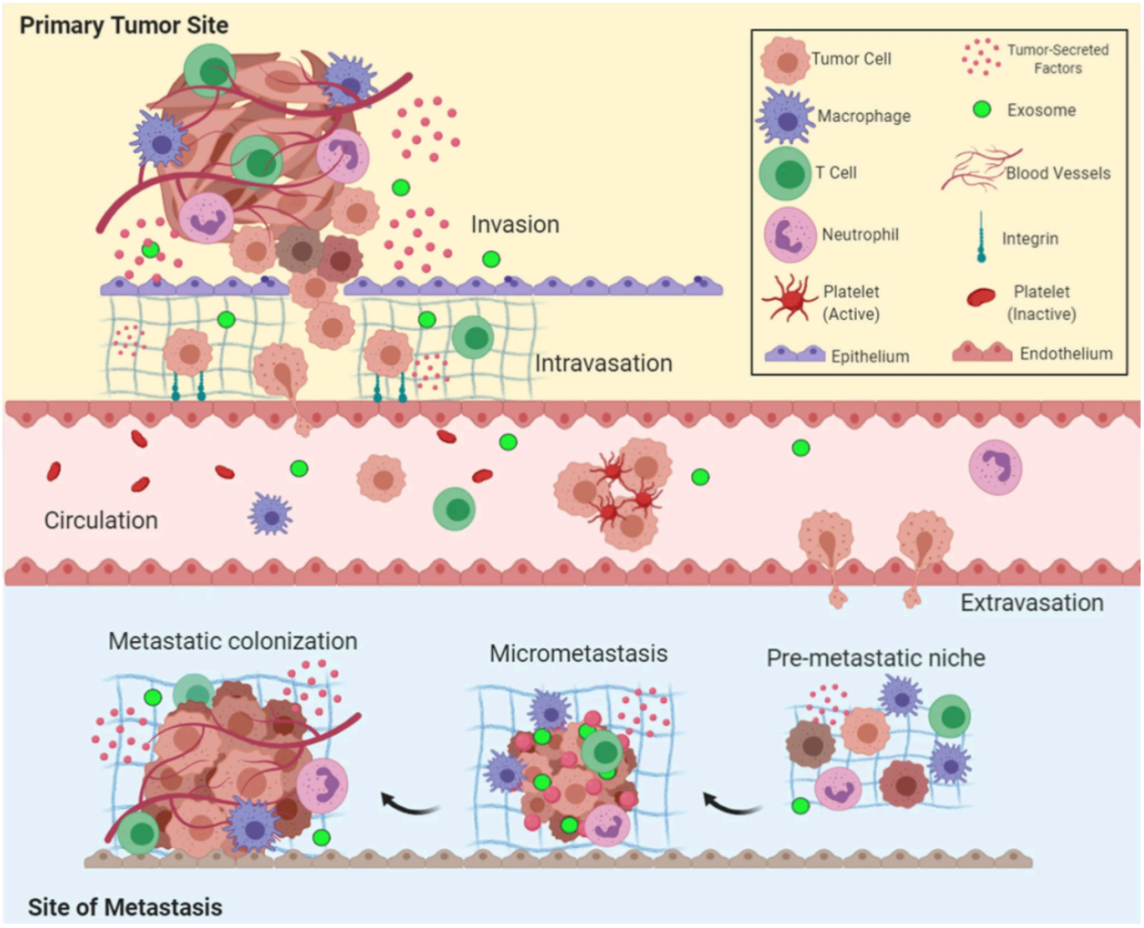


Figure 4.1. Five steps that cause metastasis of a primary tumor. There are 5 main steps that contribute to the metastasis of a tumor of a secondary site. (1) Invasion: tumor cell invasion is the initial step of the metastasis cascade, (2) intravasation: tumor cells invade the endothelium to enter circulation in the bloodstream or lymphatic vessels, (3) circulation: tumor cells circulate throughout the body, (4) extravasation: circulating tumor cells invade endothelium to enter a new tissue location, (5) colonization: tumor cells colonize a new site away from the primary site of tumor. The invasion of tumor cells is the first key step in the metastasis cascade and occurs throughout the successive steps, resulting in metastasis. Reprinted with permission from Springer Nature: Signal Transduction and Targeted Therapy (Fares et al. 2020), Copyright © 2020, The Authors: https://s100.copyright.com/AppDispatchServlet?title=Molecular%20principles%20of%20metastasis%3A%20a%20hallmark%20of%20cancer%20revisited&author=Jawad%20Fares%20et%20al&contentID=10.1038%2Fs41392-020-0134x&copyright=The%20Author%28s%29&publication=2059-3635&publicationDate=2020-03-12&publisherName=SpringerNature&orderBeanReset=true&oa=CC%20BY.

