Investigating chemopreventive and chemotherapeutic applications of cannabinoids in prostate cancer

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DECLARATION

I hereby certify that this thesis submitted for the degree of Doctor of Philosophy (PhD) to the University College Dublin, has not been previously submitted for a degree or diploma to this or any other University. The work presented is entirely my own, except where stated.

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Eve O'Reilly

May 2022

ABSTRACT

Prostate cancer is the 5th leading cause of cancer death in men, responsible for more than 375,000 deaths worldwide in 2020. For patients with localised prostate cancer, the 5-year survival rate is close to 100%. However, metastatic prostate cancer remains a lethal disease, with a 5-year survival rate of just 30%. Therefore, novel therapeutic strategies are urgently needed to improve clinical outcomes for patients with advanced prostate cancer.

Cannabinoids, chemical components of the cannabis plant, pose a possible solution. Substantial preclinical evidence demonstrates that cannabinoids can modulate key hallmarks of cancer, including cell death, proliferation, migration and invasion, and angiogenesis. However, few studies have investigated the anti-cancer potential of cannabinoids in prostate cancer. Our overall hypothesis was that plant-derived cannabinoids (phytocannabinoids) have chemotherapeutic and chemopreventive effects in prostate cancer. To test this hypothesis, our specific aims were to assess the effects of cannabinoids on various hallmarks of cancer, to investigate the mechanisms underlying the observed phenotypic effects, and to determine whether phytocannabinoids have antioxidant or chemopreventive effects in non-cancerous prostate cells.

First, we used cell line models of prostate cancer to measure the effects of cannabinoid treatment on cell viability, survival, proliferation, apoptosis, and migration and invasion. The phytocannabinoid GL1a inhibited prostate cancer cell viability and proliferation, with no significant increase in apoptosis. GL1a reduced the invasiveness of highly metastatic PC-3 cells. We also found some evidence that combinations of cannabinoid compounds produce enhanced anti-cancer activity. However, GL1a also reduced viability and induced apoptosis in non-cancerous prostate cells and further investigation into possible off-target effects is warranted.

Having shown that GL1a reduced cell proliferation and invasion, our next goal was to investigate the underlying mechanisms. Specifically, we aimed to identify the receptor targets of GL1a in prostate cancer cells and to measure the effects of GL1a treatment on the expression of cell cycle proteins, the phosphorylation of protein kinases, and the expression and secretion of proteins involved in cell invasion. GL1a reduced the expression of the key cell cycle proteins cyclin D3, CDK2, CDK4, and CDK1, and reduced the phosphorylation and activation of the protein kinase AKT. Additionally, we

found some evidence that GL1a may increase the expression of E-cadherin, an adhesion protein associated with a non-invasive phenotype. The effects of GL1a on cell viability were not blocked by CB_1 or CB_2 antagonists, TRPV ion channel blockers, or a GPR55 agonist, suggesting that GL1a acts independently of these targets in prostate cancer cells.

Finally, we assessed the antioxidant and cytoprotective potential of low-dose cannabinoids in non-cancerous prostate cells. Hydrogen peroxide was used to induce oxidative stress. Phytocannabinoid treatment at the doses tested produced no significant cytoprotective or antioxidant effects. However, drug exposure times and doses may require further optimisation.

This study provides novel insights into the phenotypic effects and mechanisms of action of cannabinoids in prostate cancer cells. GL1a reduces prostate cancer cell proliferation and invasion. The underlying mechanisms include altered expression of key cell cycle regulators and modulation of AKT phosphorylation. Future studies should aim to identify the receptor target(s) of GL1a and further investigate its mechanisms of action, as well as testing the effects of cannabinoid treatment in more biologically relevant experimental models. Ultimately, clinical trials will be needed to determine whether the observed phenotypic effects of GL1a *in vitro* can translate to therapeutic benefits and improved outcomes in patients with prostate cancer.

For My Family

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ABBREVIATIONS

2-AG	2 arachidonoyl glycerol
ADT	androgen deprivation therapy
AFS	anterior fibromuscular stroma
AOM	azoxymethane
AR	androgen receptor
ARE	androgen response element
ATCC	American Type Culture Collection
ATF4	activating transcription factor 4
ATM	ataxia telangiectasia-mutated gene
BCA	bicinchoninic acid
Bcl-2	B-cell lymphoma 2
cAMP	cyclic adenosine monophosphate
CBC	cannabichromene
CBD	cannabidiol
CBG	cannabigerol
CDK	cyclin dependent kinase
CHOP	C/EBP homologous protein
CI	combination index
CRPC	castration-resistant prostate cancer
CTPE	cadmium-transformed prostate epithelial
DDR	DNA damage response
DMSO	dimethyl sulfoxide
DSMZ	German Collection of Microorganisms and Cell Cultures
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-related kinase
FAAH	fatty acid amide hydrolase
FBS	foetal bovine serum
GSH	glutathione
H_2O_2	hydrogen peroxide
HDI	human development index

HER2	human epidermal growth factor receptor 2
HNSCC	head and neck squamous cell carcinoma
HRP	horse radish peroxidase
HSPC	hormone-sensitive prostate cancer
HUVEC	human umbilical vein endothelial cell
iCAM-1	intercellular adhesion molecule 1
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LPI	lysophosphatidylinositol
MAGL	monoacylglycerol lipase
MMP	matrix metalloprotease
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetyl cysteine
NASEM	National Academies of Sciences, Engineering, and Medicine
NFκB	nuclear factor kappa B
Nrf2	nuclear factor erythroid 2-related factor 2
OS	overall survival
PAI-1	plasminogen activator inhibitor 1
PARP	poly ADP ribose polymerase
PBS	phosphate buffered saline
PDL	poly-D-lysine
PERK	protein kinase R-like ER kinase
PFS	progression-free survival
PI	propidium iodide
PIN	prostatic intraepithelial neoplasia
PPARγ	peroxisome proliferator activated receptor gamma
PSA	prostate-specific antigen
PUMA	p53 upregulated modulator of apoptosis
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RB	retinoblastoma protein
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFN	sulforaphane
SOC	standard of care

STAT3	signal transducer and activator of transcription 3
THC	tetrahydrocannabinol
TIMP-1	tissue inhibitor of metalloproteinases 1
TNFα	tumour necrosis factor alpha
TRAIL	TNF-related apoptosis inducing ligand
TRIB3	tribbles pseudokinase 3
TRPA1	transient receptor potential ankyrin 1
TRPM8	transient receptor potential metastatin 8
TRPV2	transient receptor potential vanilloid 2
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
WIN	WIN55,212-2

PUBLICATIONS RELATED TO THIS THESIS

Cannabidiol inhibits the proliferation and invasiveness of prostate cancer cells (manuscript in preparation)
O'Reilly, E., Khalifa, K., Cosgrave, J., Azam, H., Prencipe, M., Simpson, J.C., Gallagher, W.M. & Perry, A.S.

Plant-derived cannabinoids as anticancer agents.
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PRESENTATIONS (ORAL)

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PRESENTATIONS (POSTER)

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"Investigating the Chemotherapeutic Potential of Cannabinoids in Prostate Cancer" – Irish Association for Cancer Research Annual Meeting, online (March 2021)

"Investigating Chemopreventive and Chemotherapeutic Applications of Cannabinoid Compounds in Prostate Cancer" – Irish Association for Cancer Research Annual Meeting, Galway (Feb 2020)

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AWARDS

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Scientific poster prize - Conway Gold Medal competition, UCD Conway Festival of Research and Innovation 2018

CHAPTER 1

Introduction

1.1. PROSTATE CANCER

1.1.1. PROSTATE CANCER INCIDENCE

Excluding non-melanoma skin cancer, prostate cancer is the fourth most commonly diagnosed cancer worldwide, accounting for 7.3% of all cancer cases, and the second most common cancer in men, accounting for 14.1% of all cases[1]. In 2020, 1,414,259 new cases of prostate cancer were diagnosed worldwide. The global age-standardised incidence rate for prostate cancer is 30.7 per 100,000, and the cumulative risk of developing prostate cancer before age 75 is 3.86%. In more than half of countries (112 countries), prostate cancer is the most frequently diagnosed cancer in men. Prostate cancer incidence is 3-fold higher in countries with a high human development index (HDI) (incidence of 37.5 per 100,000) than in countries with a lower HDI (incidence of 11.3 per 100,000). This is likely due to differences in testing practices, such as increased use of prostate-specific antigen (PSA) testing in higher HDI countries, in addition to differences in lifestyle and environmental risk factors. Incidence is highest in Northern Europe, with an age-standardised rate of 83.4 per 100,000 (**Figure 1.2**). Ireland has the highest national age-standardised rate for prostate cancer incidence worldwide, at 110.7 per 100,000[1,2].

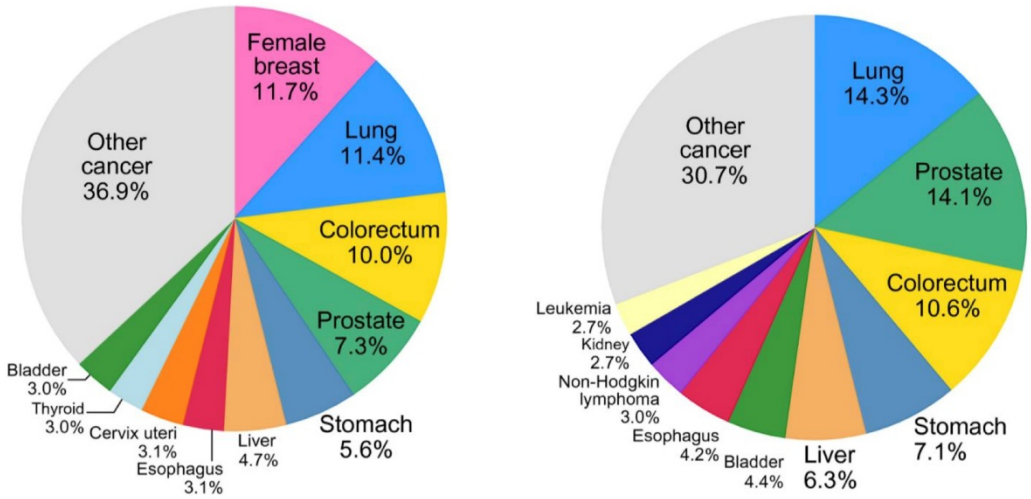


Figure 1.1. Worldwide prostate cancer incidence, overall (left) and in males (right). Source: Globocan 2020.

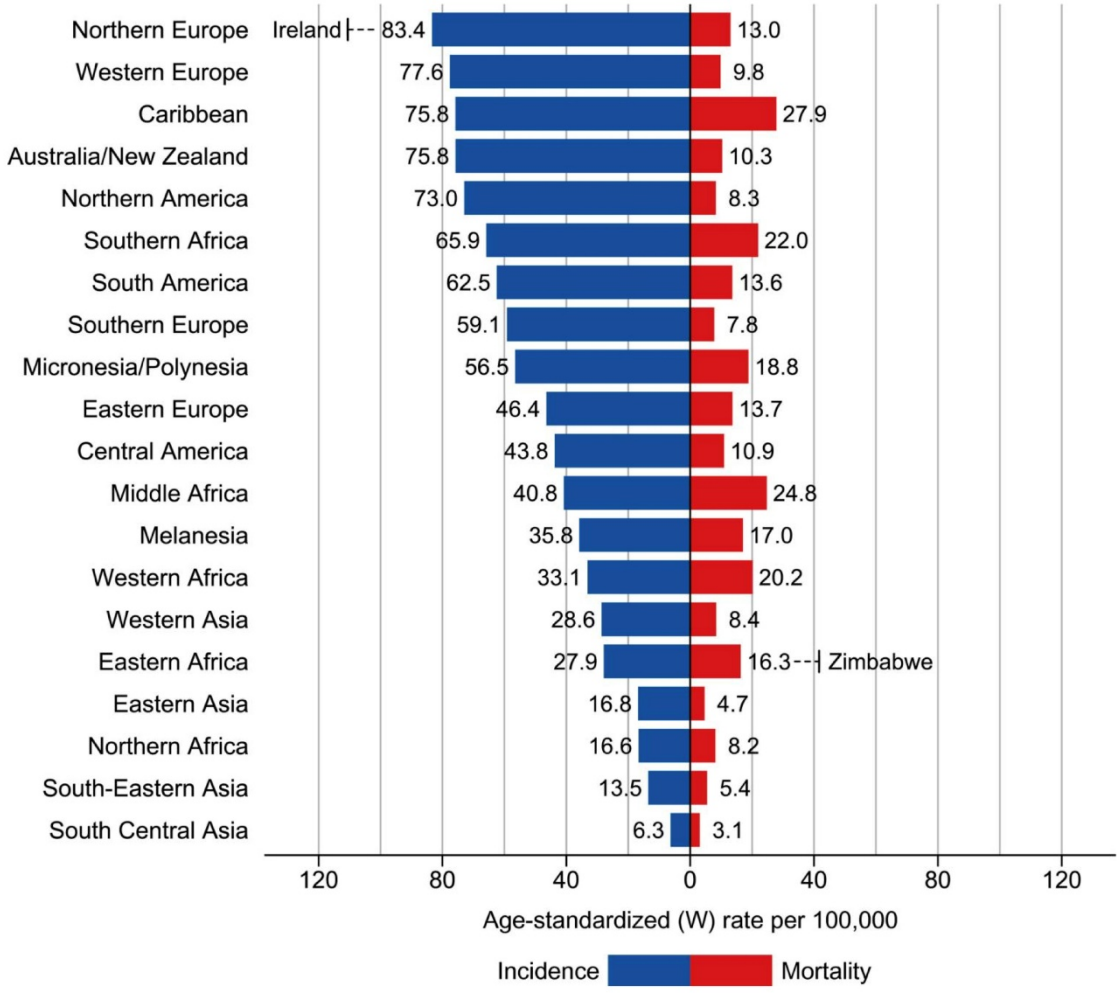


Figure 1.2. Prostate cancer incidence and mortality by region. Source: Globocan 2020.

In Ireland, excluding non-melanoma skin cancer, prostate cancer is the most common cancer, both in males and overall (**Figure 1.3**)[1,3]. According to the NCRI Annual Report for 2020, an estimated 3,890 new cases of prostate cancer were diagnosed in Ireland per year between 2018 and 2020, with an estimated incidence rate of 143.4 per 100,000[3]. The Globocan 2020 report estimated that 4,503 new cases of prostate cancer were diagnosed in Ireland in 2020, accounting for 14.1% of all new cancer cases, and 25.8% of new cancer cases in men[2]. Irish men have a 1 in 8 cumulative risk of developing prostate cancer before age 75, and a 1 in 6 lifetime risk of prostate cancer[3].

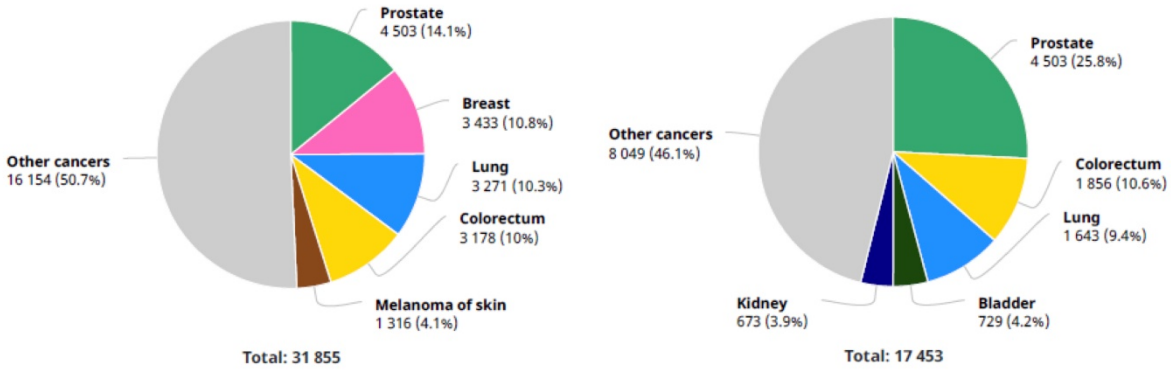


Figure 1.3. Prostate cancer incidence in Ireland, overall (left) and in males (right). Source: Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. Available from: https://gco.iarc.fr/today, accessed [30 April 2022].

Prostate cancer incidence is expected to increase globally by 71.6% from 2020 to 2040, from 1.4 million cases in 2020 to 2.4 million cases in 2040 (**Figure 1.4**)[4]. In Ireland, prostate cancer incidence is expected to increase by 54.8%, from 4,500 cases in 2020 to almost 7,000 cases in 2040.

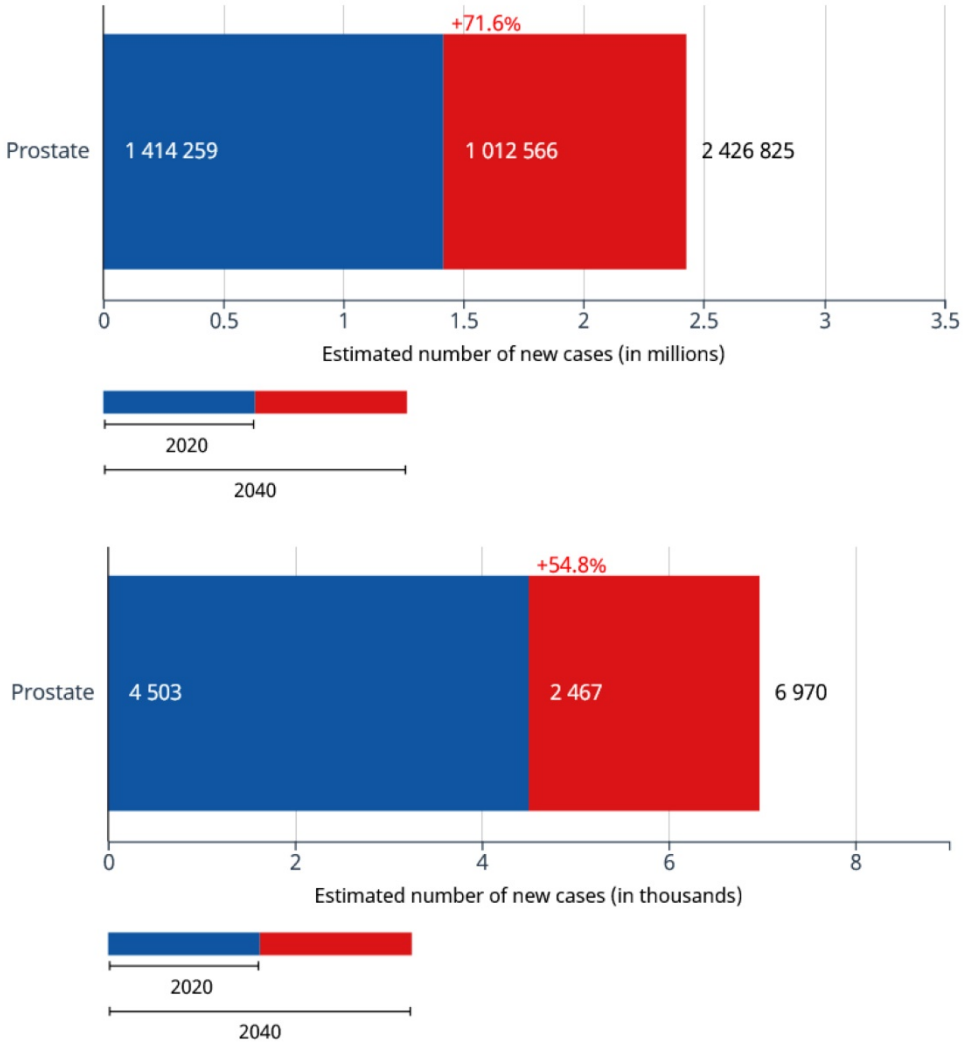


Figure 1.4. Projected prostate cancer incidence 2020-2040 worldwide (top) and in Ireland (bottom). Source: Ferlay J, Laversanne M, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F (2020). Global Cancer Observatory: Cancer Tomorrow. Lyon, France: International Agency for Research on Cancer. Available from: https://gco.iarc.fr/tomorrow, accessed [03 April 2022]

1.1.2. PROSTATE CANCER MORTALITY

Worldwide, prostate cancer is the 5th leading cause of cancer death in men, and the 7th leading cause of cancer death overall[1]. In 48 countries, prostate cancer is the leading cause of cancer death in men. In 2020, prostate cancer was responsible for more than 375,000 deaths worldwide, accounting for 3.8% of all cancer deaths. In high HDI countries, the prostate cancer mortality rate is 8.1 per 100,000, compared to 5.9 per 100,000 in lower HDI countries. In Northern Europe the age-standardised mortality rate

is 13.0 per 100,000 (**Figure 1.2**). The prostate cancer mortality rate is highest in the Caribbean, at 27.9 per 100,000[1].

In Ireland, prostate cancer was responsible for an estimated 569 deaths in 2020, making it the 5th leading cause of cancer death, accounting for 5.5% of all cancer deaths[2]. The cumulative risk of dying from prostate cancer before age 75 was 0.64%. The NCRI reported that prostate cancer was the 3rd most common cause of cancer death in Irish men from 2015-2017, with an estimated mortality rate of 20.5 per 100,000[3].

Global prostate cancer mortality is expected to almost double by 2040, from 375,000 deaths in 2020 to 740,000 deaths in 2040. In Ireland, prostate cancer mortality is expected to increase by 114.8%, with more than 1,200 prostate cancer deaths predicted in 2040[4].

1.1.3. ANATOMY OF THE PROSTATE GLAND

The prostate is a fibromuscular gland located below the bladder and surrounding the urethra. The gland is shaped like an inverted pyramid, with a narrow apex at the bottom, and a wider base at the top. The seminal vesicles and the vas deferens converge at the ejaculatory ducts, which enter the prostate at the base and ultimately converge with the urethra at the seminal colliculus in the mid-prostate[5,6]. The external sphincter muscle surrounds the urethra and regulates ejaculatory and urinary flow.