(non-THC/CBD) that we used in the present study have not been investigated for their anti-invasive capabilities in any cancer studies and our findings that CBC, CBV, CBN and THCV reduce invasion of breast cancer cells are novel.

Very little is known about the potential mechanisms by which cannabinoids exert their anti-invasive properties on cancer cells. The inhibition of matrix metalloproteinase (MMP) expression and function has been associated with the anti-invasion actions of cannabinoids. Matrix metalloproteinases are a family of endopeptidases that are capable of breaking down components of the extracellular matrix (ECM) that surrounds a solid tumor and they have been implicated to play an important role in the invasion of cancer cells (Reunanen & Kahari, 2000-2013). THC exposure reduced invasion and inhibited the expression of MMP-1 and MMP-2 in glioma cells (Blazquez et al., 2008). In cervical cancer cells, THC was found to reduce invasion by increasing the expression of tissue inhibitor of MMP-1 (TIMP-1), which further supports that cannabinoids may inhibit the invasive capabilities of cancer cells in part by inhibiting MMPs (Ramer et al., 2008). In breast cancer, epithelial-mesenchymal transition (EMT) has also been related to cannabinoids' anti-invasive properties. The EMT is a process that causes epithelial cells to transition into mobile mesenchymal cells, giving breast tumor cells invasive and metastatic properties (Foroni et al., 2012). Recently, CBD has been shown to inhibit EMT in aggressive breast cancer cells, which led to a reduction of invasion (Garcia-Morales et al., 2020). CBD was also shown to inhibit EMT of lung cancer cells, restore the epithelial phenotype, and reduce invasion (Milian et al., 2020). More studies are needed to determine how other cannabinoids inhibit cancer cell invasion and if the mechanism is the same or different than those described for THC and CBD.

4.4 Do cannabinoids kill non-cancerous breast epithelial cells?

An important consideration in the treatment of any cancer is the impact of treatment on healthy, non-cancerous cells in the body. In an ideal world, chemotherapy would only target and kill cancerous cells and leave healthy cells unaffected, however this is often not the case with the use of many chemotherapy agents. Chemotherapy is a type of systemic therapy that travels through the bloodstream to affect cancer cells and unfortunately, healthy cells (Canadian Cancer Society, 2021). The killing of healthy non-cancerous cells is why patients undergoing chemotherapy experience a variety of negative side effects, including hair loss, cardiotoxicity and reduced immune function. During the drug screening process in *in vitro* cancer studies, potential anti-cancer agents are often tested for cytotoxicity against non-tumorigenic cells. In breast cancer research, MCF-10A cells are used as a representative "normal" breast cell model (Qu et al., 2015). It is important to acknowledge that although these cells are non-tumorigenic, they have been immortalized and are not the same as primary mammary cells. A 2015 study by Qu et al. looked at the reliability of MCF-10A cells as a model of normal mammary epithelial cells and found that when cultured under 2-dimensional conditions as they were in the present study, they showed phenotypes that are consistent with normal breast cells.

It has been suggested that cannabinoids (phyto and synthetic) may be more targeted to cancerous cells relative to normal cells (Schoeman et al., 2020; Chakravarti et al., 2014; Casanova et al., 2003; Tomko et al., 2019). One study showed that a mixture containing THC, CBG, CBN and CBD totalling 40 μM did not significantly reduce the cell viability of MCF-10A cells (Schoeman et al., 2020). Another group at Newcastle University in collaboration with Australian Natural Therapeutics Group found that a

high-CBD content cannabis strain was able to kill leukaemia cells and did not kill normal bone marrow or white blood cells, however these findings remain to be published (University of Newcastle, 2020). The same group is investigating what makes cancer cells sensitive and normal cells seemingly resistant to cannabinoids as the next phase of their study. A study in our lab using abnormal CBD and its analog O-1602 also found that they did not affect the cell viability of MCF-10A cells (Tomko et al., 2019). Another study with synthetic cannabinoid WIN-55,212,2 found that it induced apoptosis in cancerous epidermal cells and not in normal epidermal cells (Casanova et al., 2003). In the present study, we examined the effects of individual cannabinoids on the cell viability of MCF-10A cells. Cells were treated at or above the concentrations that produced maximal effects on breast cancer cell viability, ranging from 10 to 75 μM. THC, CBD, CBC, CBV, CBN, THCV and CBG did not significantly reduce cell viability of MCF-10A cells at the concentrations used (Figure 3.3), reinforcing that cannabinoids may preferentially kill cancerous cells. It is not clear why cancer cells may be more susceptible to cannabinoids than healthy cells, however there are a few possible reasons that could be investigated further.