The prostate gland is composed of ducts with an inner epithelial layer surrounded by stromal tissue (**Figure 1.5**)[7]. The epithelial cells and stromal cells are separated by a basement membrane composed of collagen fibres containing extracellular matrix proteins[8]. The epithelial layer consists of luminal cells, basal cells, and neuroendocrine cells[9]. Prostate cancer can arise from either luminal or basal cells, and there is conflicting evidence about which cell of origin produces the more aggressive cancer phenotype[9-11]. Neuroendocrine cells may arise through differentiation of luminal-derived prostate cancer cells[12]. Neuroendocrine differentiation can occur in response to therapy, and neuroendocrine cells are treatment-resistant. Neuroendocrine differentiation is correlated with poor prognosis and disease progression[12].

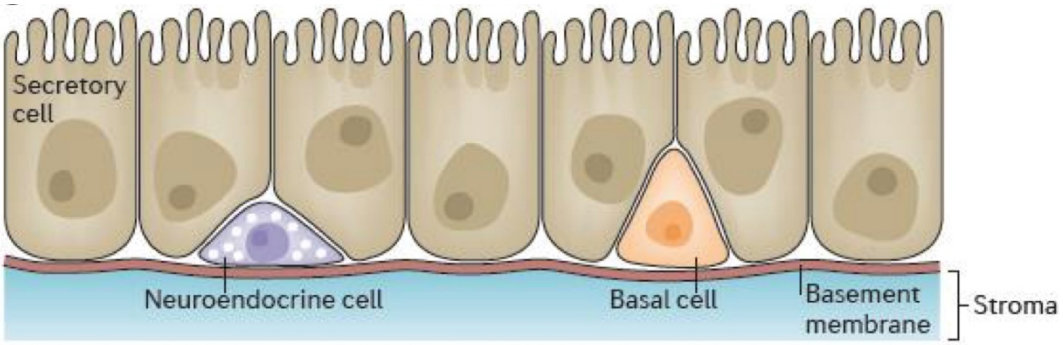


Figure 1.5. Cellular composition of the prostate gland. Source: Verze, P. et al. (2016) The role of the prostate in male fertility, health and disease. Nat Rev Urol 13, 379-386. 10.1038/nrurol.2016.89

The prostate is divided into histologically distinct zones (**Figure 1.6**), first described by McNeal through examination of autopsy specimens[13,14]. The central zone, transition zone, and peripheral zone are glandular, while the anterior fibromuscular stroma (AFS) is a non-glandular region[15]. The zones are enclosed by a fibromuscular capsule. The outermost peripheral zone is the main glandular component of the prostate, comprising approximately 65% of the normal prostate mass, and surrounds most of the central zone, with ducts extending from the urethra to the prostate apex[15]. The peripheral zone is the most common site of prostate cancer initiation, with approximately two in three prostate cancers arising in this zone[9,16]. The central zone surrounds the ejaculatory ducts, extending from the mid-prostate to the prostate base, and comprises approximately 20-30% of the normal prostate mass[7,15]. The transition zone surrounds a portion of the urethra proximal to the ejaculatory ducts, and typically comprises approximately 5-10% of the prostate mass[7,15]. In older men, hypertrophy causes the transition zone to become more dominant, and the other zones become compressed[17]. The AFS is a thick layer of fibromuscular tissue forming the anterior surface of the prostate gland[14].

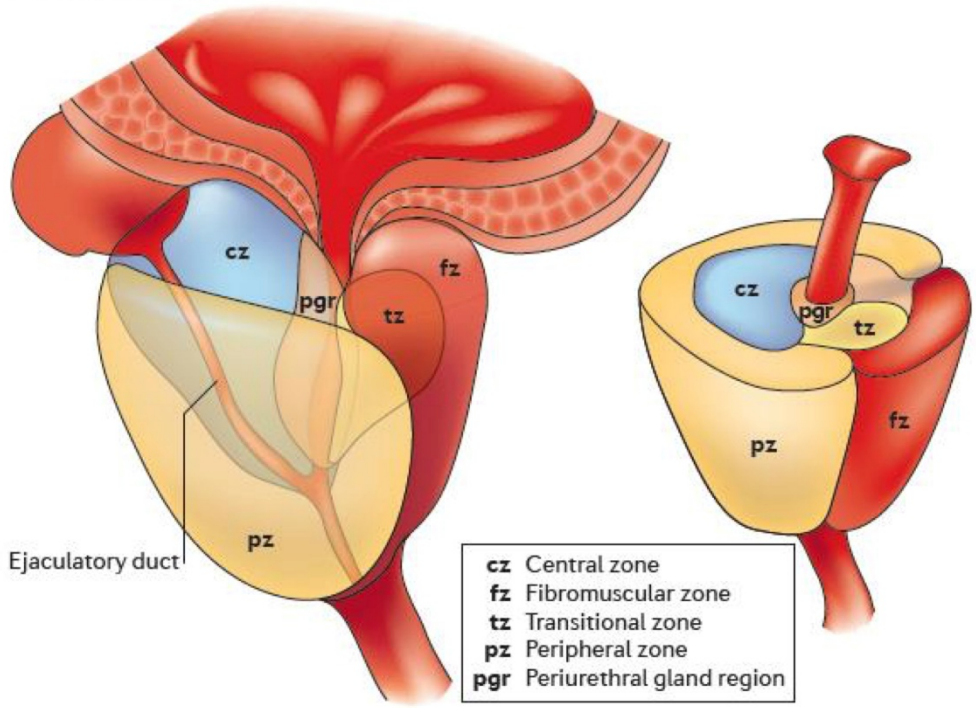


Figure 1.6. Zonal anatomy of the prostate gland. Source: Verze, P. et al. (2016) The role of the prostate in male fertility, health and disease. Nat Rev Urol 13, 379-386. 10.1038/nrurol.2016.89

1.1.4. FUNCTION OF THE PROSTATE GLAND

The prostate gland is an important accessory gland of the male reproductive system. The primary function of the prostate is to contribute to the production of the seminal fluid. The luminal epithelial cells of the prostate secrete an alkaline fluid which coats sperm cells[8]. The prostatic fluid protects the sperm cells in the acidic environment of the vagina, increasing the lifespan of the sperm and improving fertility. Additionally, the fluid provides proteins and enzymes that nourish the sperm, activate sperm maturation, and regulate ejaculation[7]. The prostatic fluid comprises between one-third and one-fifth of the total ejaculate volume[7]. The neuroendocrine cells of the prostate secrete neuropeptides and growth factors needed for growth of luminal cells[8]. The basal epithelial cells adhere to the basement membrane and separate the luminal epithelial cells from the stroma[8,18]. The main function of the stromal compartment is to produce signals that maintain the homeostasis of the epithelial cell microenvironment[7].

1.1.5. DEVELOPMENT OF PROSTATE CANCER

Prostate cancer most commonly arises in the epithelial cells of the outermost peripheral zone of the prostate gland[8,19]. Debate still exists regarding the cell of origin of prostate cancer, though it appears that prostate cancer can develop from either luminal or basal epithelial cells[10,11,20]. Factors such as chronic inflammation and microbial infection cause increased oxidative stress which drives prostate carcinogenesis[19]**.** Oxidative stress occurs when an imbalance exists between the production of reactive oxygen species (ROS) and the ability of the body to neutralise reactive species and repair resulting oxidative damage[21]. At high levels, ROS induce protein, lipid, and DNA damage, and increase cancer susceptibility[21,22]. Increased oxidative stress is associated with prostate cancer development, progression, and treatment response[21]. As such, several antioxidants have been investigated for the prevention of prostate cancer development and progression with some promising results, including reduced expression of genes involved in prostate cancer development and reduced prostate cancer incidence in specific subgroups of patients[23-26].

Prostate cancer develops in multiple stages, progressing from pre-cancerous lesions to localised disease to metastatic prostate cancer and finally to castration-resistant prostate cancer. Prostatic intraepithelial neoplasia (PIN) are pre-neoplastic lesions believed to be precursors to prostate cancer[19,27]. PIN is characterised by hyperproliferation of luminal cells, dysplasia of epithelial cells, and loss of basal cells[8,19]. The presence of PIN indicates that epithelial cells are vulnerable to malignant transformation, and 23-35% of high-grade PIN develops into prostate cancer[27]. Progression from localised to locally advanced prostate cancer occurs as cancerous cells spread outside of the prostate gland to the lymph nodes following degradation of the basal cell layer[9]. Metastasis develops with further spread of cancerous cells to the bone, liver, lungs, and other tissues, and can occur in both treatment-naïve and castration-resistant cancers[9,28]. Metastatic prostate cancer has a high mortality rate, with a 5-year survival rate of less than 30%[28]. Androgen deprivation therapy is the standard of care for metastatic prostate cancer, often in combination with other hormone treatments or with chemotherapy[28]. Prostate cancer cells initially respond to androgen deprivation therapy. However, resistance to therapy eventually develops, leading to castration-resistant prostate cancer, which is considered a lethal diagnosis.

The major risk factors for prostate cancer development include age, race, and family history, with some evidence that diet and lifestyle also play an important role. Age is the

most important risk factor for prostate cancer. In 60- to 69-year-old men the prevalence of prostate cancer is 35%, increasing to 46% at 70 to 81 years[29]. Race is also a risk factor for prostate cancer. Data from the SEER database from 2008-2012 indicate that the age-standardised incidence of prostate cancer is 64% higher in African Americans compared to non-Hispanic Whites[30]. Even adjusting for socioeconomic, clinical, and pathologic factors, the risk of diagnosis with advanced prostate cancer is higher for African Americans than for non-Hispanic Whites[31]. Worldwide, prostate cancer incidence is highest in men of African descent in the USA and the Caribbean[32]. Family history of prostate cancer is an additional risk factor. The risk of developing prostate cancer is twice as high for those with a first-degree relative diagnosed with prostate cancer[33]. Prostate cancer risk is substantially increased for individuals with 2 or 3 first-degree relatives diagnosed with prostate cancer before 60 years of age[34]. Additionally, increased incidence and more aggressive disease is observed in those with mutations in known cancer susceptibility genes such as *BRCA1*, *BRCA2*, and other DNA damage repair genes[8,9,19,27,33].

Some evidence indicates a role for environmental factors in prostate cancer development. For example, a much lower prostate cancer incidence is observed in Asian countries compared to in the USA and Europe[1]. Furthermore, increased rates of prostate cancer are observed in Asian Americans born in the USA, indicating the involvement of environmental factors such as diet and lifestyle[8]. Several studies indicate that dietary factors affect prostate cancer risk, possibly due to their antioxidant activity[8,23,25,26].

While high-frequency *de novo* genetic changes are relatively rare in prostate cancer, several genetic alterations are observed in substantial proportions of patients. For example, *TMPRSS2-ERG* gene fusions are the most common chromosomal aberrations in prostate cancer, observed in 40-60% of patients[19]. *PTEN* deletions and *TP53* mutations are observed in 10-20% of localised prostate cancers, with frequency increasing to 40-50% in advanced prostate cancer[19]. *Wnt* activation, *MYC* overexpression, and *RB1* alterations are found in approximately 20-30% of patients with metastatic castration-resistant prostate cancer[19]. Loss-of-function mutations in *SPOP* are observed in 5-15% of patients, while gain-of-function mutations in *FOXA1* are observed in 3-5% of patients[19]. Epigenetic changes are a more common feature of prostate cancer. For example, hypermethylation of the *GSTP1* gene, which plays a role in DNA damage repair, is observed in more than 90% of prostate cancers[35].

1.1.6. ANDROGEN SIGNALLING IN PROSTATE CANCER

Androgen signalling drives prostate cell proliferation, both during normal development of the prostate gland and in prostate carcinogenesis[36]. In 1941, Huggins and Hodges first reported the importance of androgen signalling in prostate cancer[37]. They observed that administration of testosterone in mouse models increased prostate growth. Furthermore, in patients, castration strongly reduced testosterone levels, leading to prostate cancer regression[37,38].

Androgen signalling is mediated through the androgen receptor (AR). The AR belongs to the nuclear steroid receptor superfamily of transcription factors[38]. In the absence of androgens, the AR is inactive and remains bound to chaperone proteins in the cytoplasm[38]. Upon binding of androgens, the AR becomes activated and translocates to the nucleus, where it binds to androgen response elements (AREs) on DNA and interacts with various co-regulators to regulate gene transcription[38,39]. Binding of the AR to AREs causes transcription of various genes that drive cell proliferation.

Alterations in the AR signalling pathway are observed in a majority of prostate cancers[9]. AR overexpression is associated with increased cell proliferation, disease progression, and shorter recurrence-free survival[40]. Furthermore, single nucleotide polymorphisms in genes encoding enzymes involved in androgen synthesis are significantly associated with increased prostate cancer risk[8]. Because of the crucial role of androgen signalling in prostate cancer, androgen deprivation therapy is the standard of care for prostate cancer that cannot be cured through surgery or radiotherapy[39]. At diagnosis, 80-90% of prostate cancers are androgen-dependent, and 80% of patients show an initial positive response to androgen deprivation[36].

However, cancer cells can overcome their dependence on androgens for growth through various mechanisms, including altered AR expression and structure, AR mutations that reduce ligand-specificity, increased expression of AR co-regulators, increased growth factor production, and enhanced response to growth factors[36,38,39], leading to the development of castration-resistant prostate cancer (CRPC). In approximately 80% of cases castration resistance occurs due to activating genetic alterations in the AR signalling pathway[41]. Once prostate cancer becomes castration-resistant, treatment options are limited, and remissions are generally short-lived[41]. Consequently, more effective treatments for CRPC are urgently needed.

1.1.7. CELL CYCLE REGULATION IN PROSTATE CANCER

The eukaryotic cell cycle consists of two main phases, interphase and mitosis. Interphase comprises 90% of the cell cycle, and can be subdivided into G1, S, and G2 phases. During the G1 phase, cells increase in size and synthesise proteins and organelles. DNA replication occurs during the S phase. Cell growth and protein synthesis continue during the G2 phase, in preparation for mitosis. Finally, during the M phase, cytokinesis occurs, and the cytoplasm is separated to form two new daughter cells. Tightly controlled checkpoints exist between the various phases of the cell cycle to ensure proper timing of events and to prevent cells with damaged DNA from undergoing mitosis[41].

The cell cycle is regulated through complex interactions between cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors. During resting conditions, the tumour suppressor retinoblastoma protein (RB) binds to the E2F transcription factor and prevents the transcription of genes involved in driving cell cycle progression from the G1 to S phase[41,42]. The G1-S phase transition is regulated by cyclin D and cyclin E, which bind to and activate the corresponding CDKs, CDK4/6 and CDK2. These CDKs promote the phosphorylation and inactivation of RB, which releases E2F, allowing the transcription of genes that promote DNA replication and cell growth[41,42]. CDK inhibitors such as p21 and p27 act as tumour suppressors, preventing CDKs from phosphorylating RB [41]. Meanwhile, the G2-M phase transition is controlled by the interaction between cyclin B and CDK1. Binding of cyclin B to CDK1 stimulates the formation of the mitotic spindle and the degradation of the nuclear envelope[41].

In prostate cancer, de-regulation of cell cycle progression pathways results in uncontrolled cell proliferation, and eventually carcinogenesis. Increased activity of mitogenic signalling pathways, such as the AR signalling pathway, causes overexpression and hyperactivation of cyclin D, leading to increased CDK4/6 activity[42]. CDK4/6 mutations and loss of CDK inhibitors like p16, p21, and p27 also contribute to prostate cancer cell proliferation[41,42]. Loss of RB is also frequently observed in prostate cancer and promotes cancer initiation and progression[42]. RB loss is more prevalent in CRPC and is associated with worse clinical outcomes [41]. Accordingly, E2F expression increases with progression from localised prostate cancer to metastatic and castration-resistant disease[41]. In addition, the expression levels of cyclin B, cyclin D, and cyclin E are increased in treatment-naïve prostate cancer, and cyclin expression is

correlated with tumour grade[41]. Similarly, expression levels of CDK1, CDK2, CDK4, and CDK6 are increased in prostate cancer, particularly in high-grade disease [41].

Due to the crucial role of cell cycle regulation in cancer development, several CDK inhibitors have been investigated as anti-cancer agents[42]. These inhibitors act by binding to CDKs and preventing the formation of the cyclin-CDK complexes that drive cell proliferation. Notably, CDK4/6 inhibitors have proven effective for the treatment of breast cancer. Clinical trials in patients with advanced breast cancer showed that the CDK4/6 inhibitors palbociclib, ribociclib, and abemaciclib improved progression-free survival and overall survival[43-45]. Following these and other successful trials, palbociclib, ribociclib, and abemaciclib were granted FDA approval for the treatment of advanced breast cancer[42]. In prostate cancer, preclinical studies indicate that palbociclib inhibits cell proliferation[46]. Several clinical trials are currently investigating the effects of palbociclib, ribociclib, and abemaciclib in patients with prostate cancer[42].

The AKT signalling pathway is one of the most important pathways in cancer, involved in the regulation of cell proliferation, cell survival, and cell death. AKT is a protein kinase which phosphorylates p21 and p27, preventing them from binding and inhibiting CDKs, in addition to modulating downstream targets such as mTOR, cyclin D1, and p53, which play important roles in cell proliferation[47]. Activation of the AKT pathway is regulated by the tumour suppressor PTEN[41]. *PTEN* inactivation is one of the most frequent genetic aberrations in prostate cancer, with *PTEN* deletions or mutations detected in up to 20% of treatment-naïve prostate cancer samples and up to 50% of CRPC samples[48].

PTEN loss increases the activation of AKT, promoting cell proliferation. Accordingly, AKT overactivation is a common feature of prostate cancer, with increased AKT1 activity observed in 50% of prostate cancers[48]. AKT hyperphosphorylation is associated with high-grade cancer and poor survival[49]. Similarly, PI3K, which is found upstream of AKT and promotes AKT phosphorylation, is over-activated in 40% of early diagnosed and 70% of advanced prostate cancers and is associated with androgen-resistance and poor outcome[49]. Several clinical trials have investigated the use of synthetic and natural AKT inhibitors for cancer treatment, with some success[48].

The activity of the cyclin-CDK and AKT signalling pathways are frequently modulated in prostate cancer, driving uncontrolled cell proliferation. Compounds such as

cannabinoids, which have been shown to target proteins in these pathways[50-54], may be effective in reducing tumour growth in prostate cancer.

1.1.8. TREATMENT OF PROSTATE CANCER

Selection of treatment approaches for patients with prostate cancer is dependent on the stage and risk group of the cancer. Localised prostate cancer has good outcomes, with a 5-year survival rate close to 100%[55], so immediate medical intervention is not always needed. For patients with advanced prostate cancer, androgen deprivation therapy is the standard of care, but resistance eventually develops. Despite advances in prostate cancer treatment in recent years (**Table 1.1**), the 5-year survival rate for metastatic prostate cancer is just 30%[55].

Table 1.1. Clinical trials of treatments for advanced prostate cancer.

TRIAL NAME	TREATMENT	CANCER TYPE	INTERVENTION	OUTCOME	REF.
TAX327	Docetaxel	Metastatic CRPC	Docetaxel + prednisone vs mitoxanthrone + prednisone	Increased survival by 2.4 months	[56]
CHAARTED	Docetaxel	Metastatic HSPC	Docetaxel + ADT vs ADT alone	Increased OS by 13.6 months, prolonged time to progression by 8.5 months	[57]
STAMPEDE	Docetaxel	HR, locally advanced, metastatic, recurrent prostate cancer	Docetaxel + SOC vs SOC alone	Increased OS by 10 months	[58]
TROPIC	Cabazitaxel	Metastatic CRPC	Cabazitaxel + prednisone vs mitoxanthrone + prednisone	Increased OS by 2.4 months and PFS by 1.4 months	[59]
COU-AA-301	Abiraterone	Metastatic CRPC (previous docetaxel)	Abiraterone + prednisone vs placebo	Increased OS by 3.9 months	[60]
COU-AA-302	Abiraterone	Metastatic CRPC (no previous chemotherapy)	Abiraterone + prednisone vs placebo + prednisone	Increased PFS by 8.2 months	[61,62]
LATITUDE	Abiraterone	Metastatic HSPC	Abiraterone + ADT vs placebo + ADT	Significantly increased OS (median not reached), increased PFS by 18.2 months	[63]
AFFIRM	Enzalutamide	CRPC (previous chemotherapy)	Enzalutamide vs placebo	Increased OS by 4.8 months	[64]
PREVAIL	Enzalutamide	Metastatic CRPC (no previous chemotherapy)	Enzalutamide vs placebo	Significantly increased PFS	[65]
PROSPER	Enzalutamide	Non-metastatic CRPC	Enzalutamide + ADT vs placebo + ADT	Increased OS by 10.7 months	[66]
ARAMIS	Darolutamide	Non-metastatic CRPC	Darolutamide + ADT vs placebo + ADT	Increased 3-year OS from 77% to 83%	[67]
SPARTAN	Apalutamide	Non-metastatic CRPC	Apalutamide + ADT vs placebo + ADT	Increased OS by 14 months	[68]
TITAN	Apalutamide	Metastatic HSPC	Apalutamide + ADT vs placebo + ADT	Significantly increased OS (median not reached)	[69]

STAMPEDE	Radiotherapy	Metastatic HSPC	Radiotherapy + SOC vs SOC	Increased PFS but not OS	[70]
ALSYMPCA	Radium-223	Metastatic CRPC	Radium-223 + SOC vs placebo + SOC	Increased OS by 3.6 months	[71]
IMPACT	Sipleucel-T	Metastatic CRPC	Sipleucel-T vs placebo	Increased OS by 4.1 months	[72]
TOPARP-B	Olaparib	Metastatic CRPC	Olaparib	Antitumour activity in participants with DDR gene aberrations	[73]
PROFOUND	Olaparib	Metastatic CRPC with DDR gene aberrations	Olaparib vs abiraterone or enzalutamide	Increased PFS by 3.8 months and OS by 3.4 months in patients with DDR alterations	[74]

Abbreviations: CRPC – castration-resistant prostate cancer, HSPC – hormone-sensitive prostate cancer, ADT – androgen deprivation therapy, OS – overall survival, SOC – standard of care, PFS – progression-free survival, DDR – DNA damage response.

Active surveillance involves foregoing immediate treatment of patients with low-risk or intermediate-risk prostate cancer in favour of close monitoring of cancer status through PSA testing, repeat biopsies, or MRI[75]. Treatment is deferred until evidence of disease progression is detected. The ProtecT trial compared active surveillance vs radical prostatectomy vs radiotherapy in patients with predominantly low-risk or intermediate-risk localised prostate cancer[76]. At a median 10-year follow-up, only 62 of the 1,643 participants had developed metastatic prostate cancer. While the incidence of metastatic prostate cancer was twice as high in the active surveillance group, the overall incidence was very low (6.3 metastatic events per 1,000 person-years). Importantly, participants in the treated groups reported substantial reductions in quality of life compared to those on active surveillance. Active surveillance allows patients with indolent prostate cancer to avoid unwanted side effects of treatment, such as sexual dysfunction, urinary incontinence, and infertility, until treatment is necessary.

Radical prostatectomy, surgical removal of the prostate gland, is an effective treatment strategy for localised and locally advanced prostate cancer[75]. The SPCG-4 trial showed that radical prostatectomy prolonged survival by 2.9 years compared to watchful waiting[77]. The PIVOT trial found that radical prostatectomy reduced mortality risk by more than 30% in patients with high PSA levels or other features of high-risk prostate cancer[78]. However, radical prostatectomy leads to side effects such as urinary incontinence and erectile dysfunction and is not an option for surgically unfit patients[19,75].

Radiation therapy is another treatment strategy for patients with localised and locally advanced prostate cancer. Clinical trials have shown that the addition of radiotherapy to

androgen deprivation therapy improves overall survival in patients with locally advanced or high-risk localised prostate cancer[79,80]. More recently, radium-223 has come under investigation for the treatment of metastatic prostate cancer. Radium-223 is an alpha-emitting radioisotope which selectively binds to bone metastases and emits alpha particles that cause targeted double-stranded DNA breaks without damaging the surrounding tissue[81]. The ALSYMPCA trial found that the addition of radium-223 to standard-of-care treatment improved overall survival and delayed the first symptomatic skeletal event in patients with bone metastases[71]. In 2013, radium-223 received FDA approval for the treatment of patients with metastatic CRPC[81].

The efficacy of androgen deprivation therapy (ADT) for the treatment of metastatic prostate cancer was first reported by Charles Huggins in 1941[82]. Since then, ADT has been the standard of care for metastatic prostate cancer. Initially, androgen deprivation was achieved through surgical castration. Today, medical castration using agonists or antagonists of luteinising hormone-releasing hormone is the most common method of androgen deprivation in developed countries[28,81]. While ADT is initially effective, resulting in remissions of 1-2 years in most patients, resistance eventually develops with progression to CRPC[81].

The next major development in the treatment of metastatic CRPC was the approval of the chemotherapeutic agent docetaxel for patients with metastatic CRPC in 2004[81]. Docetaxel is a taxane that binds to tubulin and prevents microtubule depolymerisation, leading to inhibition of AR nuclear transport and AR signalling[28,81,83]. Several clinical trials showed that docetaxel improves overall survival in patients with metastatic CRPC[56-58]. Furthermore, in patients with hormone-sensitive prostate cancer, the addition of docetaxel to ADT improves progression free survival and overall survival[57,58,84]. Based on the above findings, docetaxel has become a standard of care for patients with high-volume metastatic disease. However, docetaxel is associated with several adverse events, including febrile neutropenia, neuropathies, alopecia, diarrhoea, and fatigue, and resistance to docetaxel treatment will eventually develop[28,81].

Further improvements in the treatment of advanced prostate cancer occurred with the finding that cabazitaxel showed efficacy for the treatment of docetaxel-resistant tumours[59]. Cabazitaxel, like docetaxel, is a tubulin-binding taxane, but has lower affinity for the efflux pump P-glycoprotein, which is associated with drug resistance[81,85]. The TROPIC trial showed that, compared to mitoxanthrone,

cabazitaxel improved overall survival by 2.4 months and progression-free survival by 1.4 months in patients showing disease progression following docetaxel treatment[59]. Reported adverse events included neutropenia, diarrhoea, and febrile neutropenia.

The addition of anti-androgen drugs such as abiraterone acetate (abiraterone) or enzalutamide to ADT can enhance ADT efficacy in metastatic prostate cancer. Abiraterone reduces androgen synthesis by inhibiting p450 c17. P450 c17 converts progesterone and pregnenolone to 17-OH progesterone and 17-OH pregnenolone, which are converted to androstenedione and DHEA, and eventually to testosterone[81]. Clinical trials have demonstrated that abiraterone improves progression-free and overall survival in patients with metastatic CRPC[60-62]. Abiraterone was granted FDA approval in 2011 for the treatment of patients with metastatic CRPC who had previously received docetaxel treatment[81]. In patients with hormone-sensitive metastatic prostate cancer, the addition of abiraterone to ADT improves overall survival, suggesting that abiraterone may also be an effective treatment for hormone-sensitive cancer[63].

Enzalutamide inhibits AR signalling by preventing the binding of androgens to the AR, inhibiting AR nuclear translocation, and preventing the activated AR from binding to DNA[81]. Clinical trials showed that enzalutamide improves overall survival in patients with metastatic CRPC[64,65]. Reported adverse events included fatigue, hypertension, diarrhoea, and hot flashes[64,65]. In 2012, enzalutamide was granted FDA approval for the treatment of patients with metastatic prostate cancer. Other AR inhibitors, such as apalutamide and darolutamide, have also shown effectiveness for the treatment of prostate cancer[66-69].

The development of immunotherapeutic treatments for prostate cancer is still in its early stages. However, sipleucel-T shows some promise in patients with metastatic CRPC. Treatment involves *ex vivo* activation of patient-derived peripheral blood mononuclear cells with prostatic acid phosphatase and granulocyte-macrophage colony-stimulating factor, before reinjection to patients[28,81]. The IMPACT trial showed that sipleucel-T improved overall survival in patients with metastatic CRPC[72]. Moderate adverse events were reported, including chills, fever, and headache. These results suggest that sipleucel-T may produce beneficial therapeutic effects in patients with metastatic prostate cancer.

PARP inhibitors have proven effective for the treatment of breast and ovarian cancers, particularly in patients harbouring genetic defects in DNA damage response genes such

as *BRCA1* and *BRCA2*[86]. PARP inhibition induces synthetic lethality in cells with defects in DNA damage response[19]. In clinical trials of patients with metastatic CRPC, PARP inhibitors such as olaparib, rucaparib, and talazoparib have produced favourable responses in patients with alterations in DNA damage response genes[73,74,87-89].

While several new treatments have produced improvements in survival for prostate cancer patients, CRPC remains incurable, and the mortality rate for patients with metastatic and castration-resistant cancer remains high, with a median overall survival of less than 2 years for patients with CRPC[90]. New, more effective treatments are urgently needed, particularly for patients with advanced prostate cancer.

1.1.9. PROSTATE CANCER CHEMOPREVENTION

Cancer chemoprevention refers to the use of agents to prevent or delay the development or progression of cancer[91-93]. The World Health Organisation estimates that 30-50% of cancers may be preventable[94]. Therefore, identifying agents that can protect against cancer development has the potential to substantially reduce cancer incidence and mortality. The chemoprevention approach has already proven effective in the context of heart disease, substantially reducing the incidence of cardiovascular diseases over the last few decades[91].

Several cancer chemopreventive agents have been granted FDA approval, including tamoxifen and raloxifen for the prevention of breast cancer[91,95]. In prostate cancer, the 5α-reductase inhibitors finasteride and dutasteride showed potential as chemopreventive agents in preclinical studies[96-98]. 5α-reductase inhibitors block the conversion of testosterone to the more active dihydrotestosterone. Finasteride and dutasteride showed some chemopreventive effectiveness in the PCPT and REDUCE trials, particularly for preventing the development of low-grade tumours[99,100]. However, due to an observed increase in the incidence of high-grade disease in the treatment arms of both studies, neither agent has been granted FDA approval[91].

Due to the important role of oxidative stress in cancer development, antioxidant compounds have been extensively studied for chemoprevention of various cancer types[101-108]. In prostate cancer, several plant-derived antioxidants have been investigated for their ability to protect against oxidative stress and inhibit prostate carcinogenesis[21]. Sulforaphane (SFN), found in cruciferous vegetables, induces

transcription factors such as nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates antioxidant defences and increases the expression of free radical scavenging enzymes[92,109]. SFN showed some promising results in chemoprevention clinical trials, attenuating the increased expression of genes involved in oncogenic pathways observed in the control arm of the study[25,26]. Additionally, the consumption of cruciferous vegetables was inversely correlated with prostate cancer progression[25]. Lycopene, found in tomatoes, has been investigated for prostate cancer prevention in several clinical trials (NCT01105338, NCT00416325, NCT00093561, NCT00006078, NCT00744549, NCT00450957, NCT01443026), with some evidence that lycopene supplementation significantly alters AR signalling and Nrf2-mediated oxidative stress response[110], though its effects on prostate cancer incidence and progression have not been assessed directly. Curcumin, found in turmeric, scavenges free radicals and induces antioxidant enzymes, leading to reduced oxidative stress[92,93]. Curcumin is currently under investigation for prostate cancer prevention (NCT03769766). Selenium is a dietary antioxidant that can inhibit malignant transformation of normal prostate epithelial cells[21]. The Nutritional Prevention of Cancer Trial showed that selenium supplementation significantly reduced prostate cancer incidence, but only in participants with low baseline selenium levels[23]. The large-scale SELECT trial investigated the use of selenium and vitamin E for prostate cancer chemoprevention in 34,887 participants. However, the study found that vitamin E supplementation significantly increased prostate cancer risk by 17%[24]. While several plant-derived antioxidants have been investigated for their ability to protect against prostate cancer development, no agents have yet received FDA approval for chemoprevention in prostate cancer.

1.2. CANNABINOIDS

1.2.1. THE CANNABIS PLANT

Cannabis sativa is among the most useful and versatile plants in the world. It is a prime example of a multi-purpose crop, which humans have exploited for a range of applications, including the manufacture of fuel, building materials, textiles, and cosmetics. The cannabis plant also has a long history of medicinal use by cultures across the world, with some evidence dating its healing practise as early as 400 A.D.[111]. Considerable preclinical evidence now indicates that cannabis bio-actives (cannabinoids) can elicit favourable therapeutic effects in a wide range of medical

conditions. The development of new cannabis-based medicines could potentially benefit many patients.

However, cannabis also generates much controversy due to its effects on brain activity. Certain cannabinoids, such as tetrahydrocannabinol (THC), elicit psychotropic effects and modulate brain functioning. Consequently, from the early twentieth century onwards, countries began to classify cannabis as an illegal drug, preventing its use in medicine[112]. Legal barriers have limited the ability of scientists to research the therapeutic properties of cannabis and cannabinoids comprehensively, and to identify the molecular mechanisms driving their medicinal effects. However, since the beginning of the twenty-first century, mounting evidence demonstrating the medicinal benefits of cannabinoids has led several countries to legalise cannabis or cannabinoids for specific medical uses[113].

A growing body of research indicates the importance of the human endocannabinoid system and cannabinoids in regulating processes related to cancer. The strongest evidence of cannabinoid anti-cancer effects comes from studies in glioma. A large number of preclinical studies indicate that cannabinoids reduce cell proliferation and increase cell death in glioma cell lines and reduce tumour growth and prolong survival in mouse models of glioma[50,51,114-119]. In 2006, a small pilot clinical study found that THC was well-tolerated in patients with glioblastoma[120]. More recently, clinical trials have assessed the use of a cannabinoid combination treatment in patients with recurrent glioblastoma multiforme, reporting some improvements in 1-year survival following cannabinoid treatment (NCT01812603, NCT01812616). While many studies, across a range of cancer types, have reported anti-cancer effects of plant-derived cannabinoids (phytocannabinoids), the current understanding of the molecular targets and the intracellular mechanisms driving these effects remains incomplete.

1.2.2. THE ENDOCANNABINOID SYSTEM

Early investigations into the pharmacological activity of the cannabis plant and, specifically, the phytocannabinoid THC, led to the discovery of the human endogenous cannabinoid (endocannabinoid) system. The endocannabinoid system is a complex signalling network that developed early in evolution and regulates crucial processes throughout the body, including pain, inflammation, energy metabolism, synaptic plasticity, cell survival, and cell proliferation[121]. The components of the

endocannabinoid system include: the major cannabinoid receptors, CB_1 and CB_2; the endogenous cannabinoid ligands *N*-arachidonoyl-ethanolamine (anandamide) and 2-arachidonoyl glycerol (2-AG); and the metabolic enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), which catalyse the degradation of endocannabinoids[122] (**Figure 1.7**). More recently, Ryberg *et al.* identified the orphan G-protein coupled receptor GPR55 as a putative cannabinoid receptor[123]. Exogenous cannabinoids, both plant-derived and synthetic, can compete with endocannabinoids for cannabinoid receptor binding sites, and modulate processes controlled by endocannabinoids.

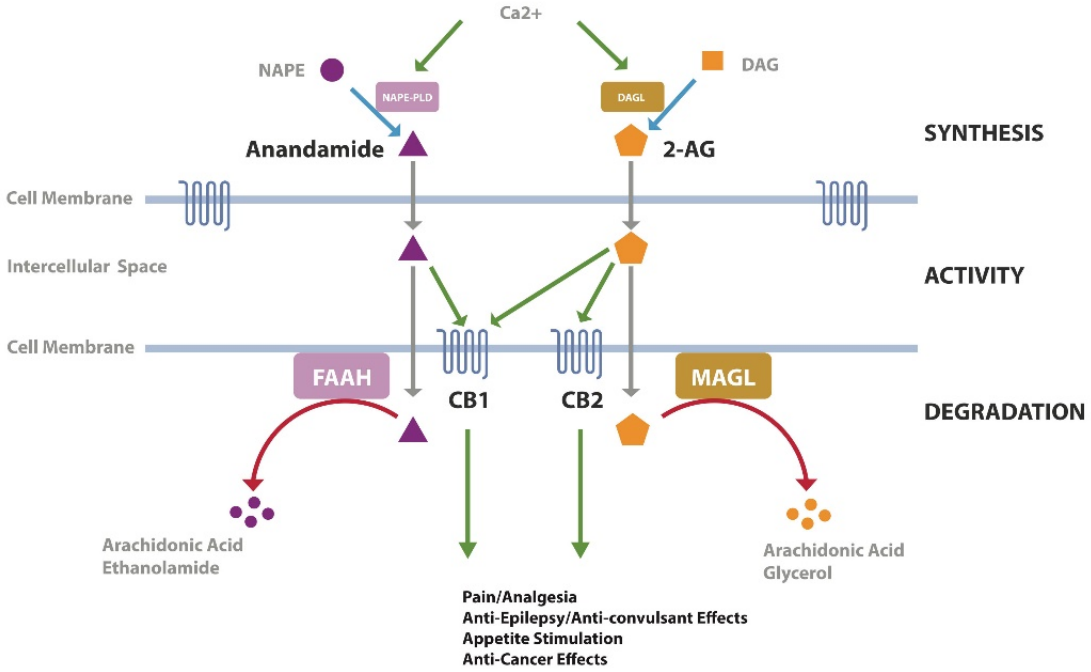


Figure 1.7. The endocannabinoid system. Synthesis, activity, and degradation of endocannabinoids. NAPE – N-arachidonoyl phosphatidyl ethanolamine, DAG – diacylglycerol, 2-AG - 2-arachidonoyl glycerol, FAAH - fatty acid amide hydrolase, MAGL - monoacyl glycerol lipase.

Cannabinoid receptors are among the most abundant neurotransmitter receptors in humans[124]. The CB_1 receptor is predominantly expressed by cells of the central nervous system, in areas that regulate learning and memory, emotion, and motor, sensory, and endocrine functions[125]. Activation of CB_1 by cannabinoids produces psychoactive effects. In contrast, the CB_2 receptor is primarily expressed outside the

central nervous system, predominantly by immune cells[124]. CB_2 agonists generally lack the psychoactivity displayed by CB_1 agonists. CB_1 and CB_2 receptors are also expressed in the lungs, liver, skin, lymph nodes, and bones, where they can regulate processes such as inflammation, immune cell recruitment, lipid metabolism, cell proliferation and differentiation, and bone formation[126-130]. GPR55 is expressed at high levels in the central nervous system, the adrenal glands, and parts of the gastrointestinal tract, and is involved in immune cell functioning, inflammation, pain regulation, cell proliferation, bone formation, and regulation of vascular tone[123,131]. Both CB_1 and CB_2 are seven-transmembrane G-protein coupled receptors, and function by interacting with $G_{i/o}$ proteins, inhibiting adenylyl cyclase, and reducing intracellular levels of cyclic adenosine monophosphate (cAMP)[132]. Meanwhile, GPR55 appears to be coupled to G_{α} proteins, though GPR55 activation has been less extensively studied [123,133].

Studies of cannabinoid receptor expression in cancer tissues show altered CB_1 and CB_2 expression in various cancer types, including prostate cancer, often associated with poor prognosis, indicating a possible involvement of the endocannabinoid system in cancer development[132,134]. Similarly, GPR55 is expressed at higher levels in cancer cells and high expression is associated with poor prognosis and increased cancer aggressiveness[131]. Components of the endocannabinoid system can regulate crucial processes in cancer, including cell proliferation, cell death, angiogenesis, and metastasis[125,132]. Therefore, modulation of the endocannabinoid system with synthetic or plant-derived cannabinoids is a promising approach for cancer treatment.

1.2.3. PHYTOCANNABINOIDS

As the cannabis plant is easy to cultivate, and contains an abundance of potential therapeutic agents, it is an attractive option as a source of plant-based medicines. *Cannabis sativa* contains more than 500 chemical compounds, including more than 100 phytocannabinoids[135,136]. Phytocannabinoids are a group of terpenophenol compounds synthesised and stored in secretory cells within the glandular trichomes in the female flowers of the cannabis plant[137]. Phytocannabinoids can target the endocannabinoid system and are responsible for most of the biological effects of cannabis. Cannabis plants are commonly classified according to their phytocannabinoid content. Drug-type plants, bred for their intoxicating properties, contain a high ratio (>1)

of psychoactive cannabinoids (THC, cannabinol) to non-psychoactive cannabinoids (cannabidiol). Meanwhile, fibre-type plants, cultivated as a source of textile fibre or oil, have a low ratio (<1) of psychoactive to non-psychoactive cannabinoids[138].

THC, the most abundant phytocannabinoid in drug-type cannabis plants, acts as a partial agonist at CB_1 and CB_2 receptors, in addition to activating the transcription factor peroxisome proliferator activated receptor gamma (PPARγ) and the transient receptor potential ion channels transient receptor potential vanilloid 2 (TRPV2) and transient receptor potential ankyrin 1 (TRPA1), and antagonising transient receptor potential metastatin 8 (TRPM8)[139-142] (**Figure 1.8**). THC produces a range of biological effects, including anti-emetic, analgesic, anti-inflammatory, neuroprotective, and anti-cancer effects[140,142-146]. However, by interacting with CB_1 in neurons, THC also modulates brain activity and produces psychotropic effects which limit its therapeutic usefulness.

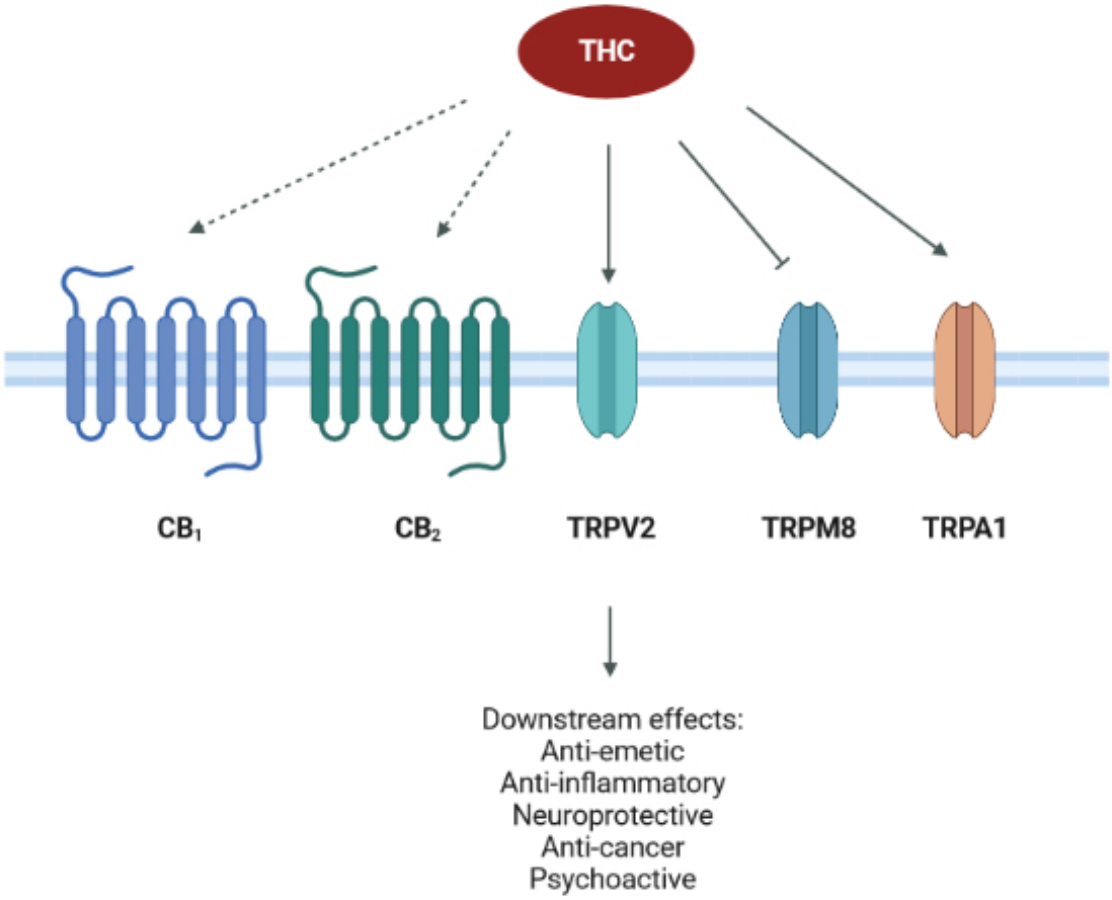


Figure 1.8. Receptor targets of THC. Solid arrows indicate activation. Solid inhibitor line indicates inhibition. Dashed arrows indicate partial agonism. TRPV – transient receptor potential vanilloid, TRPM – transient receptor potential metastatin, TRPA – transient receptor potential ankyrin. Figure created using BioRender.com.