As briefly mentioned earlier, cancer cells have differential cannabinoid receptor expression compared to their normal counterparts, which could be a key contributing factor to cancer cells having increased sensitivity to cannabinoids. CB1 and CB2 are overexpressed in some prostate, colorectal, hepatocellular carcinoma, astrocytoma, glioma, and breast cancers (Chung et al., 2009; Gustafsson et al., 2011; Xu et al., 2006; Sanchez et al., 2001; de Jesus et al., 2010; Caffarel et al., 2010). Other targets that cannabinoids may exert their effects through are also differentially expressed in cancer

cells compared to normal cells. Transient receptor potential channels (TRP) have been shown to be involved in proliferation, migration and invasion of cancer cells and cannabinoids can be ligands at these receptors (Daris et al., 2019; Kiskova et al., 2019). TRPs are involved in the regulation of intracellular calcium which can be directly related to dysregulated gene transcription and calcium-dependent proliferative or anti-apoptotic pathways in cancer cells (Weber et al., 2016). TRPV1 has been shown to be up-regulated in breast cancer tissue compared to healthy breast tissue and CBD activation of TRPV1 induces ROS accumulation and ER stress in breast cancer cells (Weber et al., 2016; de la Harpe et al., 2021). It is therefore possible that the sensitivity of cancer cells to cannabinoids occurs as a result of differential receptor profiles compared to their normal phenotype.

Another interesting avenue that could contribute to the differential action of cannabinoids in cancer vs. non-cancer cells is the effect of cannabinoids on cholesterol and lipid raft integrity in the cell membrane. Unfortunately, the interaction between drugs and the cell membrane is often overlooked during drug screening studies (Knobloch et al., 2018). Cholesterol has been shown to play a significant role in the development of cancer and is a major component of lipid rafts in the cell membrane (Ding et al., 2019). Lipid rafts are lipid domains that are high in cholesterol and sphingolipid content and serve as platforms mediating cellular signal transduction. Lipid raft structure depends on the cholesterol and sphingolipid content, and changes in these components has been shown to impact cancer progression (Luo et al., 2017). It is well-established that cancer cells have a higher concentration of cholesterol and several agents have been shown to suppress the growth of tumor cells by disrupting lipid raft integrity through the alteration

of lipid rafts containing cholesterol (Beloribi-Djefaflia et al., 2016), and some literature suggests that cannabinoids may exert anti-cancer effects in this manner. A 2012 study by Wu et al. found that CBD induced apoptosis of microglial cells through a lipid raft-dependent mechanism. Upon further investigation, it was found that CBD resulted in lipid raft disruption through coalescence. It has been shown that elevated levels of cholesterol-rich lipid rafts in cancer cells (compared to their normal counterpart) is directly correlated with their sensitivity to apoptosis induced by cholesterol-targeting agents (Li et al., 2006). A large array of signaling proteins and receptors that regulate pro-oncogenic and apoptosis pathways reside in lipid rafts, making them a desirable target in cancer treatment. The potential action of cannabinoids at the membrane level could explain why MCF-10A and other normal cells do not appear as sensitive to the effects of cannabinoids, since cancer cells contain increased levels of cholesterol-containing lipid rafts susceptible to disruption (Li et al., 2006; Beloribi-Djefaflia et al., 2016). While there is some evidence that cannabinoids may mediate some beneficial anti-cancer effects via membrane disruption involving cholesterol and lipid rafts, this remains poorly understood.

4.5 Potential for cannabinoids in combination with chemotherapeutic agents

The combination of cannabinoids (THC and/or CBD) with established anti-cancer chemotherapeutic agents has been evaluated in a few studies. In breast cancer, it was recently shown that the combination of CBD (as a solution and encapsulated in polymeric nanoparticles) with paclitaxel or doxorubicin produced synergistic anti-proliferative effects *in vitro* (Fraguas-Sanchez et al., 2020). Two studies have demonstrated that combined treatment with THC and temozolomide (TMZ) was able to synergistically

reduce the growth of xenotransplated glioblastoma multiforme (GBM) tumors (Lopez-Valero et al., 2018; Lopez-Valero et al., 2018). In a two-part clinical study, the effects of TMZ with Sativex (1:1 THC:CBD) in GBM patients was further explored (NCT01812603; NCT01812616). It was found that the combination of TMZ with Sativex was able to increase the 1-year survival rate of GBM patients by 39 percent. In leukaemia, THC and/or CBD have been shown to sensitize the cells to anti-cancer agents, including cytarabine and vincristine (Scott et al., 2017; Liu et al., 2008; Holland et al. 2006). THC was able to decrease P-glycoprotein expression in multidrug resistant leukaemia cells, which sensitized the cells to vinblastine (Holland et al., 2006). Given previous literature that suggests enhanced anti-cancer effects following combination treatment with THC and/or CBD and chemotherapeutic agents, we wanted to evaluate the combination of chemotherapeutic agents with cannabinoids in breast cancer.