Non-intoxicating phytocannabinoids, such as cannabidiol (CBD), do not produce the characteristic 'high' associated with cannabis and may be more suitable than THC as therapeutic agents. CBD is the most abundant phytocannabinoid in fibre-type cannabis plants[137]. Unlike THC, CBD appears to have low affinity for CB_1 and CB_2 receptors[141], but displays a wide range of pharmacological effects, mediated through numerous mechanisms (**Figure 1.9**). Thomas *et al.* reported that CBD can potently antagonise the activity of synthetic CB_1 and CB_2 agonists[147]. Other studies identified CBD as a negative allosteric modulator of CB_1, altering receptor conformation and ligand-binding activity[148,149]. While some studies reported that CBD acts as an inverse agonist at CB_2, others identified CBD as a partial CB_2 agonist[147,149]. CBD can also enhance endocannabinoid signalling, by inhibiting FAAH activity and anandamide reuptake[150,151]. Furthermore, CBD may act as an antagonist at the putative cannabinoid receptor GPR55[123]. Like THC, CBD can modulate the activity of TRP ion channels. Substantial evidence indicates that CBD can increase the mRNA and protein expression of TRPV2 and promote TRPV2 activation[50,114,152]. CBD can also produce anti-cancer effects through the activation of TRPV1[153,154]. Meanwhile, CBD antagonises TRPM8 activity and reduces TRPM8 mRNA expression[140,155]. Additionally, CBD can activate PPARγ[153] and the serotonin receptor 5-HT_{1a}[143].

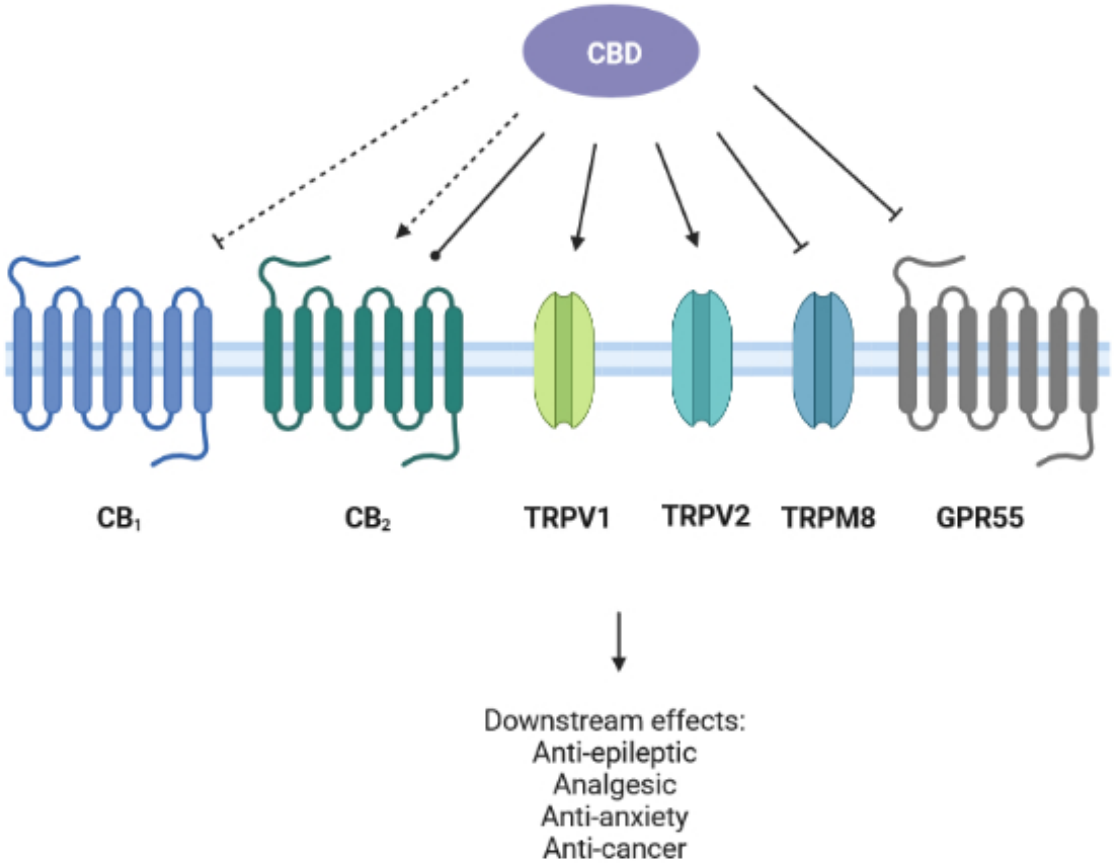


Figure 1.9. Receptor targets of CBD. Solid arrows indicate activation. Solid inhibitor lines indicate inhibition. Dashed arrow indicates partial agonism. Dashed inhibitor line indicates negative allosteric modulation. Dot line indicates inverse agonism. TRPV – transient receptor potential vanilloid, TRPM – transient receptor potential metastatin. Figure created using BioRender.com.

Furthermore, the cannabis plant contains many additional, less abundant cannabinoids displaying weak or no psychoactivity, including cannabigerol (CBG), cannabichromene (CBC), tetrahydrocannabivarin, cannabidivarin, and the cannabinoid acids tetrahydrocannabinolic acid and cannabidiolic acid. The relative ratios of phytocannabinoids vary between different strains of the cannabis plant and appear to be determined by genetic factors, specifically the allelic variants of the enzymes involved in cannabinoid synthesis[156]. Using conventional breeding methods, De Meijer *et al.* were able to produce cannabis strains containing high levels of specific phytocannabinoids, including THC, CBD, CBG, and CBC[156-158]. Determining which phytocannabinoids and phytocannabinoid combinations provide the greatest therapeutic benefits could allow for the development of new strains with cannabinoid ratios tailored for specific medical uses.

The current study investigates the anti-cancer potential of the phytocannabinoids GL1a and GL4a. GL1a is a purified form of CBD, extracted from the flowers and leaves of the hemp plant. GL4a is a purified form of CBG extracted from the hemp plant. Both GL1a and GL4a were provided by my enterprise partners, GreenLight Pharmaceuticals. Both CBD and CBG have previously shown chemotherapeutic potential in various cancer types, including prostate cancer[155].

1.2.4. MEDICAL USES OF PHYTOCANNABINOIDS

Scientific interest in the medicinal potential of the cannabis plant is growing. Increasingly, research indicates potential benefits of cannabis-based medicines for the treatment and management of a broad range of conditions. In particular, the analgesic, anti-emetic, and anti-convulsant properties of phytocannabinoids are now widely accepted[159]. In 2017, the National Academies of Sciences, Engineering, and Medicine (NASEM) reported that "conclusive or substantial" evidence supports the use of cannabinoids for the treatment of pain, multiple sclerosis-associated spasticity, and chemotherapy-induced nausea and vomiting[160]. Additional studies report anti-inflammatory[161], antioxidant[161], anxiolytic[162], antidepressant[163], and antimicrobial[137] properties of cannabinoids, though further investigation into these effects is required. With growing evidence of cannabinoid therapeutic benefits, many countries have approved cannabis-based medicines for the treatment of specific medical conditions. For example, Epidiolex, a purified form of CBD, is used to treat two severe paediatric seizure disorders, Lennox-Gastaut syndrome and Dravet syndrome[113]. Many countries have approved nabiximols (Sativex), an oral spray of THC and CBD, for the treatment of spasticity and related symptoms in multiple sclerosis[113]. Furthermore, synthetic analogues of phytocannabinoids, such as the THC analogues dronabinol and nabilone, act as anti-emetics and are prescribed for the management of chemotherapy-induced nausea and vomiting and AIDS-associated anorexia[113].

While many *in vitro* and *in vivo* studies demonstrate anti-cancer potential of phytocannabinoids, little is known about whether the beneficial effects seen in preclinical studies can translate to therapeutic benefits for cancer patients. Few clinical trials have investigated the anti-tumour effects of cannabis-based medicines, and no large-scale trials have yet shown a therapeutic benefit of phytocannabinoids in cancer. Additionally, most preclinical studies have focused on the effects of cannabinoids in glioma, and the

effects in other cancer types are understudied. Consequently, the NASEM report describes the evidence for anti-cancer effects of cannabinoids as “insufficient”. Further investigations into the effects of cannabinoids in multiple cancer types, and the subsequent design of clinical trials to evaluate cannabinoid anti-tumour effects are thus needed.

1.2.5. PHYTOCANNABINOIDS AND CANCER

1.2.5.1. RECEPTOR EXPRESSION

The altered expression of cannabinoid receptors in malignant tissue identifies these receptors as potential therapeutic targets[132]. Although several studies show differential expression of cannabinoid receptors between cancerous and normal tissues, no clear consensus exists regarding the function of the endocannabinoid system in tumour development and progression. High CB_1 mRNA and protein expression is associated with poor prognosis in prostate[134], pancreatic[164], ovarian[165], and renal cancers[166]. CB_2 overexpression is linked to tumour grade and poor prognosis in breast cancer[167,168] and astrocytoma[169]. Furthermore, expression of heteromers of CB_2 and the human epidermal growth factor receptor 2 (HER2) correlates with reduced survival and increased metastasis in breast cancer[170]. Similarly, high levels of GPR55 expression are associated with poor prognosis and disease progression in breast cancer, pancreatic cancer, and glioma[131,171]. Additionally, high expression of the TRPV channels TRPV1, TRPV2, and TRPV4 correlates with tumour grade and reduced survival in cancer patients[172,173]. Therefore, phytocannabinoids, which modulate the activity of cannabinoid receptors and TRPV channels, may have potential as anti-cancer agents.

1.2.5.2. ANTI-PROLIFERATIVE EFFECTS

Cell proliferation is a fundamental hallmark of tumour growth and disease progression. Accordingly, anti-proliferative agents are potential anti-cancer therapies. *In vitro* studies have demonstrated growth inhibition by phytocannabinoids in multiple cancer cell lines, commonly accompanied by modulation of cancer-related signalling cascades, including the extracellular signal-related kinase (ERK), AKT, epidermal growth factor receptor

(EGFR), ROS, and p38 mitogen-activated protein kinase (MAPK) pathways. An early study of the effects of CBD in glioma cells showed that CBD inhibited proliferation by downregulating the ERK and AKT pro-survival pathways[51]. In breast cancer cells, CBD reduced EGF-induced proliferation by inhibiting the recruitment of EGF-secreting tumour-associated macrophages, leading to decreased phosphorylation and activation of EGFR, ERK, and AKT[52]. Additionally, CBD suppressed tumour growth in mouse models of breast cancer, which was accompanied by reduced phosphorylation of EGFR, ERK, and AKT in tumour cells. Similarly, CBD reduced the proliferation of lung cancer cells by downregulating *EGFR* expression[174]. In glioma stem cells, which are resistant to standard therapies, CBD inhibited self-renewal through increased production of ROS, inhibition of AKT phosphorylation, and activation of the p38 MAPK pathway[119]. Activation of p38 MAPK led to downregulation of the stem cell regulators Sox-1, Id-1, and phosphorylated signal transducer and activator of transcription 3 (STAT3). Furthermore, CBD treatment significantly prolonged survival in glioma-bearing mice. Recent RNA-seq analysis in head and neck squamous cell carcinoma (HNSCC) cells indicates that CBD modulates the expression of genes from pathways involved in the regulation of cell proliferation[175].

Several studies point to the involvement of the ion channel TRPV2 in the anti-proliferative effects of CBD. TRPV2 can regulate important cancer-related processes including tumour growth and progression by regulating levels of intracellular calcium and modulating the activation of pro-survival signalling pathways[176-178]. In glioma cells, the inhibition of proliferation by CBD was associated with increased TRPV2 expression and reduced AKT signalling[114]. TRPV2 activation stimulated the upregulation of the transcription factor Aml-1a, which regulates a range of cellular processes, including proliferation. CBD also reduced proliferation through the activation of TRPV2 in multiple myeloma cells[50]. The proliferation inhibition was accompanied by reduced activation of ERK, AKT, and nuclear factor kappa B (NFκB), and reduced expression of the cell cycle regulator cyclin D1. Notably, the effects were enhanced in TRPV2-positive cells and reverted by a TRP channel blocker. Similarly, in leukaemia cells, CBD inhibited cell proliferation and induced cell cycle arrest, effects that were blocked by a TRPV2 antagonist[179].

Several studies have shown that CBD can modulate the activity of cell cycle regulators, through various mechanisms. One potential target mediating the effects of CBD on the cell cycle is the putative cannabinoid receptor GPR55. GPR55 promotes cell proliferation and tumour growth and is upregulated in various cancer cell types[180]. Ferro *et al.*

reported that CBD induced cell cycle arrest and reduced proliferation in pancreatic cancer cells by antagonising GPR55[53]. GPR55 antagonism by CBD led to decreased ERK phosphorylation and reduced levels of ribonucleotide reductases, needed for DNA synthesis. Additionally, CBD reduced the expression of cyclin D and the phosphorylation of RB. In gastric cancer cells, CBD inhibited cell proliferation and colony formation through upregulation of ataxia telangiectasia-mutated gene (ATM)[54]. ATM upregulation led to modulation of p21 and p53 expression, resulting in the inhibition of CDK2/cyclin E complex formation and the induction of cell cycle arrest. The growth-inhibitory actions were accompanied by increased production of ROS. The modulation of cell cycle regulators and the inhibition of cell proliferation by CBD suggest that CBD-based treatments could potentially reduce tumour growth in cancer patients.

The anti-proliferative effects of THC have been less extensively researched. Like CBD, THC reduced cell proliferation in lung cancer cells by downregulating EGFR[174]. THC also reduced proliferation of breast cancer cells by increasing the expression of p21 and downregulating CDK1, and reduced tumour growth in mice through activation of CB_2 and inhibition of AKT phosphorylation[168,181]. In hepatocellular carcinoma, THC reduced cell proliferation by modulating the transcription factor PPARγ[182,183]. THC increased the intracellular expression of PPARγ and upregulated PPARγ signalling pathways[182]. Furthermore, THC treatment inhibited tumour growth in a mouse xenograft model of liver cancer, which was accompanied by increased PPARγ mRNA levels in tumours. The *in vitro* and *in vivo* effects of THC were reversed by the addition of a PPARγ antagonist. More recently, some evidence suggests that THC can inhibit the proliferation of glioblastoma cells through activation of GPR55[184].

The key mechanisms of action underlying the anti-proliferative and anti-cancer effects of phytocannabinoids are summarised in **Figure 1.10**.

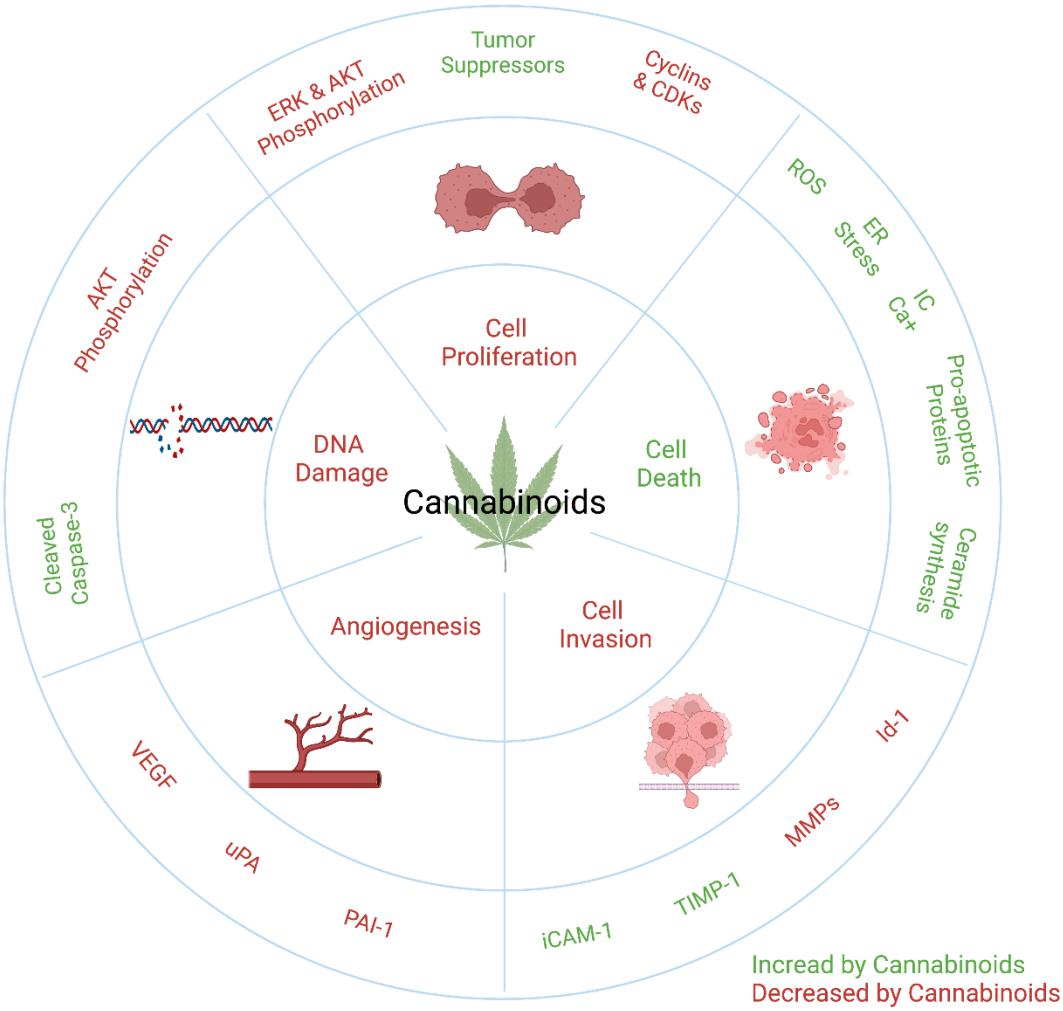


Figure 1.10. Phenotypic effects and underlying mechanisms of phytocannabinoids in cancer cells. ERK – extracellular signal-related kinase, CDK – cyclin dependent kinase, ROS – reactive oxygen species, ER – endoplasmic reticulum, IC Ca^{2+} - intracellular calcium, VEGF – vascular endothelial growth factor, uPA – urokinase-type plasminogen activator, PAI-1 – plasminogen activator inhibitor 1, iCAM-1 – intercellular adhesion molecule 1, TIMP-1 – tissue inhibitor of metalloproteinases 1, MMP – matrix metalloprotease, Id-1 – inhibitor of differentiation 1. Source: O'Reilly *et al.* (2022). Plant-derived cannabinoids as anti-cancer agents. *Trends Cancer*. 10.1016/j.trecan.2022.01.017

1.2.5.3. INDUCTION OF CELL DEATH

Substantial evidence supports a role for phytocannabinoids in the induction of cell death through apoptosis and/or autophagy in a range of cancer types, although the precise mechanisms are still under investigation.

CBD-induced apoptosis has been reported in many cancer types, including glioma, breast cancer, colorectal cancer, lung cancer, multiple myeloma, prostate cancer, leukaemia, gastric cancer, and neuroblastoma. Many of the mechanisms underlying the anti-proliferative effects of CBD also contribute to its pro-apoptotic activity (**Figure 1.10**). For example, in glioma and multiple myeloma cells, CBD induced cell death through increased activation of TRPV2[50,114]. In leukaemia cells, CBD-induced apoptosis was associated with reduced phosphorylation of the survival-related proteins mammalian target of rapamycin (mTOR), AKT, and S6[185]. CBD increased the phosphorylation of the stress kinases c-Jun N-terminal kinase (JNK) and p38 MAPK in glioma cells, leading to apoptosis through decreased activation of ERK and AKT[186]. Additionally, CBD increased the expression of the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα) and the death ligand TNF-related apoptosis inducing ligand (TRAIL), as well as the expression of the death receptors TNFR1 and TRAIL-R2. Alharris *et al*. showed that CBD increased apoptosis in neuroblastoma cell lines by activating TRPV1 and the serotonin receptor 5-HT_{2A}[154]. The study also identified the involvement of miRNA in CBD-induced apoptosis, with downregulation of has-let-7a leading to increased expression of caspase-3 and has-mir-1972 upregulation leading to decreased expression of genes involved in proliferation and survival.

The pro-apoptotic effects of CBD in cancer cells commonly involve increased ROS production and ER stress. CBD promoted cell death in gastric cancer cells through increased ROS production, leading to apoptosis through the upregulation of Bad and Bax, downregulation of B-cell lymphoma 2 (Bcl-2), loss of mitochondrial membrane potential, release of cytochrome c, and activation of caspases[54]. CBD increased ROS production leading to increased apoptosis in cisplatin-resistant lung cancer cells, through activation of TRPV2 and inhibition of Nrf2 expression[187]. A further study in colorectal cancer showed that CBD-induced ER stress promoted apoptosis by increasing the expression of the pro-apoptotic protein Noxa. Furthermore, CBD induced Noxa and increased apoptosis *in vivo*, leading to inhibition of tumour growth[188]. In a mouse model of glioma, CBD induced apoptosis through increased ROS production, resulting in inhibition of tumour progression and prolonged survival[119].

Several studies indicate that CBD can induce cell death by modulating both autophagy and apoptosis. In breast cancer cells, CBD induced ER stress, leading to reduced AKT signalling, decreased phosphorylation of mTOR and 4EBP1, and reduced expression of cyclin D1[189]. The CBD-induced cell death required increased ROS production and occurred through modulation of the cross-talk between autophagy and apoptosis. In glioma cells, CBD induced autophagy through activation of TRPV4, causing changes in calcium flux leading to increased ER stress, mitochondrial dysfunction, and altered AKT signalling[173]. Additionally, CBD induced cell death through increased autophagy and apoptosis in endometrial cancer and HNSCC cells[172,175]. A recent study showed that inhibition of autophagy blocked the pro-apoptotic effects of CBD in breast cancer cells[190]. Interestingly, inhibitors of early-stage autophagy blocked the effects of CBD in glioma cells, while inhibitors of late-stage autophagy enhanced CBD effects[191], highlighting the complexity of CBD-induced cell death in cancer.

Meanwhile, the induction of cancer cell death by THC is largely mediated through increased synthesis of the sphingolipid ceramide and increased ER stress, leading to the stimulation of autophagy and/or apoptosis. The induction of cell death by THC has been demonstrated in glioma, melanoma, pancreatic cancer, hepatocellular carcinoma, and leukaemia, and in transformed embryonic fibroblasts. Studies in glioma in the early 2000s reported that THC induced apoptosis by stimulating ceramide synthesis and accumulation in cancer cells[115,116]. Carracedo *et al.* showed that THC increased ceramide synthesis in both glioma and pancreatic cancer cells, causing apoptosis through upregulation of the stress protein p8 and the ER stress targets activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), and tribbles pseudokinase 3 (TRIB3)[117,118]. An additional study in glioma showed that THC-induced TRIB3 upregulation promoted autophagy through inhibition of the AKT/mTOR pathway[192]. THC also inhibited AKT and mTOR activation through the upregulation of TRIB3 in transformed embryonic fibroblasts, stimulating autophagy-mediated cell death[193]. In hepatocellular carcinoma cells, THC stimulated both autophagy and apoptosis through activation of PPARγ[182]. The THC-induced cell death was associated with increased activity and expression of PPARγ, which was dependent on TRIB3. More recent studies showed that THC also induced cell death in melanoma cells through a TRIB3-dependent mechanism[194]. In glioma cells, modulation of ceramide synthesis by THC stimulated the permeabilization of the lysosome membrane, the release of cathepsin and cytochrome c, and the activation of apoptosis[195]. THC also increased the expression of enzymes involved in the synthesis of sphingolipids, and

THC-induced autophagy was dependent on increased sphingolipid synthesis. Furthermore, THC promoted autophagy and reduced tumour growth in a melanoma mouse model, and induced apoptosis, reduced tumour growth, and prolonged survival in animal models of glioma[115,194]. Taken together, the above results indicate that THC induces cancer cell death by promoting ceramide synthesis, leading to increased ER stress, inhibition of the AKT/mTOR signalling pathway, and the induction of autophagy and/or apoptosis.

Little is known about the ability of other phytocannabinoids to induce cancer cell death. However, one study by Borrelli *et al.* showed that CBG induced apoptosis in colorectal cancer cells through increased ROS production and the upregulation of CHOP[196]. The effects were dependent on the antagonism of TRPM8 and the activation of CB_2, and occurred independently of the TRP ion channels TRPA1, TRPV1, and TRPV2. Notably, CBG also reduced tumour growth in a mouse xenograft model of colorectal cancer.

1.2.5.4. ANTI-METASTATIC EFFECTS

One of the major challenges in cancer treatment is preventing cancer cells from metastasising. Several *in vitro* and *in vivo* studies show that phytocannabinoids can inhibit the migration and invasion of cancer cells by inhibiting the epithelial-mesenchymal transition (EMT) and by modulating the expression of various of pro- and anti-metastatic proteins, including Id-1, matrix metalloproteases (MMPs), and tissue inhibitor of metalloproteinases 1 (TIMP-1) (**Figure 1.10**).

The transition from an epithelial to a mesenchymal phenotype is a key characteristic of invasive cancer cells. Garcia-Morales *et al.* showed that CBD can restore an epithelial phenotype in breast cancer cells by re-localising β-catenin and E-cadherin at the adherens junctions, leading to reduced cell migration and invasion[197]. The anti-invasive effects were accompanied by reduced AKT phosphorylation, leading to inhibition of β-catenin nuclear translocation and the resulting overexpression of various pro-metastatic genes.

MMPs promote cell invasion by degrading the extracellular matrix, while TIMP-1 regulates and inhibits MMP activity. CBD reduced the invasiveness of cervical cancer and lung cancer cells by upregulating TIMP-1 through increased phosphorylation of p38 MAPK and ERK[198]. The anti-invasive effects of CBD were dependent on the activation

of CB_1, CB_2, and TRPV2. Similarly, in lung cancer cells, CBD reduced invasion by increasing TIMP-1 levels, in this case through modulation of ERK and upregulation of intercellular adhesion molecule 1 (iCAM-1)[199]. CBD reduced the migration and invasion of breast cancer cells by inhibiting the activation of EGFR, ERK, AKT, and NFkB, and inhibiting the nuclear translocation of NFkB, leading to reduced secretion of MMP-2 and MMP-9[52]. The reduced motility was associated with reduced recruitment of tumour-associated macrophages, which facilitate the migration and invasion of cancer cells. Additionally, several studies have shown that CBD reduces cancer cell invasion through the downregulation of Id-1, a metastatic regulator associated with an invasive phenotype [178,200,201], in addition to reducing the expression of other pro-metastatic proteins including vimentin, fibronectin, and Slug[202,203]. Notably, numerous studies have also demonstrated anti-metastatic activity of CBD *in vivo*. For example, in mouse models of breast cancer and lung cancer, CBD treatment reduced the number of metastatic foci by 50-60% and the size of metastatic foci by 30-40%[52,178,187].

The anti-metastatic effects of other phytocannabinoid compounds have not been thoroughly investigated. However, one recent study reported that CBG reduced cell invasiveness by 55-80% in various glioblastoma cell lines[204]. Additionally, recent studies have explored the anti-metastatic effects of combinations of phytocannabinoids. In multiple myeloma cells, CBD and THC, either alone or in combination, reduced cell migration by 30-60% by downregulating the chemokine receptor CXCR4 and the plasma membrane glycoprotein CD147[205]. A THC/CBD combination treatment also reduced EGF-induced migration in lung cancer cells by inhibiting the epithelial mesenchymal transition. The cannabinoid treatment restored the epithelial phenotype in cells, as evidenced by increased expression of *CDH1* and decreased expression of *CDH2* and vimentin[174]. Although the THC/CBD combinations reduced cell migration, the combination treatments were not significantly more effective than treatment with either agent alone.

1.2.5.5. INHIBITION OF ANGIOGENESIS

Formation of new blood vessels through angiogenesis is a crucial process in cancer, supplying growing tumours with necessary oxygen and nutrients. Some evidence suggests that phytocannabinoids may display anti-angiogenic properties. In human umbilical vein endothelial cells (HUVECs), CBD inhibited HUVEC sprouting *in vitro* and

angiogenesis *in vivo* by reducing protein expression levels of various pro-angiogenic molecules, including MMPs, urokinase-type plasminogen activator (uPA), and plasminogen activator inhibitor 1 (PAI-1)[206]. In contrast, Ramer *et al.* observed an up to 70% increase in sprout formation in HUVECs following direct treatment with THC and CBD[207]. However, when recombinant TIMP-1 was added to HUVECs to mimic the established cannabinoid-induced production of TIMP-1 by cancer cells, the THC/CBD treatment significantly reduced tube and sprout formation by 50-75% in the endothelial cells. A recent study identified a potential mechanism underlying the anti-angiogenic effects of cannabinoids in breast cancer cells, where CBD inhibited angiogenesis by increasing the ubiquitination and degradation of hypoxia-inducible factor 1 alpha (HIF-1a) by ubiquitin ligases, leading to decreased secretion of vascular endothelial growth factor (VEGF) and reduced blood vessel formation[203]. Additionally, THC reduced tumour blood vessel formation in a mouse model of breast cancer and reduced angiogenesis in pulmonary tumours in mice, as evidenced by reduced staining of CD-31[181,208]. Moreover, THC treatment reduced VEGF protein expression by 50-75% and inhibited VEGFR-2 activation in tumours from two patients with glioblastoma multiforme who were participating in a clinical trial for anti-cancer effects of THC[209].

1.2.5.6. SYNERGY BETWEEN PHYTOCANNABINOIDS

A possible benefit of the cannabis plant as a source of medicines is the potential synergy between different components of the plant to produce enhanced therapeutic effects. In glioma cells, a combination treatment of THC and CBD reduced cell proliferation, modulated the cell cycle, and induced apoptosis through increased ROS production, effects not observed when cells were treated with either compound in isolation[210]. THC and CBD also acted synergistically to reduce the viability of multiple myeloma cells[205]. The THC:CBD combination arrested cell cycle progression and induced autophagy-dependent necrosis more effectively than treatment with THC or CBD alone. Furthermore, in a mouse model of glioma, a combination treatment of THC and CBD reduced the growth of xenografts more effectively than treatment with either individual compound[211].

The enhanced therapeutic effects produced by combining phytocannabinoids suggests that whole plant extracts may be more effective than single isolated compounds as anti-cancer agents. In prostate cancer cells, several whole cannabis extracts were more

effective than pure cannabinoids at reducing cell viability[155]. In glioma cells, a whole-plant extract containing high levels of THC was more effective than pure THC at reducing cell viability, although the opposite effect was observed with an extract high in CBD[212]. Baram *et al.* measured the anti-cancer effects of whole cannabis extracts containing varying ratios of phytocannabinoids in a range of cancer cell types[135]. The whole plant extracts reduced cell survival and proliferation more effectively than THC alone. Additionally, a whole plant extract inhibited tumour growth in a mouse model of breast cancer, an effect not seen when treating with the same dose of pure THC[213]. Furthermore, non-cannabinoid components of the cannabis plant, such as terpenoids, may contribute to the anti-cancer activity of whole plant extracts. For example, administering cannabinoids with co-related terpenoid compounds enhanced the effects of cannabinoids in breast and colon cancer cells[214]. Notably, the anti-cancer effects were greatest when the cannabinoid ratios used were most similar to those found naturally in the plant.

1.2.5.7. SYNERGY WITH EXISTING ANTI-CANCER AGENTS

Substantial research indicates that cannabinoids show synergistic activity (a combined effect greater than the expected additive effect) when combined with existing anti-cancer therapies. Phytocannabinoids can sensitise cancer cells to commonly used chemotherapeutic agents. In 2008, Liu *et al.* demonstrated that treatment of leukaemia cells with THC sensitised the cells to the anti-leukaemic agents cytarabine, doxorubicin, and vincristine[215]. THC acted synergistically with each agent to reduce cell viability, as evidenced by combination index (CI) values <1 for each combination treatment. The sensitising effects were dependent on THC-induced inhibition of ERK phosphorylation. Nabissi *et al.* showed that CBD increased the uptake of the chemotherapeutic agents temozolomide, carmustine, and doxorubicin by glioma cells and enhanced their cytotoxic activity, by increasing the expression and activity of TRPV2[152]. Similarly, in multiple myeloma, CBD acted synergistically with bortezomib to reduce cell proliferation and induce cell death, with a stronger effect in cells expressing TRPV2[50]. Additional studies showed that CBD synergistically enhanced the effects of paclitaxel and doxorubicin on cell viability in breast cancer, and enhanced the effects of cisplatin, 5-fluoracil, and paclitaxel in HNSCC[175,202,216]. Co-treatment of colorectal cancer cells with CBD and TRAIL produced synergistic pro-apoptotic effects, including increased expression of CHOP, protein kinase R-like ER kinase (PERK) and death receptor 5 (DR5)[217].

Notably, the combination treatment also caused upregulation of CHOP and DR5 *in vivo*, leading to increased apoptosis and reduced tumour growth. In prostate xenograft tumours, combining bicalutamide with a cannabis extract high in CBD produced a significantly greater reduction in tumour volume after 35 days compared to treatment with bicalutamide alone. Additionally, the combination treatment significantly prolonged survival compared to treatment with vehicle or bicalutamide alone[155]. CBD also enhanced the effects of cisplatin and temozolomide on glioblastoma and HNSCC tumour growth *in vivo*[175,218].

Combinations of phytocannabinoids can also enhance the effects of anti-cancer treatments. In multiple myeloma cells, a THC/CBD combination enhanced the effectiveness of carfilzomib at reducing cell migration and promoting cell death[205]. Leukaemia cells were also killed more effectively by combinations of cannabinoids than by individual components, and further synergy occurred when cannabinoids were combined with existing chemotherapeutic agents[219]. Pre-treatment of glioma cells with a combination of THC and CBD prior to radiation therapy increased the sensitivity of the cells to the therapy through reduced ERK and AKT phosphorylation and increased autophagy and apoptosis[212]. Treatment with the THC:CBD combination sensitised the cells more effectively than treatment with either compound alone. Additionally, *in vivo*, combining THC and CBD with irradiation enhanced the anti-tumour effects of the therapy. In glioma xenografts, several studies showed that combining THC or Sativex with temozolomide reduced tumour growth[211,220,221]. The cannabinoids enhanced autophagy and apoptosis, even in tumours resistant to temozolomide treatment[211]. The synergy increased as the proportion of CBD increased[221], indicating the importance of identifying the relative contributions of individual cannabinoids to the therapeutic effects of cannabinoid-based treatments. Growing evidence suggests that phytocannabinoids can enhance the effects of existing anti-cancer agents, both *in vitro* and *in vivo*. The addition of phytocannabinoids to cancer treatment could reduce the treatment doses required and potentially reduce the toxic side effects experienced by patients.

1.2.5.8. CLINICAL TRIALS OF PHYTOCANNABINOIDS FOR CANCER TREATMENT

While substantial preclinical evidence supports a role for cannabinoids as anti-cancer agents, no large-scale clinical trials have assessed the use of phytocannabinoids for

cancer treatment. Clinical trials of phytocannabinoids in cancer have largely focussed on palliative effects such as pain and symptom relief in patients with advanced cancer[222]. One small pilot clinical study assessed the effects of THC in patients with recurrent glioblastoma[120]. THC was well tolerated, with no overt psychoactive effects. Notably, in cultured patient tumour cells, THC treatment reduced the percentage of Ki67-positive cells from 8.5% to 2.5%. Recently, a phase 1b trial in patients with glioblastoma showed that a THC:CBD combination treatment increased survival to 83% after one year, compared to 44% with placebo treatment[223] (**Table 1.2**). Reported adverse events included vomiting, dizziness, fatigue, nausea, and headache. Ongoing trials in glioblastoma, pancreatic cancer, and prostate cancer will assess the safety of cannabinoid-based treatments, alone or in combination with existing therapies, as well as their effects on progression-free and overall survival (NCT04428203, NCT03984214, NCT03529448). Further clinical trials, particularly large-scale trials, will be needed to determine whether the observed preclinical anti-cancer properties of phytocannabinoids can translate to therapeutic benefits for cancer patients.

Table 1.2. Clinical trials of phytocannabinoids for cancer treatment.

Cancer Type	Phase	Intervention	Outcomes measured	Results	Trial ID	Ref.
Glioblastoma	Ib	Sativex (THC:CBD) vs placebo	Primary: adverse events Secondary: PFS, OS	Higher survival rates in Sativex group (83% vs 44% at 1yr), no change in PFS. Adverse events: vomiting, dizziness, fatigue, nausea, headache.	NCT01812603 NCT01812616	[223]
Prostate	I/Ib	Epidiolex (CBD)	Primary: Adverse events Secondary: PSA levels, PSA velocity, testosterone levels, quality-of-life	NYA (completed)	NCT04428203	NA
Pancreatic	III	Dronabinol (THC) vs placebo	Primary: quality-of-life Secondary: PFS, OS, prognostic score, adverse events	NYA (recruiting)	NCT03984214	NA
Glioblastoma	Ib	TN-TC11G (THC:CBD) + TMZ + radiation therapy	Primary: maximum tolerated dose, adverse events Secondary: anti-tumour activity, PFS, OS	NYA (not yet recruiting)	NCT03529448	NA

TMZ – temozolomide, PFS – progression free survival, OS – overall survival, PSA – prostate specific antigen

1.2.5.9. PHYTOCANNABINOIDS AND CHEMOPREVENTION

The World Health Organisation estimates that up to 50% of cancers may be preventable[94]. Therefore, identifying novel agents that can reduce an individual's risk of developing cancer could substantially reduce cancer-related mortality. Some studies indicate the potential of phytocannabinoids as chemopreventive agents. In cancers driven by inflammation, such as colorectal cancer, the anti-inflammatory properties of cannabinoids may be beneficial in protecting against carcinogenesis. Aviello *et al.* showed that CBD slowed the proliferation of colorectal cancer cells *in vitro* and protected DNA from oxidative damage[153]. Furthermore, treatment of mice with CBD prior to treatment with the carcinogenic agent azoxymethane (AOM) reduced the AOM-induced formation of aberrant crypt foci, polyps, and tumours. Intracellularly, AOM increased the phosphorylation of AKT and reduced protein levels of cleaved caspase-3, effects that were counteracted by pre-treatment with CBD. The protective effects of CBD were mediated through the activation of CB_1, TRPV1, and PPARγ. An additional study from the same research group showed that a CBD-rich plant extract also inhibited colorectal cancer cell proliferation[224]. The effects of the plant extract were mediated by cannabinoid receptors and were cancer cell specific. In mice, the extract prevented AOM-induced formation of preneoplastic lesions and polyps and reduced tumour growth. Further research is needed to understand the protective effects of CBD in colorectal cancer, and to determine whether cannabinoids have chemopreventive potential in other cancer types.

1.2.5.10. PHYTOCANNABINOIDS IN PROSTATE CANCER

Despite the high incidence of prostate cancer and lack of treatments for aggressive disease, as well as the well-established anti-cancer effects of cannabinoids in other cancer types, few studies have investigated the chemotherapeutic potential of cannabinoids in prostate cancer.

There is limited evidence showing that expression levels of cannabinoid receptors and TRPV ion channels are higher in prostate cancer cells than in normal prostate epithelial cells[225], identifying them as potential therapeutic targets. An additional study found that high CB_1, TRPV1, and TRPV2 expression in prostate cancer correlated with disease progression and poor prognosis[134].

Some evidence indicates that cannabinoids show anti-cancer activity in prostate cancer. De Petrocellis *et al.* showed that CBD inhibited prostate cancer cell viability, with enhanced effects in serum-deprived cells[155]. In androgen-sensitive LNCaP cells, the pro-apoptotic effects of CBD were partly mediated through antagonism of the ion channel TRPM8, which is involved in AR-dependent prostate cancer cell survival. The CBD-induced apoptosis was accompanied by increased ROS production, increased levels of PUMA, CHOP, and intracellular calcium, activation of p53, and downregulation of the AR. CBD also increased the expression of the cell cycle regulators p21 and $p27^{kip}$. Furthermore, CBD enhanced the effects of docetaxel and bicalutamide on prostate cancer cell viability. Notably, in prostate xenograft tumours, a whole plant extract enriched in CBD potentiated the effects of bicalutamide and docetaxel on tumour growth and combining bicalutamide with the plant extract increased survival more effectively than treatment with either agent alone.

Sharma *et al.* showed that CBD reduced the viability of PC-3 and LNCaP cells, with less potent effects in non-cancerous PrEC and BPH-1 cells[226]. Furthermore, a plant extract containing CBD, CBN, and THC inhibited cell viability in LNCaP cells, accompanied by reduced expression of CB_1, CB_2, AR, VEGF, and PSA. The plant extract had no effect on the viability of non-cancerous cells.

Sreevalsan *et al.* showed that CBD induced apoptosis in LNCaP cells by increasing phosphatase expression, leading to cleavage of poly ADP-ribose polymerase (PARP) and caspase-3[227]. The pro-apoptotic effects of CBD were blocked by CB_1 or CB_2 receptor antagonists.

Research into cannabinoid effects in prostate cancer has largely focussed on the androgen-sensitive LNCaP cell line. The phenotypic effects of cannabinoids in the more treatment-resistant DU145 and PC-3 cell lines have not been thoroughly investigated. Additionally, while cannabinoids show some anti-cancer potential in prostate cancer, the underlying mechanisms of action have not yet been extensively investigated.

1.3. HYPOTHESIS AND AIMS

Substantial preclinical evidence demonstrates anti-cancer properties of cannabinoids in many cancer types. Cannabinoids can inhibit cancer cell proliferation, induce cell death, reduce cell migration and invasion, and inhibit angiogenesis. However, few studies have investigated the anti-cancer potential of cannabinoids in prostate cancer. Our overall hypothesis was that the phytocannabinoids GL1a, GL1b, and GL4a have chemotherapeutic and chemopreventive properties in prostate cancer. To test this hypothesis, we aimed to measure the phenotypic effects of these cannabinoids in cell line models of prostate cancer, to investigate the mechanisms of action underlying the observed phenotypic effects, and to assess the antioxidant and chemopreventive potential of phytocannabinoids in non-cancerous prostate cells. These aims were addressed in three results chapters:

Chapter 3: Phenotypic effects of cannabinoids in prostate cancer cell lines

In Chapter 3, we assessed the chemotherapeutic potential of GL1a and GL4a by performing a phenotypic screen in cell line models of prostate cancer. The specific aims were to determine the effects of cannabinoids on cell viability, cell proliferation, apoptosis, cell survival, and migration and invasion. Additionally, we assessed whether combination cannabinoid treatments produced enhanced anti-cancer activity.

Chapter 4: Mechanisms of action of cannabinoids in prostate cancer

In Chapter 4, we investigated the mechanisms of action underlying the phenotypic effects of GL1a. Specifically, we aimed to identify the receptor target(s) of GL1a in prostate cancer cells, to determine whether GL1a alters the expression of proteins involved in cell cycle regulation and cell invasion, and to investigate whether GL1a modulates the activity of the key cancer-related AKT and ERK signalling pathways.

Chapter 5: Chemopreventive potential of cannabinoids in prostate cancer

In Chapter 5, we conducted an exploratory investigation into the antioxidant and chemopreventive potential of phytocannabinoids by measuring their ability to protect non-cancerous cells from oxidative damage. The specific aims were to assess the ability of cannabinoids to protect non-cancerous cells from hydrogen peroxide-induced cytotoxicity and oxidative stress.