As a preliminary study, we first combined the individual cannabinoid dose-response curves with a single concentration of paclitaxel (470 nM) in PR MDA-MB-231 and PR MCF-7 cells to determine if the IC_{50}s could be reduced. We found that the addition of paclitaxel to CBD, THC, CBC, CBV, CBN, THCV or CBG curves did not cause desirable curve shifts or reduce IC_{50} values in either PR cell line (Figure 3.6–3.7). We then moved on to a much more comprehensive method for evaluating potential drug combinations using the previously described matrix assay. Doxorubicin is a commonly used anti-cancer agent in the anthracycline family of drugs. It is often used in the treatment of breast cancer and causes type 1 (irreversible) cardiotoxicity (Thomas, 2017). Due to the severity of doxorubicin-induced cardiotoxicity, we chose to explore the synergistic potential of doxorubicin in combination with cannabinoids. We evaluated its

cytotoxic ability in combination with four cannabinoids: THC, CBD, CBC and CBV. Chemo-sensitive MDA-MB-231 and MCF-7 cells as well as PR MDA-MB-231 cells were used throughout the matrix assays to assess the combination of doxorubicin and cannabinoids. We first produced dose-response curves for doxorubicin in each of the 3 cell lines to determine an appropriate dose range to be used in the matrix assays (Figure 3.10.1).

We found that the PR MDA-MB-231 cells were resistant to doxorubicin relative to the chemo-sensitive cells. Upon further characterization, the PR cells were found to over-express mRNA levels for *ABCB1* by 95,000-fold relative to chemo-sensitive cells (Hall et al., 2017). The *ABCB1* gene encodes P-glycoprotein ABC efflux transporter and based on its significant upregulation in the paclitaxel-resistant cells, it is responsible for conferring resistance. The Hall study (2017) also confirmed that the PR MDA-MB-231 cells were resistant to doxorubicin with an IC_{50} significantly higher at ~4μM than in the chemo-sensitive cells (IC_{50} ~0.5 μM). This was beneficial because it allowed us to evaluate doxorubicin in combination with cannabinoids in a resistant breast cancer model. We found that the combination of doxorubicin with THC, CBD, CBC or CBV exerted predominantly additive effects in all three cell lines used, however, depending on the concentrations, some synergistic or antagonistic effects also occurred (Figures 3.10.2–3.13). The majority of synergy scores produced across all four combinations fell between -10 and 10, which Synergy Finder (2.0) defines as likely indicative of additivity between drugs. Out of the combinations tested, doxorubicin with CBD or CBC produced the best overall effects and highest synergy scores across all three cell lines, while doxorubicin with THC or CBV produced overall lower synergy scores.

To our knowledge, there have been no other published studies that have evaluated the effects of doxorubicin with cannabinoids at such a large array of dose combinations with a matrix assay, let alone in a MDR model. While one study did examine the synergistic effects between doxorubicin and CBD in breast cancer cells, they only looked at three concentrations of doxorubicin and CBD, and the concentrations of CBD were very high ranging from 5 to 20 μM (Fraguas-Sanchez et al., 2020). They found some moderate synergistic effects between doxorubicin and CBD in MDA-MB-231 and MCF-7 cells. Interestingly, the same study found that the combination of doxorubicin with CBD had a more pronounced synergistic effect in estrogen receptor-positive MCF-7 cells compared to triple-negative MDA-MB-231 cells (Fraguas-Sanchez et al., 2020), which is consistent with what we found (Table 3.1). We also found that the combinations of doxorubicin with THC, CBC or CBV produced overall higher synergy scores in MCF-7 cells than in MDA-MB-231 cells. Intriguingly, the combination of doxorubicin with CBD, THC or CBC yielded higher synergy scores in the PR MDA-MB-231 cells than in either chemo-sensitive cell line. Although doxorubicin with cannabinoids produced primarily additive (rather than synergistic) effects on cell viability, these findings are important as there are still benefits to drug additivity. Importantly, we showed that doxorubicin and cannabinoids produce additive inhibitory effects in MDR breast cancer cells, which could also permit reductions in individual drug dosing to achieve the same anti-cancer effects. In addition, lower doxorubicin dosing would reduce doxorubicin-induced cardiotoxicity (Fraguas-Sanchez et al., 2020; Fouad et al., 2013). Our results also point to the potential for cannabinoids to sensitize MDR cancer cells to chemotherapeutic agents, which are otherwise difficult to treat.

4.6 Potential for cannabinoid combinations

In breast cancer, the effects of individual cannabinoids versus combinations of multiple cannabinoids has been recently explored. Current literature looking at the anti-cancer effects of cannabinoid combinations is mostly focused on combinations involving THC with CBD or THC with another cannabinoid. In 2018, Blasco-Benito and colleagues aimed to investigate the entourage effect that has been proposed between THC and other cannabinoids to result in an overall superior effect compared to THC alone. They found that a whole botanical cannabis extract containing THC, THCA and CBG was more potent at inhibiting breast tumor growth *in vivo* than was THC alone. In glioblastoma, the addition of CBD to THC treatment increased the efficacy of THC and produced synergistic anti-cancer effects (Marcu et al., 2010). More recently, THC, CBD, CBN and CBG as a single combination (C6) was found to exert synergistic dose-reductions in MDA-MB-231 and MCF-7 cells while leaving non-tumorigenic MCF-10A cells unaffected (Schoeman et al., 2020). Upon further investigation, it was determined that C6 exerted its effects on MCF-7 cells by causing cell cycle arrest and subsequent induction of apoptosis. Another recent study showed that THC and CBC together had synergistic properties against bladder urothelial carcinoma and lead to cell cycle arrest, reduced migration and induced apoptosis (Anis et al., 2021). A recent study in our lab also showed that CBD and THC display synergistic properties when combined with other cannabinoids like CBC or CBV in bladder cancer cells, however this study has not yet been peer reviewed (Tomko et al., 2021).

The final part of this work evaluated the synergistic anti-cancer potential of cannabinoid combinations on breast cancer cells. We first combined the dose-response

curves for CBC, CBV, CBN and THCV with a single concentration of CBD (2.5 μM) in all four of our cell lines. Cannabidiol was selected for this preliminary evaluation of cannabinoid combinations in part because some other studies have shown that cannabinoids, like THC, exert superior anti-cancer effects when combined with CBD (Marcu et al., 2010; Schoeman et al., 2020; Tomko et al., 2021). Another reason why we chose to combine curves with cannabidiol is because it lacks the intoxicating psychoactive effects that THC has and may have more clinical implications as a result (Afrin et al., 2020). The addition of 2.5 μM CBD to CBC, CBV, CBN and THCV dose-response curves was able to reduce IC_{50} values (Figures 3.8.1–3.9.2). The shifts in IC_{50} values due to the addition of CBD were statistically significant in all cases except THCV + CBD in PR MDA-MB-231 and MCF-7 cells (Figure 3.8.2 D, Figure 3.9.1 D). Following our investigation of the effect of CBD on cannabinoid dose-response curves, we combined the following cannabinoids in MDA-MB-231 cells using matrix assays to comprehensively assess dose combinations: CBD + CBC, CBD + CBV, CBD + CBN, THC + CBC, and THC + CBV. The combination of CBD with CBV produced the highest synergy scores, reaching a synergistic score of 41.52 at a high dose of CBD (Figure 3.14 C, Table 3.5). Each of the other cannabinoid combinations that we looked at also produced some scores indicative of synergy, and these scores tended to fall at higher concentrations of either CBD or THC (Figure 3.14–Figure 3.15). Another study also found that synergistic effects on cell survival of bladder cancer cells appeared to occur at higher phytocannabinoid concentrations (Anis et al., 2021). In our results, it was apparent that different drug interactions (eg. antagonism, additivity, synergism) occurred between cannabinoids depending on the doses of each drug. These results were consistent with

another recent breast cancer study showing that depending on the concentrations of cannabinoids used in each combination, effects varied between antagonism, additivity, and synergism (Schoeman et al., 2020).

4.7 Interpretation of synergy scores

It is important that synergy scores generated by Synergy Finder (2.0) for drug combinations be interpreted with caution. Synergy Finder makes it clear that their range of scores provided to indicate antagonism, additivity or synergism between two doses of drugs are likely indicative of these interactions and are not definitive. Currently, there is not a specific threshold that defines a "good" synergy score (SynergyFinder, 2021). The purpose of using matrix assays and programs like Synergy Finder (2.0) is to screen the potential of combined drugs over a large array of dose combinations to provide insight on which combinations may be effective and could be explored further, more rigorously. Part of the process in screening drugs for synergy is selecting one of several reference models that is used to calculate an expected response and compare it with the observed response. The Bliss Independence model has been widely used to assess drug combinations during the screening process (Zhao et al., 2014; Liu et al., 2018), and it is the reference model we chose to use in the present study. The Bliss Independence model assumes that the drugs being tested work independently through different mechanisms (Zhao et al., 2014). For example, doxorubicin and cannabinoids mediate their anti-cancer effects through different pathways; doxorubicin intercalates into DNA and cannabinoids act on GPCRs and channels to promote anti-cancer responses. The Bliss Independence model has also recently been used in studies to assess the synergistic potential between cannabinoids in bladder cancer and lymphoma, and between cannabinoids and

cannabinolic acids in colorectal cancer (Anis et al., 2021; Mazuz et al., 2020; Nallathambi et al., 2018). Unfortunately, there is no well-defined method to statistically assess the significance of synergy and due to some subtle differences in the reference model assumptions, there is a general lack of consensus regarding how to select an appropriate model and how to assess results in a statistically meaningful manner. It is important to recognize that the selected reference model can also change as knowledge of drugs changes.