CHAPTER 2

Materials and Methods

2.1. MATERIALS

2.1.1. CELL LINES

For this study, 3 metastatic prostate cancer epithelial and 2 non-cancerous epithelial prostate cell lines were used, specifically DU145 and PC-3 (androgen-independent prostate cancer cells), LNCaP (androgen-dependent prostate cancer cells), RWPE-1 (prostate epithelial cells from histologically normal prostate), and PWR-1E (prostate epithelial cells from a normal prostate with mild hyperplasia) (**Table 2.1**). PC-3 cells appear to be more representative of basal than luminal prostate cancer, but also display characteristics typical of neuroendocrine cells[228,229]. DU145 cells are also more similar to basal than luminal cells, while LNCaP cells display a luminal phenotype[228,230]. Both PWR-1E and RWPE-1 cells express predominantly luminal cell markers[231]. The non-cancerous prostate cells were immortalised using HPV-18 (RWPE-1) or an adenovirus 12-sv40 hybrid virus (PWR-1E)[232]. Both non-cancerous cell lines express the androgen receptor. CCD-18Co colon fibroblast cells were also included as a non-cancerous cell line. DU145, PC-3 and LNCaP cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). PWR-1E, RWPE-1 and CCD-18Co cells were purchased from the American Type Culture Collection (ATCC).

Table 2.1. Characteristics of cell lines used in this study.

Name	*Tissue of origin*	*Cancerous (Yes/No)*	*Metastatic (Yes/No)*	*AR status*	*PSA production*	*P53 status*	*Immortalisation method*	*Ref.*
DU145	Prostate cancer brain metastasis	Yes	Yes	Negative	No	Positive (mutation P223L, mutation V274F)	-	[233] [234] [235] [232]
PC-3	Prostate cancer bone metastasis	Yes	Yes	Negative	No	Negative	-	[236] [234] [232]
LNCaP	Prostate cancer lymph node metastasis	Yes	Yes	Positive (mutation T878A, mutation F877L)	Yes	Wild-type	-	[237] [234] [232] [238]
PWR-1E	Human prostate epithelium	No	-	Wild-type	Yes	Wild-type	HPV-18	[239] [232]

RWPE-1	Human prostate epithelium	No	-	Wild-type	Yes	Wild-type	Adenovirus12-SV40	[231] [234] [232]
CCD-18Co	Human colon fibroblast	No	-	-	-	Wild-type	Finite cell line	[240] [241]

DU145, PC-3, and LNCaP cells were cultured in Roswell Park Memorial institute (RPMI) 1640 medium with GlutaMAX™ (Gibco), supplemented with 10% foetal bovine serum (FBS) (Gibco). PWR-1E and RWPE-1 cells were cultured in Keratinocyte Serum-Free medium supplemented with L-glutamine, bovine pituitary extract (50mg/L) and epidermal growth factor (5µg/L) (Gibco). CCD-18Co cells were maintained in Eagle's Minimum Essential medium (EMEM) (ATCC), supplemented with 10% FBS. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator (HERAcell™ 150i). All cells were routinely tested for mycoplasma contamination using the MycoAlert™ mycoplasma detection kit (Lonza).

2.1.2. CANNABINOID TREATMENTS

The phytocannabinoid compounds GL1a (purified plant-derived CBD), GL1b (purified plant-derived CBD), and GL4a (purified plant-derived CBG) were provided by my enterprise partner, GreenLight Pharmaceuticals. The compounds were extracted from the hemp plant using a proprietary technology that allows for the sequential isolation of cannabinoids from raw plant material. The purity data for GL1a are shown in **Appendix Figure A7.1.** The purity data for GL1b and GL4a were not available to me. 40mM stock solutions were prepared by dissolving the compounds in cell culture grade dimethyl sulfoxide (DMSO) (PanReac AppliChem). Aliquots of stock solution were stored at -80°C.

2.1.3. OTHER DRUG TREATMENTS

SR141716, SR144528, capsazepine, L-A-lysophosphatidylinositol (LPI), hydrogen peroxide 30 (w/w) H_2O (H_2O_2), N-acetyl-L-cysteine (NAC), chloroquine diphosphate, staurosporine, and L-sulforaphane (SFN) were purchased from Sigma-Aldrich. Palbociclib monohydrochloride was purchased from MedChemExpress. SR141716, SR144528, and capsazepine were dissolved using DMSO. LPI, NAC, and chloroquine

were dissolved using sterile dH_2O. All drug compounds were stored according to the manufacturer's instructions.

2.1.4. WESTERN BLOT ANTIBODIES

The antibodies used for Western blot experiments are summarised in **Table 2.2**.

Table 2.2. Details of antibodies used for Western blot experiments.

Name	Supplier	Cat. No.	Source	Dilution	MW
ß-actin	Sigma-Aldrich	A5316	Mouse	1:4000	42
α-tubulin	Abcam	AB52866	Rabbit	1:4000	52
GAPDH	Sigma-Aldrich	MAB374	Mouse	1:4000	38
Rabbit	Cell Signaling	7074	Goat	1:2000	-
Mouse	Cell Signaling	7076	Horse	1:2000	-
Cyclin D3	Cell Signaling	2936	Mouse	1:2000	31
Cyclin E1	Cell Signaling	4129	Mouse	1:1000	48
CDK1	Cell Signaling	9116	Mouse	1:1000	34
CDK2	Cell Signaling	2546	Rabbit	1:1000	33
CDK4	Cell Signaling	12790	Rabbit	1:1000	30
CDK7	Cell Signaling	2916	Mouse	1:2000	40
Phospho-ERK	Cell Signaling	9101	Rabbit	1:1000	42,44
ERK	Cell Signaling	9102	Rabbit	1:1000	42,44
Phospho-AKT	Cell Signaling	4060	Rabbit	1:2000	60
AKT	Cell Signaling	9272	Rabbit	1:1000	60
E-cadherin	Cell Signaling	14472	Mouse	1:1000	135
Vimentin	Cell Signaling	5741	Rabbit	1:1000	57

MW – molecular weight

2.2. METHODS

2.2.1. CELL CULTURE

2.2.1.1. PASSAGING CELLS

The cell lines used in this study were adherent. Cells were grown in 75cm^2 Bio-One CELLSTAR cell culture flasks (Greiner) and passaged once they had grown to 70-80% confluence. The culture medium was removed by aspiration and the cells were washed gently with 3mL Dulbecco's phosphate-buffered saline (PBS) (Gibco). 3-4mL Trypsin-EDTA (0.05%) (Gibco) was added to the flask and the cells were incubated at 37°C for 2-5 min, depending on the cell line, until the cells began to detach. The trypsin was then neutralised by the addition of 5mL complete medium (DU145, PC-3, LNCaP, CCD-18Co) or 2% FBS in PBS (PWR-1E, RWPE-1). The cell suspension was centrifuged at 1,200rpm for 4 min (Scanspeed 416 centrifuge, LaboGene). After discarding the supernatant, the cell pellet was resuspended in 1mL fresh culture medium and transferred to a new flask containing 10mL culture medium. Cells were maintained at 37°C and 5% CO_2.

2.2.1.2. CRYOPRESERVATION OF CELLS

Cells were frozen at the end of the log phase of growth when the flasks were 80-90% confluent. Cells from three 75cm^2 flasks were detached and pelleted, as described in **Section 2.2.1.1.** The cell pellets were resuspended in 10mL freezing medium according to the supplier's instructions: complete growth medium with 10% FBS and 10% DMSO for DU145, PC-3, and LNCaP; complete growth medium with 5% DMSO for CCD-18Co; complete growth medium with 7.5% DMSO for PWR-1E and RWPE-1. 1mL aliquots of cell suspension were transferred to 2mL Bio-One cryovials (Greiner) and placed in a Nalgene Mr. Frosty freezing container (Analab) at -80°C overnight. Cells were then transferred to a -150°C freezer for long-term storage.

2.2.1.3. THAWING CELLS

Vials were thawed quickly in a water bath at 37°C. The cell suspension was transferred to a 25cm^2 Bio-One CELLSTAR cell culture flask (Greiner) containing 10mL pre-warmed culture medium. The following day, the freezing medium containing DMSO was removed and replaced with fresh culture medium. When flasks were 70-80% confluent, the cells were detached and transferred to a 75cm^2 flask.

2.2.1.4. COUNTING CELLS

Cells were trypsinised and resuspended in fresh culture medium as described in **Section 2.2.1.1.** The cell suspension was mixed well and 10µL cell suspension was added to the counting chamber of a haemocytometer. Each 1mm square on the haemocytometer represents a volume of 1x10^{-4}mL. The cells were visualised using an inverted microscope (Olympus CK2). The number of cells in the four corner squares and the central square were counted and averaged to determine the number of cells per 1x10^{-4}mL. The number of cells counted was multiplied by 1x10^4 to determine the number of cells per mL.

2.2.1.5. COATING PLATES WITH POLY-D LYSINE

To promote adhesion of LNCaP cells, all plates used to seed LNCaP cells were coated with poly-D-lysine (PDL) hydrobromide (Sigma-Aldrich). 5mg PDL was resuspended in 17mL sterile dH_2O to create a 10X stock solution and stored in 1mL aliquots at -20°C. Plates were incubated overnight at 37°C with 50µL 1X PDL per well (96-well plate) or 700µL PDL per well (6-well plate). Before seeding cells, PDL was removed, and wells were washed with PBS.

2.2.2. 3-(4,5-DIMETHYLTHIAZOLE-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) ASSAY

Cell viability was assessed using the MTT assay, which measures the mitochondrial activity of cells, as indicated by the reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) to formazan (**Figure 2.1**). Cells were seeded in flat-bottomed 96-well CELLSTAR® Cell Culture Microplates (Greiner) at densities of $1x10^3$ (DU145, PC-3), $2x10^3$ (PWR-1E, CCD-18Co), $4.5x10^3$ (RWPE-1), or $6x10^3$ (LNCaP) cells per well, in 100µL culture medium. Cell densities were optimised for each cell line (see **Chapter 3, Figure 3.2**). After 24h adhesion, drug treatments were applied in triplicate in 100µL culture medium, to give a total volume of 200µL per well. Thiazolyl Blue Tetrazolium Bromide (MTT) reagent (Sigma-Aldrich) was prepared in PBS (5mg/mL). At the experiment endpoint, 20µL MTT solution was added to each well and the plates were incubated at 37°C for 3h. The culture medium and MTT reagent were then discarded. 200µL DMSO (Sigma-Aldrich) was added to each well to dissolve the formazan. Plates were placed on a plate shaker (Gyro-Rocker, Stuart Scientific) for 5 min at 30 rev/min. Absorbance was measured at a wavelength of 570nm using a CLARIOstar microplate reader (BMG Labtech). Background absorbance levels were measured from wells containing culture medium only and subtracted from the sample absorbance levels. The percentage viability was calculated relative to the vehicle control.

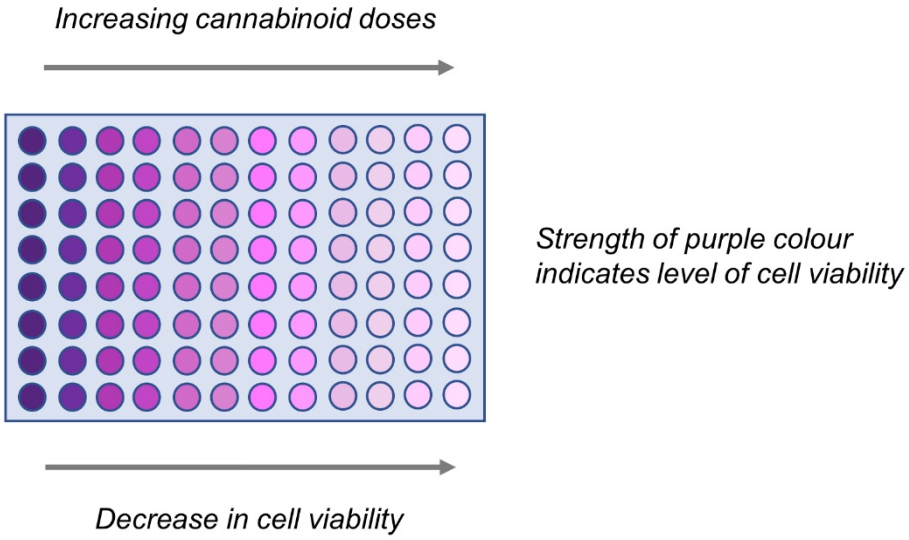


Figure 2.1. Example of an MTT plate. Viable cells reduce MTT reagent to formazan, indicated by a strong purple colour.

2.2.3. MEASUREMENT OF APOPTOSIS USING THE ACCURI FLOW CYTOMETER

Apoptosis was measured by flow cytometry using the Vybrant™ Apoptosis Assay Kit #4 (Invitrogen). The kit uses YO-PRO and propidium iodide (PI) as markers of early and late apoptosis, respectively. YO-PRO selectively permeates the membranes and stains the DNA of cells in early apoptosis. PI stains the DNA of dead cells whose membranes have been ruptured (**Figure 2.2**).

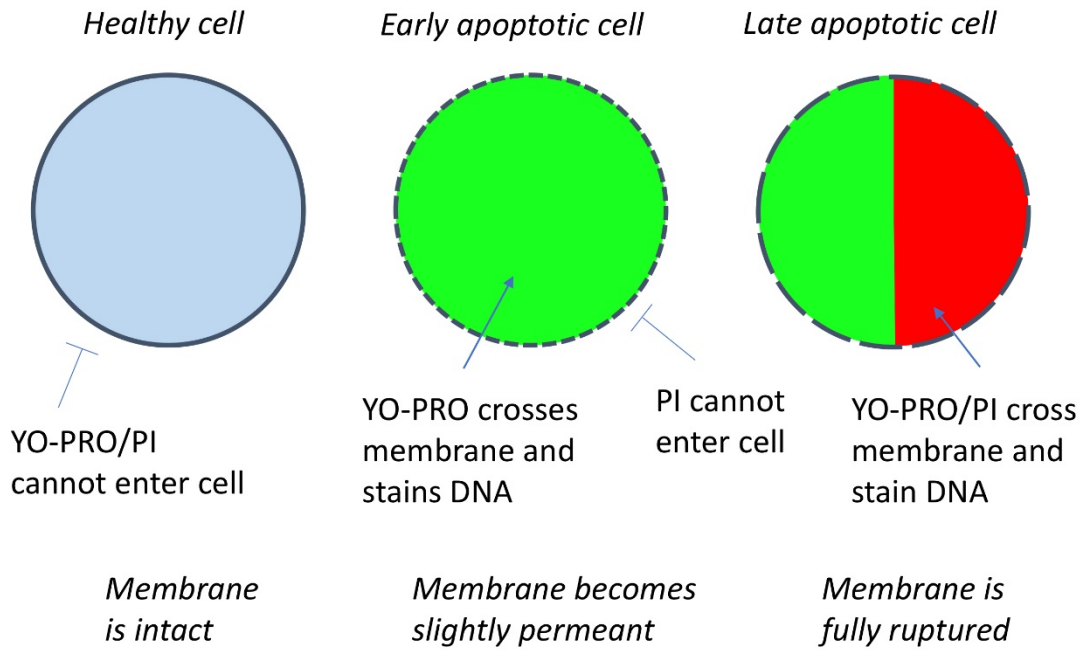


Figure 2.2. YO-PRO and PI staining of cells. YO-PRO selectively stains DNA of cells in early apoptosis, where the cell membrane has been compromised. PI stains DNA of late apoptotic cells, where the membrane has been fully ruptured.

To determine the optimal cell seeding density for the assay, cells were seeded in 6-well plates at various densities ($2x10^4$-$5x10^4$ cells per well) and visually inspected after 72h. Wells seeded with cell densities of $4x10^4$ cells per well (DU145) or $3x10^4$ cells per well (PC-3) were 70-80% confluent after 72h and these were selected as the optimal seeding densities. After drug treatment, the cell supernatant was collected from each well and the remaining adherent cells were detached from the plate by the addition of 1mL trypsin for 1-2 min at room temperature. The supernatant and the detached cell suspension were combined and centrifuged at 1,200rpm for 4 min. After the supernatant was

discarded, the remaining cell pellet was resuspended in ice-cold PBS containing YO-PRO (1:1000) and PI (1:1000). The samples were analysed using an Accuri C6 flow cytometer (BD Biosciences). Samples were run at slow speed to ensure that data were obtained from single cells. Samples were run until 15,000 events had been recorded or for a maximum of 5 min. Forward scatter and side scatter gating were used to remove aggregates and debris from the analysis, before using YO-PRO and PI staining to gate for alive cells, early apoptotic cells, late apoptotic cells, and DNA fragmentation.

2.2.4. MEASUREMENT OF APOPTOSIS AND PROLIFERATION USING THE CYTOFLEX FLOW CYTOMETER

Cell proliferation and apoptosis were measured using flow cytometry. Cell proliferation was measured using the CellTrace™ Violet Cell Proliferation kit (Invitrogen), according to the manufacturer's instructions. Live cells are stained with an extremely bright dye which is diluted through cycles of proliferation. The fluorescence intensity of the CellTrace dye indicates the rate of cell proliferation (**Figure 2.3**).

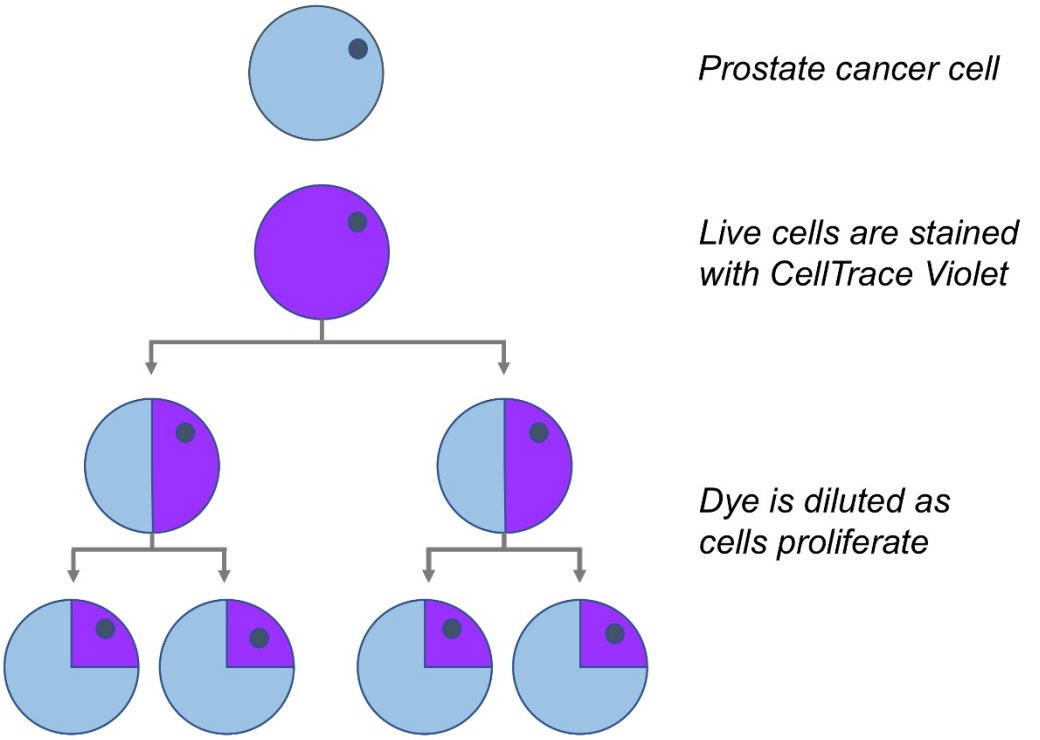


Figure 2.3. CellTrace Violet staining of cells. Live cells are stained with an extremely bright dye. As cells proliferate, the dye is diluted and the fluorescence intensity decreases.

PC-3 cells were seeded in 6-well plates at an optimised cell density of $3x10^4$ cells per well (**see Section 2.2.3**). After 24h adhesion, cells were serum-starved for 18h to synchronise the cell cycle. To prepare the CellTrace Violet solution, 20µL DMSO was added to a vial of CellTrace violet, which was then diluted further in 20mL pre-warmed PBS. After serum starvation, the culture medium was removed and replaced with 2mL CellTrace Violet solution. The cells were incubated with CellTrace Violet at 37°C for 20 min. The CellTrace Violet solution was removed, and the cells were washed twice with 500µL culture medium, before applying the drug treatments. At the experiment endpoint, the cell supernatant from each sample was collected and the remaining adherent cells were detached from the plate by the addition of 1mL trypsin for 1-2 min at room temperature. The supernatant and the detached cell suspension were combined and centrifuged at 1,200rpm for 4 min. The supernatant was discarded, and the remaining cell pellet was resuspended in complete culture medium containing YO-PRO (1:1000) and PI (1:1000). The fluorescence intensities of CellTrace Violet, YO-PRO, and PI were measured at 0h and 48h timepoints. The samples were analysed using a CytoFLEX flow cytometer (Beckman Coulter). Samples were run at slow speed to ensure that data were

obtained from single cells. Samples were run until 5,000 events (0h samples) or 20,000 events (24h samples) had been recorded or for a maximum of 10 min. Forward scatter and side scatter gating were used to remove aggregates and debris from the analysis, before using YO-PRO and PI to gate for alive cells, early apoptotic cells, and late apoptotic cells. Proliferation analysis was conducted on the live cell population.

2.2.5. INCUCYTE LIVE CELL IMAGING

Cell proliferation was assessed using the Incucyte Live Cell Imaging System (Essen Bioscience). The Incucyte imaging system is placed within the cell culture incubator, allowing for real-time, non-invasive measurement of cell proliferation over time, and ensuring that optimal and stable conditions are maintained throughout the experiment. PC-3 cells were seeded and treated as described above for the MTT assay. Following treatment, cells were photographed (2 images per well) at 100X magnification every 2h for 72h. Cell confluence was analysed using the Incucyte analysis software.

2.2.6. FLUORESCENCE MICROSCOPY

Cell proliferation and apoptosis were assessed using fluorescence microscopy. YO-PRO was used as a marker of early apoptosis. PI was used as a marker of late apoptosis. Nuclei were stained using Hoechst 33342 (Sigma). DU145, PC-3, and PWR-1E cells were seeded in black-walled PhenoPlate™ 96-well microplates (PerkinElmer) at densities of $2x10^3$ cells per well. The cell densities used were higher than those used for the 72h MTT assay to ensure the presence of a sufficient number of cells for statistical analysis after 24h treatment. After 24h or 72h drug treatment, the treatment medium was removed and replaced with complete medium containing YO-PRO (1:1000), PI (1:1000), and Hoechst (1:5000). The cells were incubated at 37°C for 30 min before imaging. Images were captured using the Opera Phenix high-content screening system (PerkinElmer) using a 20X air objective. The cells were maintained at 37°C and 5% CO_2 throughout. For each well, 25 fields of view were captured. The images were analysed using the Harmony high-content imaging and analysis software. An imaging analysis pipeline was developed. Nuclei and cytoplasm were segmented based on Hoechst staining using the 'FindNuclei' and 'FindCytoplasm' building blocks built into the software. Border objects were excluded from the analysis. The YO-PRO and PI positivity

thresholds were set based on the mean and sum intensity values per cell in the positive and negative control groups.

2.2.7. CLONOGENIC ASSAY

Cells were treated with IC_{50} doses of cannabinoids for 48h, then detached and re-seeded in 6-well plates at low cell densities (250 DU145 cells per well, 500 PC-3 cells per well) without treatment. Cells were maintained at 37°C and 5% CO_2 for 7 days, with the culture medium replaced every 2-3 days. After 7 days, the culture medium was removed, and the cells were washed twice with 1mL PBS. The colonies were fixed and stained by the addition of 1mL glutaraldehyde/crystal violet solution (3.6mL Glutaraldehyde, 25% aqueous solution (Thermo Fisher Scientific) + 3.26mL 2.3% crystal violet solution (Sigma-Aldrich) + 8.14mL H_2O) for 30 min. After fixing/staining, the cells were rinsed thoroughly with tap water and the plates were left to dry overnight. Colonies containing >50 cells were identified using a microscope and colonies were counted using ImageJ image analysis software.

2.2.8. SCRATCH WOUND-HEALING ASSAY

Cell migration was measured using the scratch wound-healing assay, which is used to assess the ability of cells to migrate and close a wound created in the cell monolayer. Cells were seeded in 12-well CELLSTAR® cell culture multiwell plates (Greiner) in complete medium at densities of $3x10^5$ (DU145, LNCaP) or $2.5x10^5$ (PC-3) cells per well. A scratch was created in each cell monolayer using a sterile P200 pipette tip, and drug treatments were applied. The scratches were photographed at 40X magnification at 0h and 72h timepoints using an inverted phase-contrast microscope (Olympus CKX41). The scratch distance was measured at three separate positions for each technical replicate using ImageJ image analysis software. Wound closure was calculated as the percentage change in scratch distance from 0h to 72h.

2.2.9. TRANSWELL MATRIGEL ASSAY

Cell invasion was measured using the transwell Matrigel assay, which measures the ability of cells to invade through a layer of Extracellular Matrix (ECM) gel (Sigma-Aldrich). 100µL of 1mg/mL ECM was added to an 8.0µm pore Falcon™ Cell Culture Insert (Analab) in a 24-well plate. The plate was incubated at 37°C for 1h to allow the gel to polymerise. Cells were seeded in the upper compartment of the Matrigel-coated insert at a density of $5x10^4$ cells per well in 500µL serum-free medium. To provide a chemotactic gradient, 500µL complete medium containing 10% FBS was added to the well underneath. Cells were incubated at 37°C for 48h. After 48h, the treatment medium was discarded, and any non-invasive cells were removed from the upper compartment using a cotton swab soaked in PBS. The invasive cells were then stained with 0.23% crystal violet for 10 min. The inserts were washed three times using dH_2O and left to dry overnight. The invasive cells were visualised and photographed at 100X magnification (Nikon Eclipse E600).

2.2.10. WESTERN BLOT:

2.2.10.1. PRINCIPLE OF WESTERN BLOT

Proteins from the cell lysate are separated on an acrylamide gel according to their molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins are transferred to a protein-binding PVDF membrane. The target protein is labelled with a primary antibody and a horse radish peroxidase (HRP)-tagged secondary antibody. The application of a chemiluminescent HRP substrate allows the detection of target protein expression using a CCD camera. Target protein expression levels can then be semi-quantified using densitometry.

2.2.10.2. WESTERN BLOT BUFFERS & REAGENTS

The preparation of the buffers, reagents, and gels used for Western blot is detailed in **Table 2.3.**

Table 2.3. Preparation of buffers, reagents, and gels for Western immunoblotting

10X Running Buffer	30g Trizma® Base (Sigma-Aldrich) 144g Glycine (Thermo Fisher Scientific) 10g Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich) Up to 1L with dH_2O
Wet Transfer Buffer	6g Trizma Base 28.5g Glycine 300mL Methanol (Sigma-Aldrich) Up to 2L with dH_2O
Wash Buffer	100mL 20X Tris Buffered Saline with Tween 20 (ChemCruz) Up to 2L with dH_2O
Lysis Buffer	100µL RIPA buffer (Sigma-Aldrich) 1µL Protease Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific)
Sample Buffer	100µL 2-Mercaptoethanol (Sigma-Aldrich) 900µL 4X Laemmli Sample Buffer (Bio-Rad)
5% Milk	5g Skim Milk Powder (Sigma-Aldrich) 100mL 1X Wash Buffer
1.5M Tris-HCl	181.65g Trizma Base 700mL dH_2O Adjust pH to 6.8 with concentrated HCl Up to 1L with dH_2O
10% SDS	10g SDS Up to 100mL with dH_2O
10% APS	1g Ammonium Persulfate (Sigma-Aldrich) 10mL dH_2O
12% Resolving Gel	6.6mL dH_2O 8.0mL 30% Bisacrylamide (Bio-Rad) 5.0mL 1.5M Tris-HCl 0.2mL 10% SDS 0.2mL 10% APS 0.008mL TEMED (Sigma-Aldrich)
5% Stacking Gel	4.1mL dH_2O 1.0mL 30% Bisacrylamide 0.75mL 1.5M Tris-HCl 0.06mL 10% SDS 0.06mL 10% APS 0.006mL TEMED

2.2.10.3. SAMPLE PREPARATION

Cells were seeded in duplicate at a density of $5x10^4$ cells per well in a 6-well plate, which yielded close to full confluence after 48h. After drug treatment, the medium was aspirated from the plate, and 100µL ice-cold lysis buffer was added to the first replicate well. The plates were kept on ice and the cells were scraped in lysis buffer using a cell scraper. The lysis buffer was then transferred to the second replicate well and the process was repeated. The lysis buffer from the 2 wells was combined in a 1.5mL Eppendorf, vortexed

for 15 seconds, then placed on ice for 10 min. Samples were then centrifuged at 4°C for 15 min at 12,000rpm (Centrifuge 5417R, Eppendorf). The supernatants were collected and stored at -20°C.

2.2.10.4. BICINCHONINIC ACID (BCA) ASSAY

A bovine serum albumen (BSA) solution (1µg/µL) was prepared by adding 10mg Bovine Serum Albumen (Sigma-Aldrich) to 10mL dH_2O. Various volumes of BSA solution (2µL, 4µL, 6µL, 8µL, 10µL, 12µL, 15µL) were added to a 96-well plate, in duplicate, to create a standard curve. 2µL of each protein sample was added to the plate, in triplicate. 25µL Protein Assay Reagent A (Bio-Rad) was added to each well. 200µL Protein Assay Reagent B (Bio-Rad) was added to each well. Plates were incubated at room temperature for 10 min before reading the absorbance at a wavelength of 595nm using a CLARIOstar microplate reader (BMG Labtech). A BSA standard curve was created using the BSA absorbance values and known BSA concentrations. The protein concentrations for each sample were calculated using the equation of the line obtained from the BSA standard curve.

2.2.10.5. WESTERN BLOT

For each experiment, 10mL 12% resolving gel was prepared (**Table 2.3**). The gel was loaded between two glass plates using a transfer pipette and allowed to set for at least 30 min. A layer of methanol (Sigma-Aldrich) was added to the top of the gel to prevent drying. For each experiment, 3mL 5% stacking gel was prepared (**Table 2.3**). The methanol was removed from the resolving gel, and the stacking gel was added using a transfer pipette. A 10-well comb (1.5mm) was inserted in the stacking gel and allowed to set for at least 30 min. Once gels were set, the plates were wrapped in damp paper towels and stored at 4°C overnight.

Samples were diluted using dH_2O to provide a final concentration of 40µg protein in a 15µL volume. 5µL sample buffer was added to each sample. Samples were heated at 99°C for 5 min using a heating block to ensure protein denaturation. The gels were assembled in a running rig filled with 1X running buffer. 20µL sample was loaded to the individual wells of the gel. Additionally, each gel was loaded with 5µL PageRuler™

Prestained Protein Ladder (Thermo Fisher Scientific). Gels were run at 80V for 15 min. The voltage was then increased to 100V, and the gels were run for a further 90 min (approximately).

When SDS-PAGE was completed, the stacking gel was removed. Immobilon®-P PVDF membranes (Millipore) were cut to approximately the size of the gel. Membranes were activated for 15s in methanol, followed by 2 min in dH_2O, followed by at least 10 min in ice-cold transfer buffer. The transfer cassette was prepared in ice-cold transfer buffer in the following order: sponge, filter paper, activated membrane, gel, filter paper, sponge. Cassettes were inserted in the transfer rig with the gel facing towards the cathode and the membrane facing towards the anode. An ice pack was added to each rig to avoid overheating. The transfer rig was filled with ice-cold transfer buffer and placed in a bucket surrounded by ice. The wet transfer was conducted in a cold room at 4°C, at 100V for 100-120 min, depending on the size of the protein to be assessed. Once the transfer was completed, the membranes were blocked in 10mL 5% milk for 1h on a roller at room temperature. The primary antibody was diluted in 4mL milk. After blocking, the milk was removed, and the membranes were incubated with the primary antibody overnight at 4°C. After primary antibody incubation, the membranes were subjected to 5 x 5 min washes in 5mL wash buffer. The complementary HRP-conjugated secondary antibodies were diluted (1:2000) in 5mL 5% milk. The membranes were incubated with the secondary antibody solution for 1h on a roller at room temperature. The membranes were washed, as above. Immobilon Forte Western HRP Substrate (Millipore) was added to each membrane to detect the HRP-conjugated antibody. The chemiluminescence was detected using an Amersham Imager 600 (GE Healthcare). After imaging, the membranes were washed briefly with wash buffer, then re-blotted for the corresponding loading control. Densitometry analysis was conducted using ImageJ image analysis software. The density values for each protein were normalised to the corresponding loading control, and then to the vehicle-treated samples.

2.2.11. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

After drug treatment, cell culture supernatants were collected and stored at -80°C. The secretion of matrix metalloproteases (MMPs) into the supernatant was measured using the Human MMP 3-Plex Ultra-Sensitive kit (Meso Scale Diagnostics). Each well of the Human MMP 3-Plex Ultra-Sensitive Plate is pre-coated with spatially distinct capture

antibodies for MMP-1, MMP-3, and MMP-9. After the addition of 25µL Diluent 2 containing 1% BSA to each well, the plate was sealed and incubated for 30 min with vigorous shaking (800rpm) at room temperature. Following incubation, 25µL of sample or calibrator standard was added to each well, in triplicate. The plate was sealed and incubated overnight at 4°C. The following day, the plate was washed three times with PBS-T, and 25µL detection antibody solution was added to each well. The detection antibody is tagged with the electrochemiluminescent compound MSD SULFO-TAG™. The plate was sealed and incubated at room temperature for 2h with vigorous shaking, followed by three further PBS-T washes. Finally, 150µL Read Buffer was added to each well. The electrochemiluminescence was measured using a SECTOR® Imager (Meso Scale Diagnostics). Data were analysed using the MSD DISCOVERY WORKBENCH® analysis software.

2.2.12. QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (QRT-PCR)

Cells were detached from 70-80% confluent T75 flasks using trypsin. Trypsin was neutralised using complete culture medium or 2% FBS in PBS. Cell pellets were obtained by centrifuging the cell suspension for 4 min at 1,200 rpm. RNA was extracted from the cell pellets using RNA STAT-60 (Amsbio CS-110), and RNA yields were quantified using the NanoDrop 330 (Thermo Fisher Scientific). RNA samples were stored at -80°C until use. For each sample, 100ng of extracted RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen). 10ng cDNA was amplified by RT-qPCR using TaqMan™ Gene Expression Master Mix (Applied Biosystems) and pre-designed gene expression assays for *CNR1* (Hs.PT.58.1734882), *CNR2* (Hs.PT.58.39632996), *TRPV1* (Hs.PT.58.39592265), *TRPV2* (Hs.PT.58.45542094), and *GPR55* (Hs.PT.58.1067283) (IDT). Samples were run in triplicate. Gene expression was normalised to the expression of the housekeeping genes *ACTB* (Hs.PT.39a.22214847) and *UBC* (Hs.PT.39a.22214853) (IDT). RT-qPCR was conducted using the QuantStudio™ 7 Flex Real-Time PCR system (Applied Biosystems). Ct values for each cell line were normalised to the Ct values of the corresponding housekeeping genes, and then to the cell line showing the lowest mRNA expression for each gene.

2.2.13. GSH-GLO ASSAY

Alterations in glutathione (GSH) concentrations are a key indicator of oxidative stress. GSH concentrations were measured using the GSH-Glo^{TM} glutathione assay (Promega), a highly sensitive, high-throughput technique. The luminescence-based assay measures the conversion of a luciferin derivative to luciferin by Glutathione S-Transferase in the presence of GSH. Cells were seeded at a density of $6x10^3$ cells per well in a white-walled 96-well plate (Greiner). After drug treatment, the treatment medium was aspirated from the plate. Glutathione was added to a second plate at a range of known concentrations to create a standard curve. 100µL GSH-Glo reagent was added to each well. Plates were mixed briefly on a plate shaker, then incubated at room temperature for 30 min. 100µL reconstituted luciferin detection reagent was added to each well. Plates were mixed briefly and incubated at room temperature for 15 min. Luminescence was measured using a CLARIOstar microplate reader. Sample GSH concentrations were calculated with reference to the glutathione standard curve.

2.2.14. STATISTICAL ANALYSIS

Data were analysed using GraphPad Prism 8 data analysis software. The IC_{50} values were determined using a non-linear regression for dose-response (inhibition). 1-Way ANOVA was used to compare the effects of a range of drug concentrations compared to the control. 2-Way ANOVA was used to compare cells grown with or without serum across different drug concentrations. Unpaired two-tailed Student's *t*-tests were used to compare mean values between vehicle-treated and cannabinoid-treated samples. *P* values <0.05 were considered statistically significant. All results are representative of at least three independent experiments, unless otherwise stated.

CHAPTER 3

Phenotypic effects of cannabinoids in prostate cancer cell lines

3.1. RATIONALE

To investigate the chemotherapeutic potential of cannabinoids in prostate cancer, our first goal was to conduct a phenotypic screen of cannabinoid effects in prostate cancer cell lines. Phenotypic screening in cancer is a discovery process that involves assessing the ability of a drug to produce desirable changes in cancer-related processes[242]. Drug activity is assessed in a relevant biological system, such as live cancer cells. Phenotypic screening is a particularly effective strategy in diseases with complex biology, such as cancer[243]. Because cancer development and progression are rarely driven by a single target, focussing on functional effects may be the most efficient method of identifying clinically effective drugs[242]. Many approved cancer drugs were discovered through phenotypic screening approaches[244].

To assess the phenotypic effects of cannabinoids in prostate cancer cells, we measured their ability to modulate key hallmarks of cancer. The hallmarks of cancer are functional changes that occur during malignant transformation that allow cells to breach normal anticancer defence mechanisms[245-247]. Many of the key hallmarks of cancer, for example, sustained cell proliferation, resistance to cell death, and activation of invasion and metastasis, can be assessed using *in vitro* phenotypic assays. Agents that can target one or, preferably, multiple cancer hallmarks have therapeutic potential in cancer. For example, existing cancer therapies, such as EGFR inhibitors, CDK inhibitors, and PARP inhibitors alter one or more of these hallmark capabilities[246].

The aim of this chapter was to investigate the phenotypic effects of the phytocannabinoids GL1a and GL4a in cell line models of prostate cancer (**Figure 3.1**). This was done by investigating the effects on:

- Cell proliferation
- Cell survival
- Cell death
- Cell migration
- Cell invasion

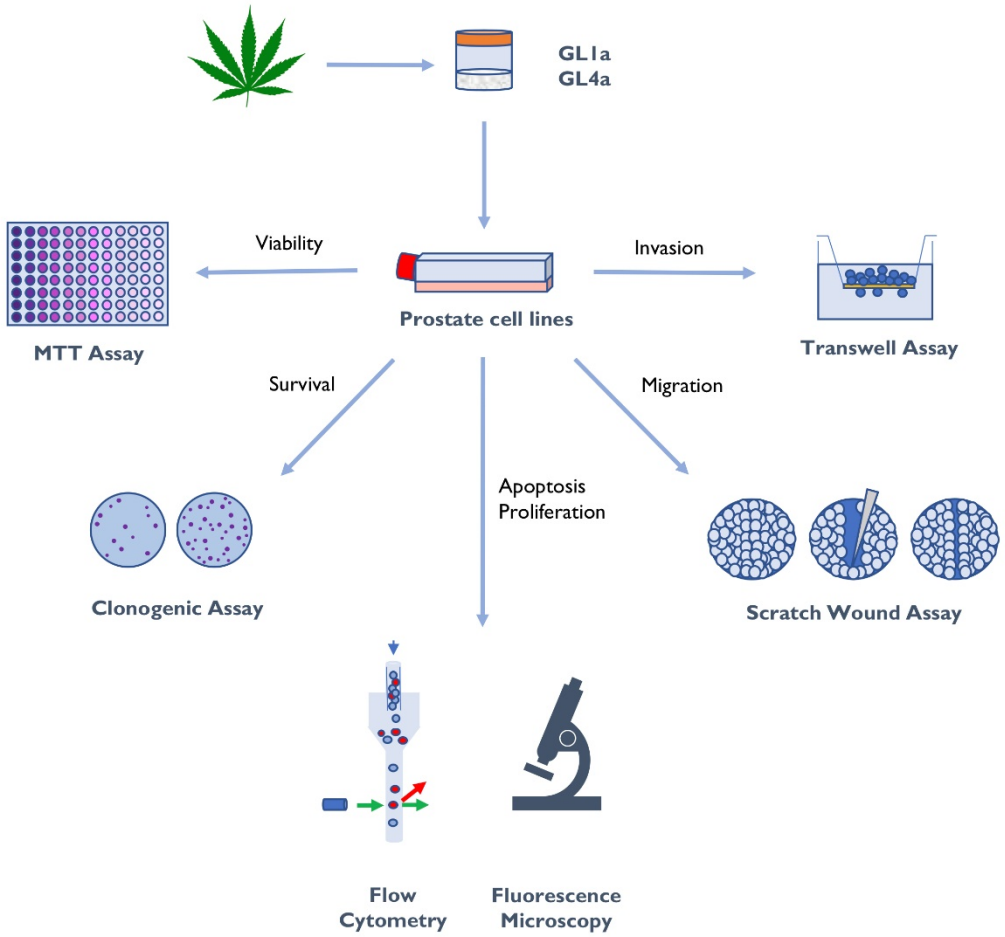


Figure 3.1. Experimental approaches used to investigate the phenotypic effects of phytocannabinoids.

3.2. RESULTS

3.2.1. OPTIMISATION OF CELL SEEDING DENSITY

Cell density titrations were performed to determine the optimal cell seeding density for MTT assay of each cell line. Cells were seeded in 96-well plates at cell densities from 0-1.6x10^4 cells per well. The MTT assay was performed 96 hours after seeding cells. Absorbance values were plotted against number of cells per well. The optimal number of cells should lie within the linear portion of this plot and yield an absorbance value of between 0.75 and 1.25 (https://www.atcc.org). This ensures that cells are within their exponential growth phase and that the absorbance values are within the limits of absorbance detection for the plate reader. For both DU145 and PC-3 cells, a cell seeding

density of approximately $1x10^3$ cells per well yielded an absorbance value of 1.0, and this was selected as the optimal cell seeding density (**Figure 3.2**). For LNCaP cells, a cell seeding density of $6x10^3$ cells per well yielded an absorbance value of 1.0. For RWPE-1 and CCD-18Co cells, optimal seeding densities were $4.5x10^3$ and $2x10^3$ cells per well, respectively. Maximal absorbance values were lower in PWR-1E cells, and a density of $2x10^3$ cells per well was selected, yielding an absorbance value of 0.8. The above optimised seeding densities were used for all subsequent MTT experiments.