The synergy scores provided by Synergy Finder can be interpreted as the excess response due to drug interactions (ie. a synergy score of 10 indicates 10 percent of response beyond expectation) (SynergyFinder, 2021). As a result, synergy scores should be looked at on an individual basis because they are relative to the overall level of inhibition achieved by combined drugs; an explanation of this premise follows. The following example depicts how not all synergistic scores indicate the same amount of absolute reduction in cell viability. Two drug combinations that yielded close synergy scores of 26 and 27 at specified doses are compared in Figure 4.2. The purpose of this figure is to illustrate that, although both cases meet the definition of synergy by the Bliss Independence model, the synergy scores are relative to the inhibition achieved by the combination of two drugs. In the case of doxorubicin (1.23 μM) with CBD (0.78 μM) that generated a synergy score of 27 (Figure 4.2 A), the overall inhibition (~45%) was less than that achieved by other drug combinations with a similar synergy scores. For example, THC (15 μM) with CBC (10 μM) yielded a synergy score of 26, yet there was a significantly larger overall reduction in cell viability (~90%) compared to the previous example with a similar synergy score (Figure 4.2 B). This highlights that not all

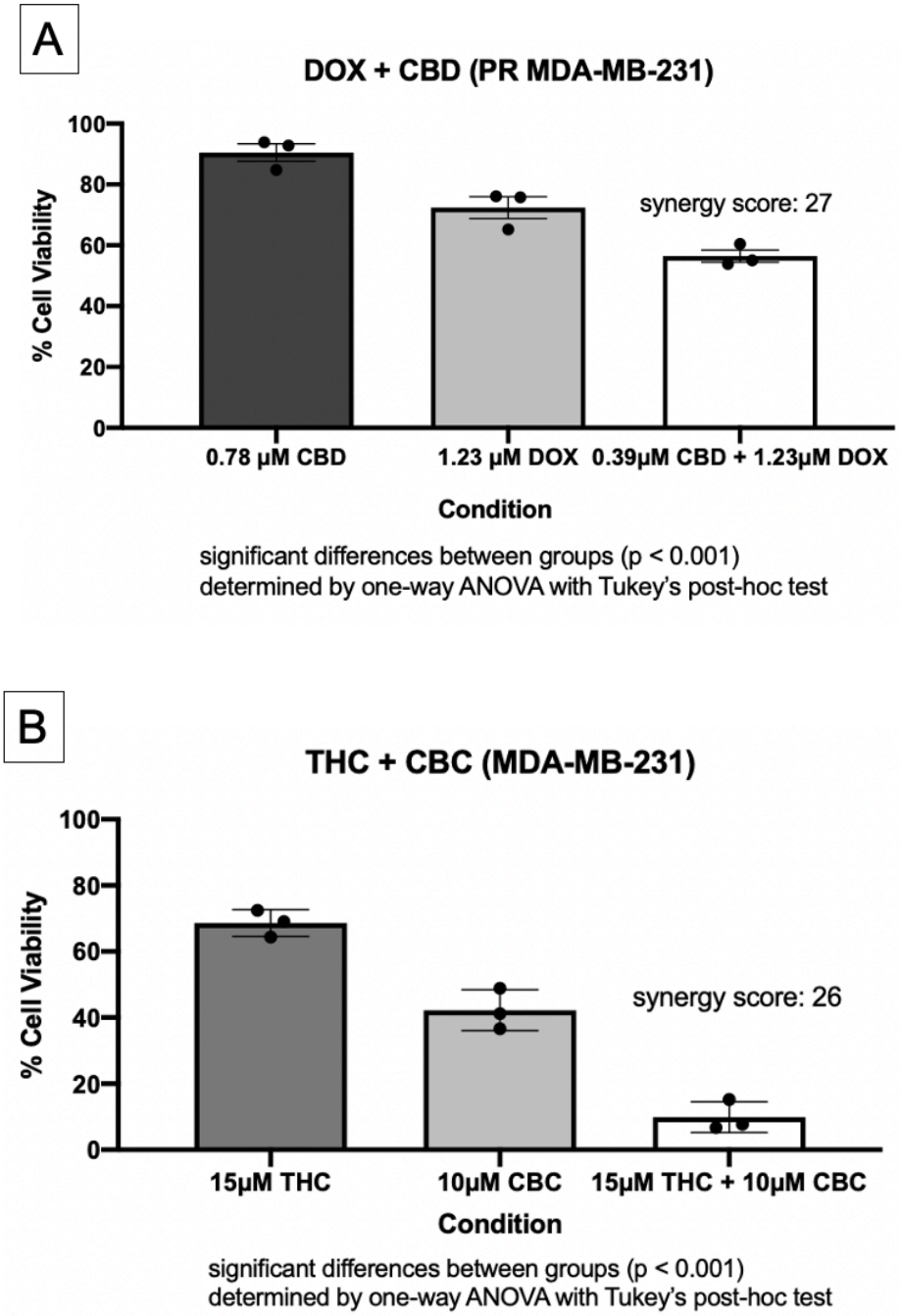


Figure 4.2. Comparison of similar synergy scores. An example to demonstrate why synergy scores should be looked at on an individual basis and how similar scores do not indicate the same absolute reduction of cell viability. (A) 1.23 µM doxorubicin with 0.78 µM CBD synergy score of 27, and (B) 15 µM THC with 10 µM CBC synergy score of 26. Although similar synergy scores, the absolute inhibition produced by each combination is significantly different.

synergistic scores reflect the same amount of absolute inhibition on cell viability and that synergy scores should be examined on an individual basis.

4.8 Study limitations and future directions

There are a few notable limitations to our study, and we recognize that we cannot make definitive conclusions based on our results alone and that further experimentation is required, which will be discussed. First, in our evaluation of apoptosis induced by cannabinoids using an Annexin V assay, we relied on accurate cell counting using a microscope. Although much more costly, an alternative method to investigate the induction of apoptosis is by performing Annexin V/PI staining followed by fluorescence activated cell sorting (FACS), which analyzes an entire sample and is less susceptible to human error than manually visualizing and counting cells (Wallberg et al., 2016). While the use of FACS would allow for a more accurate rate of apoptosis, it would not change our overall conclusion that cannabinoids induce apoptosis. Another limitation to our study was that although we covered a wide range of drug combinations in different breast cancer cell lines, we did not evaluate the effects of these same combinations in MCF-10A cells. We only examined the effects of individual cannabinoids in MCF-10A cells, however, even if we had subjected these cells to the drug combinations tested in this study, it would still not likely be reliably indicative of the effects *in vivo*, therefore it was not our priority. We simply wanted to exemplify that cannabinoids may preferentially kill cancerous cells as has been suggested by other studies (Schoeman et al., 2020; Chakravarti et al., 2014; Tomko et al., 2019; University of Newcastle 2020). A second limitation to our studies with MCF-10A cells is that we did not include a positive control to induce cell death; our lab has however found that other cannabis constituents

(eg. flavonoids) can reduce MCF-10A cell viability, indicating that they can be killed successfully. In the future, like other studies have done, treatment with camptothecin could be added as a positive control for growth inhibition of MCF-10A cells (Schoeman et al., 2020).

As previously discussed, synergy scores must be interpreted with caution and on an individual basis, and there is no well-defined method to statistically assess this type of synergy data. I propose that perhaps a simple way to assess the statistical significance of differences between individual and combined treatments is to perform one-way ANOVA followed by Tukey's post-hoc test (Figure 4.2). This could provide at least some degree of insight on whether or not specific combinations yielding high synergy scores are meaningful and could be focused on in future experiments. Another inherent limitation to our study is that we used *in vitro* models of breast cancer in entirety, and although this is an essential step in the preclinical screening of anti-cancer drug candidates, it does not recapitulate the tumor setting *in vivo*. We could further characterize the anti-proliferative effects of cannabinoids using 3D spheroid *in vitro* culture, which provides a slightly more complex environment that facilitates 3D cell-cell interactions and biochemical gradients that more closely mimic the tumor microenvironment *in vivo* (Edmondson et al., 2014).

Ultimately, *in vivo* studies are required to rigorously evaluate the anti-cancer benefits of cannabinoids, cannabinoid combinations, and cannabinoids in combination with anti-cancer agents. Our lab has previously done work to look at the anti-tumor effects of abnormal CBD and some terpenes using a zebrafish xenograft model of breast cancer (Tomko et al., 2019). This would be an ideal next step in our investigation of cannabinoids and different combinations in breast cancer. Zebrafish provide a high-

throughput, cost-effective model to evaluate the effects of novel agents on tumor proliferation before moving into a higher, more costly, and time-consuming animal model such as a murine model. Once a few potential cannabinoids and combinations are identified, *in vivo* murine models using patient-derived xenografts (PDX) could be done; PDX is superior to cell lines because it recapitulates the heterogeneity of tumors in humans (Cassidy et al., 2015). Another added benefit to murine models is that xenografts can be done in breast tissue (rather than in the yolk sac of zebrafish), making the tumor more physiologically relevant. *In vivo* studies need to be done in order to further characterize the potential therapeutic anti-cancer effects of cannabinoids and to determine off-target effects as well, since cannabinoid receptors are expressed in various tissues throughout the body. To date, THC, CBD and one study with CBG have shown anti-tumor effects in *in vivo* models (Hirao-Suzuki et al., 2019; Blasco-Benito et al., 2018; Caffarel et al., 2010; Elbaz et al., 2015; Borrelli et al., 2014), however the other cannabinoids tested in the present study have not been used in any published *in vivo* work.

A little further in the future may come clinical trials to verify the anti-cancer effects of cannabinoids, cannabinoid combinations and cannabinoids in combination with chemotherapeutic agents. To date, there have been a few clinical trials that have evaluated the efficacy of cannabinoid treatment in promoting anti-cancer effects. Temozolomide in combination with Sativex was able to increase the 1-year survival rate of GBM patients in a two-part clinical study (NCT01812603; NCT01812616). Another very recent study suggested that cannabidiol may prolong the survival of patients suffering from GBM and supported it as a co-medication in these patients (Likar et al.,

2021). One of the benefits to the treatment of cancer patients with cannabinoids is that CBD (Epidiolex), a THC-isomer (Dronabinol), and a combination of both THC and CBD (Sativex) have already been formulated as pharmaceutical preparations and are FDA or Health Canada approved, therefore it may not be a far reach to explore other cannabinoids in clinical cancer studies.

A common question that arises for the use of cannabinoids in *in vivo* and clinical studies is: can the necessary dose for anti-cancer effects of these compounds be achieved? We are by no means suggesting that the inhalation of cannabis would result in serum levels of cannabinoids high enough to observe anti-cancer effects, therefore other delivery methods are necessary. One study showed that in rats injected with 50 mg/kg THC showed a serum concentration of 10 μM within 10 hours of administration and tumor incidence was reduced (Chan et al., 1996). Additionally, despite such a high dose of THC, rats subjected to THC exposure survived for a longer period than controls (Chan et al., 1996). Although delivery of cannabinoids to cancerous breast tissue may be challenging in humans, there are some avenues that could be used to help faciliate it. For example, some studies have coupled cannabinoids to nanoparticles for delivery and found that it resulted in prolonged anti-proliferative activity (Fraguas-Sanchez et al., 2020). The coupling of cannabinoids to nanoparticles to enhance delivery would be a very interesting area to explore. Research has demonstrated that micelle-conjugated synthetic cannabinoids are stable at physiological pH, facilitating relatively slow release of the drug, which could allow the cannabinoids to reach the tumor site before releasing while minimizing systemic drug effects (Greish et al., 2018; Xian et al., 2015). Another added benefit to loading cannabinoids on nanoparticles is that it would allow for the intravenous

administration without the addition of toxic solubilizing adjuvants (Oerlemans et al., 2010).

Finally, it would be interesting to explore the molecular basis by which cannabinoids exert their anti-cancer effects in breast cancer cells. As an extension to our Annexin V apoptosis assay, we could perform western blot analysis to determine the role of caspase-3 and ROS assays to see if cannabinoids induce ROS in breast cancer cells. Other studies have shown caspase-3 involvement in cannabinoid-mediated apoptosis as well as the accumulation of ROS (De Petrocellis et al., 2013; Aviello et al., 2012; Blasco-Benito et al., 2018; Shrivastava et al., 2011). Some studies have also indicated that cannabinoids (THC and CBD) induce cancer cell death via autophagy, and this could be explored as well (Salazar et al., 2009; Vara et al., 2011; Armstrong et al., 2015). We could also study whether the anti-cancer effects of cannabinoids are mediated through CB1/CB2 (or other) receptors by co-treatment with receptor antagonists, which is what other studies have done to determine the potential role or lack of for CB1/CB2 (Caffarel et al., 2006; Sanchez et al., 1998). As previously described, it has been hypothesized that cannabinoids may exert their anti-cancer actions at the level of the membrane by altering cholesterol and disrupting lipid rafts. One study has shown that pre-treatment with methyl-β-cyclodextrin (MCD), an agent known to disrupt membrane lipid rafts, potently blocked CBD-induced apoptosis of microglial cells (Wu et al., 2012). It would be interesting to pre-treat breast cancer cells with MCD followed by successive cannabinoid treatment to see if their effects are attenuated to explore lipid raft involvement.

4.9 Conclusions

This research could hopefully contribute to a deeper investigation and the development of novel treatment options for breast cancer. New treatments are needed for patients who display or develop resistance to common chemotherapeutic agents to increase their chance of survival. In addition to this, if used in combination with currently existing chemotherapeutic drugs, cannabinoids may allow for a reduced dose of traditional treatment and thus decrease the likelihood of developing resistance as well as reducing the severity of adverse side effects. The present study showed that phytocannabinoids, including those lesser characterized compared to THC and CBD, were able to reduce the viability, induce cell death via apoptosis and reduce the invasion of breast cancer cells *in vitro*. We also showed that some cannabinoid combinations as well as cannabinoids with doxorubicin produced additive and synergistic reductions in cell viability at specific dose combinations. Although extensive research needs to be done in this area to validate findings in *in vivo* models as well as in clinical trials before more definitive conclusions can be made, this study provides insight as to which individual cannabinoids and combinations with each other or with doxorubicin may be effective in the treatment of breast cancer, including resistant forms.

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