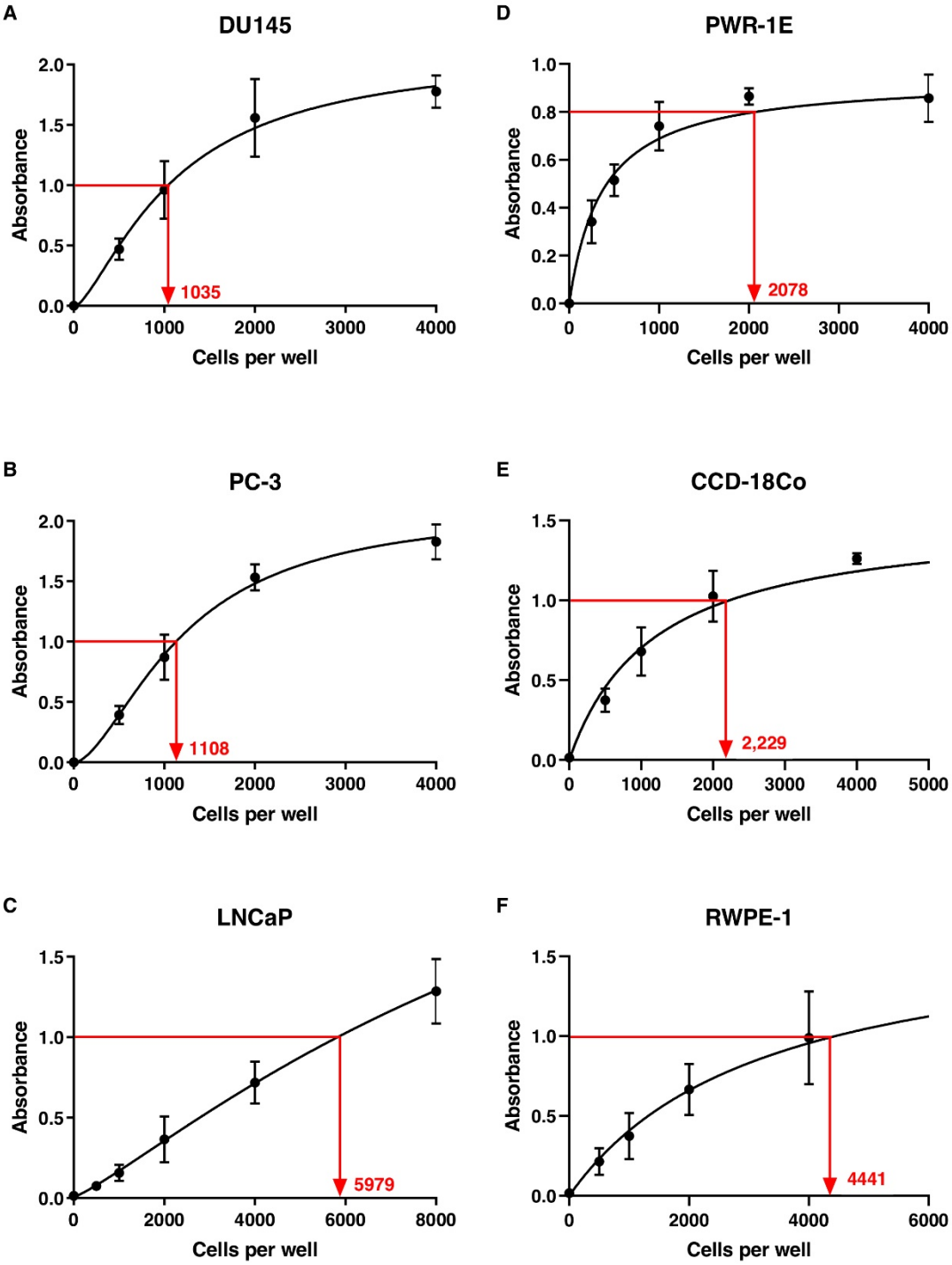


Figure 3.2. Optimisation of cell seeding density for MTT assay. (A-C) Prostate cancer (DU145, PC-3, LNCaP) and (D-F) non-cancerous (RWPE-1, PWR-1E, CCD-18Co) cells were seeded in triplicate in 96-well plates at various cell densities. After 96h, MTT reagent was added to the cells and absorbance values were read using a spectrophotometer. Cell densities that produced absorbance values between 0.75 and 1.25 were selected as the optimal seeding densities (shown in red). All cell lines were seeded at densities up to 1.6×10^4 cells per well. For ease of visualisation, only graph portions showing optimal seeding densities are shown. Results are representative of 3 biological replicates.

3.2.2. OPTIMISATION OF SOLVENT CONCENTRATION

Before beginning to investigate the effects of GL1a and GL4a on the viability of cancerous and non-cancerous prostate cell lines, the maximum tolerable solvent concentration first had to be established for each cell line. Cell viability analysis was carried out to ensure that the solvent concentrations used in subsequent experiments were not toxic to prostate cells. The MTT assay was used to measure the effect of DMSO on the viability of DU145, PC-3, and LNCaP prostate cancer cells, as well as the non-cancerous prostate epithelial cell lines PWR-1E and RWPE-1, and the non-cancerous colon cell line CCD-18Co. Cells were seeded in 96-well plates and treated with increasing concentrations of DMSO (0-5% in culture medium) for 72h. DMSO had no significant effect on viability at concentrations up to 0.2% in any of the cell lines tested (**Figure 3.3**). DMSO concentrations less than 0.2% were used for all subsequent experiments.

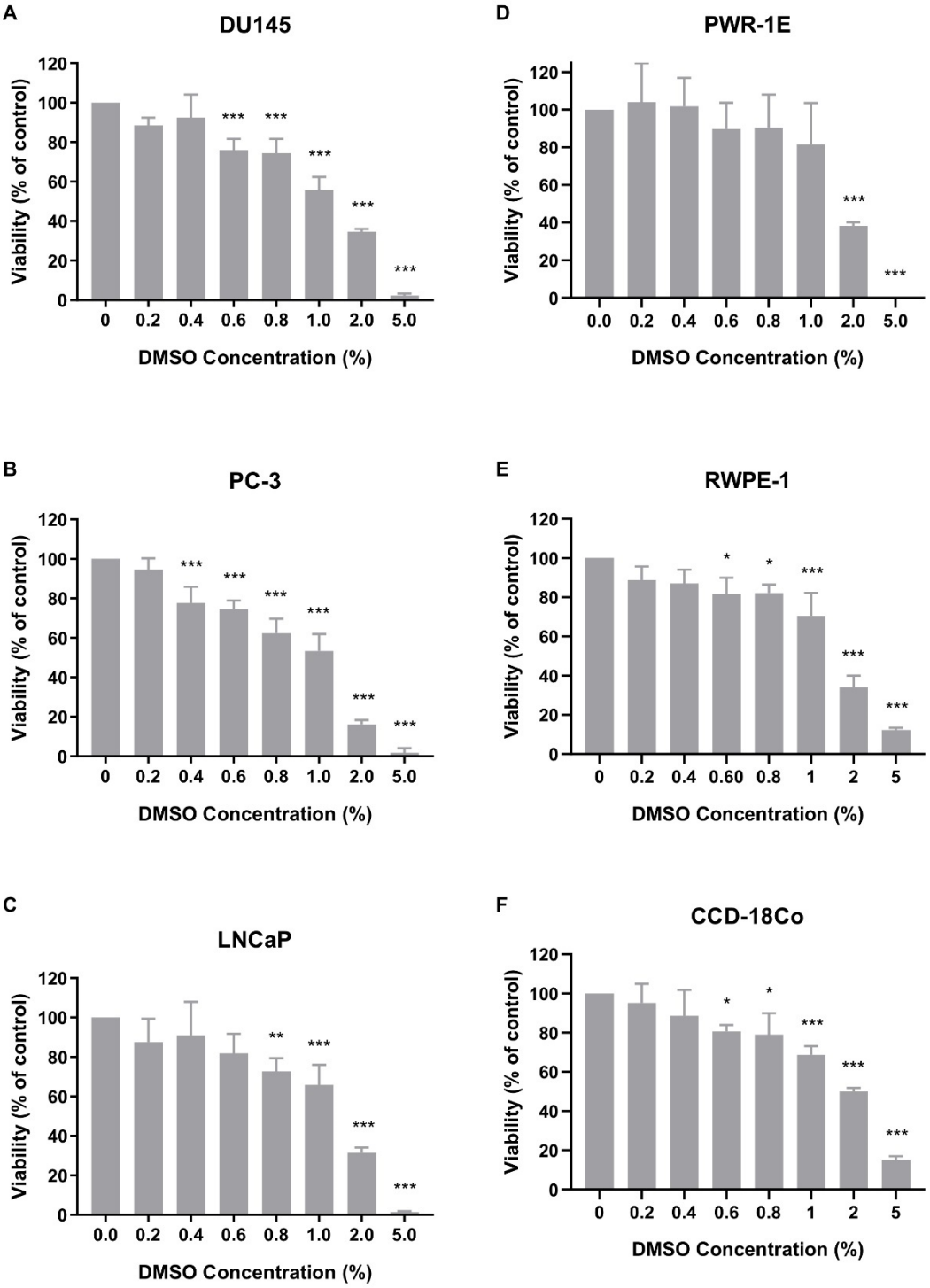


Figure 3.3. Optimisation of solvent concentration. (A-C) Prostate cancer (DU145, PC-3, LNCaP) and (D-F) non-cancerous (RWPE-1, PWR-1E, CCD-18Co) cells were treated with DMSO (0-5% in culture medium) for 72h. Cell viability was measured using the MTT assay. DMSO at a concentration of 0.2% had no significant effect on viability in any of the cell lines (1-way ANOVA, *p<0.05). Results are representative of 3 biological replicates and 3 technical replicates.

3.2.3. GL1A REDUCES PROSTATE CANCER CELL VIABILITY

The MTT assay was performed to evaluate the effect of the phytocannabinoid GL1a on the viability of DU145, PC-3, and LNCaP prostate cancer cells. After 24h adhesion, cells were treated with increasing concentrations of GL1a (0-100μM) for 72h, in the presence or absence of serum. GL1a significantly inhibited the viability of DU145, PC-3, and LNCaP cells (**Figure 3.4**). GL1a reduced cell viability by 50% at doses of 12.3μM in DU145 cells, 10.5μM in PC-3 cells, and 18.0μM in LNCaP cells, in the presence of serum. GL1a effects were strongest in the androgen-independent cell lines DU145 and PC-3. IC_{50} values for GL1a were substantially lower when the cells were treated in the absence of serum: 1.5μM in DU145 cells, 2.9μM in PC-3 cells, and 2.6μM in LNCaP cells. Overall, GL1a induced a dose-dependent decrease in the viability of prostate cancer cells after 72h treatment, an effect that was enhanced under serum deprivation conditions.

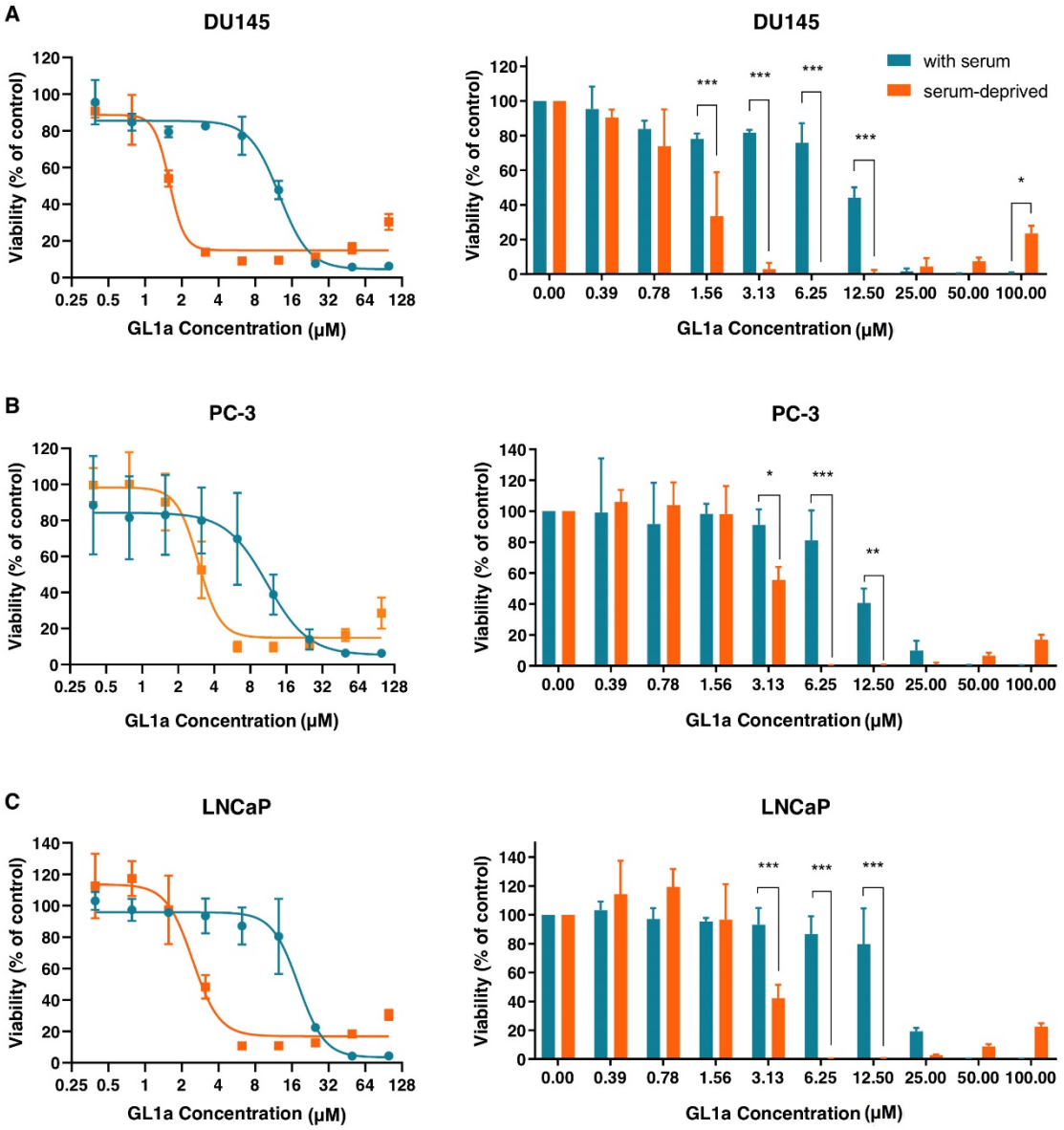


Figure 3.4. GL1a reduces prostate cancer cell viability with enhanced effects in serum-deprived cells. DU145, PC-3, and LNCaP cells were treated with vehicle (DMSO) or GL1a (0-100μM) for 72h in the presence or absence of serum. Cell viability was measured using the MTT assay. (A-C) GL1a reduced the viability of all three cell lines. GL1a effects were significantly enhanced in serum-deprived cells (2-way ANOVA, *p<0.05 **p<0.01 ***p<0.001) Results are representative of 3 technical replicates and at least 3 biological replicates.

3.2.4. GL1A REDUCES VIABILITY OF NON-CANCEROUS CELLS

To determine the cancer-specificity of GL1a, the MTT assay was used to measure the effect of GL1a on the viability of three non-cancerous cell lines PWR-1E, RWPE-1 and CCD-18Co. PWR-1E and RWPE-1 are human prostate epithelial cell lines. CCD-18Co

cells are human fibroblasts derived from normal colon tissue, whose viability is reportedly unaffected by 24h CBD treatment[188]. 72h treatment with GL1a significantly inhibited the viability of PWR-1E (IC_{50}: 0.9μM) and RWPE-1 (IC_{50}: 1.1μM) cells (**Figure 3.5**). Notably, PWR-1E and RWPE-1 cells are routinely grown in serum-free media, which is known to enhance the effects of cannabinoids[155,248]. However, even comparing cells grown in serum-deprived conditions, the IC_{50} values observed in the non-cancerous prostate cells were lower than those seen in the cancerous cell lines. CCD-18Co cells were more resistant to GL1a treatment but did show reduced viability after 72h (IC_{50}: 16.8μM). Additionally, effects in CCD-18Co cells were enhanced under serum-deprived conditions (IC_{50}: 2.7μM). GL1a IC_{50} values are summarised in **Table 3.1**. Together, these results suggest that the effects of GL1a on cell viability are not specific to cancer cells.

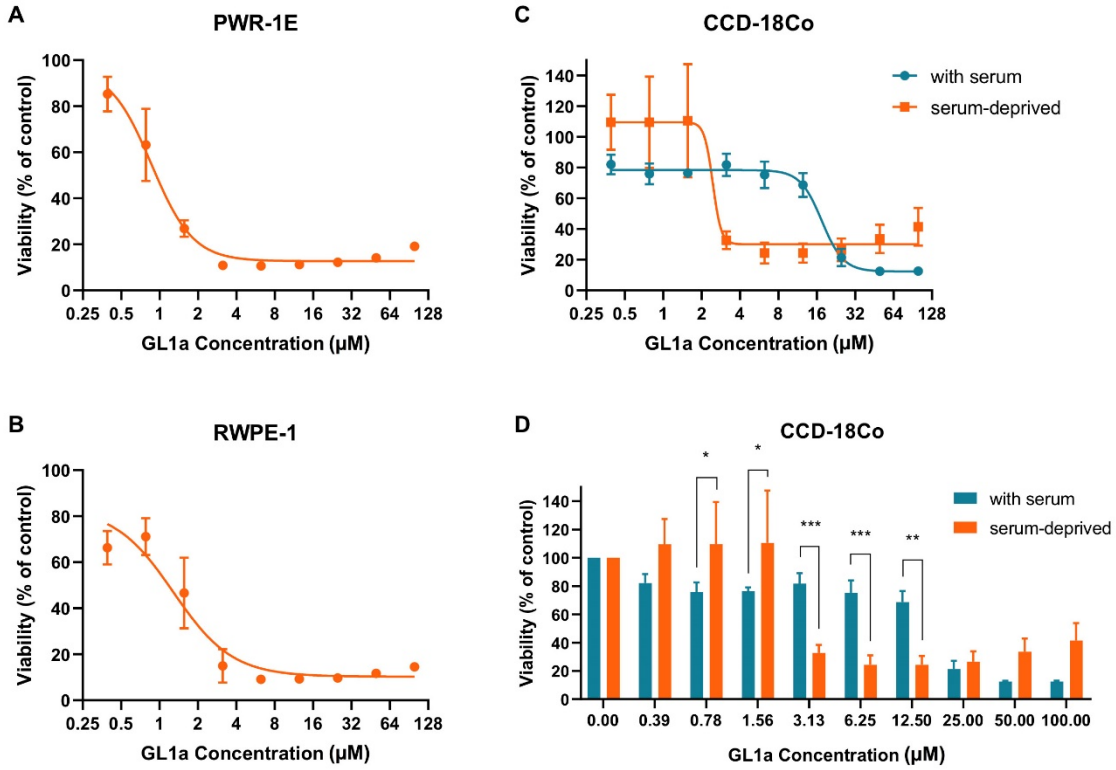


Figure 3.5. GL1a reduces the viability of non-cancerous cells. PWR-1E and RWPE-1 non-cancerous prostate cells were treated with vehicle (DMSO) or GL1a (0-100μM) for 72h in serum-free media. CCD-18Co non-cancerous colon cells were treated with GL1a (0-100μM) for 72h in the presence or absence of serum. Cell viability was measured using the MTT assay. (A-C) GL1a reduced the viability of all three cell lines. (D) In CCD-18Co cells, serum deprivation significantly enhanced the effects of GL1a at 3.13μM-12.5μM doses (2-way ANOVA, $^{**}p<0.01$ $^{***}p<0.001$). Results are representative of 3 biological replicates and 3 technical replicates.

Table 3.1. IC_{50} values for inhibition of cell viability by GL1a in prostate cancer and non-cancerous cell lines. Data represent the mean ± standard deviation obtained from at least three independent experiments.

GL1a IC_{50} Values (µM)		
	with serum	serum-deprived
DU145	12.3 ± 1.6	1.5 ± 0.1
PC-3	10.5 ± 3.9	2.9 ± 0.4
LNCaP	18.0 ± 2.4	2.6 ± 0.4
PWR-1e	NA	0.9 ± 0.2
RWPE-1	NA	1.1 ± 0.6
CCD-18Co	16.8 ± 3.2	2.7 ± 6.3

3.2.5. ASSESSMENT OF EFFECT OF GL1A ON CELL PROLIFERATION USING LIVE CELL IMAGING

To further investigate the phenotypic effects of GL1a on prostate cancer cells, the Incucyte Live Cell Imaging System was used to visualise and quantify the effect of 72h GL1a treatment on cell confluence. The Incucyte instrument captures images of cells at regular timepoints throughout an experiment and measures the phase object confluence (area of the field of view covered by cells) as an indicator of cell proliferation. PC-3 cells were selected for analysis as the most aggressive of the prostate cancer cell lines, and the cell line most sensitive to GL1a treatment based on IC_{50} values obtained from the MTT assay. Cells were treated with GL1a (0-100µM), in the presence or absence of serum. Cells were maintained in an incubator at 37°C and 5% CO_2 throughout. Images of the cells were captured from 2 separate regions per well at 2-hour intervals for 72h, using a 10x objective (**Figure 3.6**). It should be noted that the results shown represent a single biological replicate. However, these results provided preliminary evidence that

GL1a may reduce the proliferation of PC-3 cells in a time- and dose-dependent manner (**Figure 3.7**).

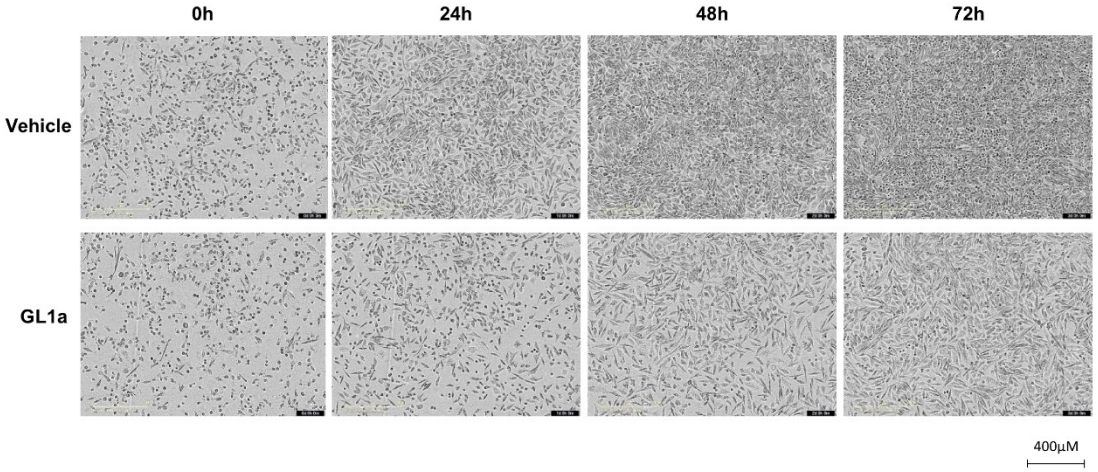


Figure 3.6. Representative images of the effect of GL1a on prostate cancer cell proliferation. Representative images showing confluence of PC-3 cells treated with vehicle alone or with 12.5µM GL1a at 0h, 24h, 48h, and 72h timepoints. Original magnification x100.

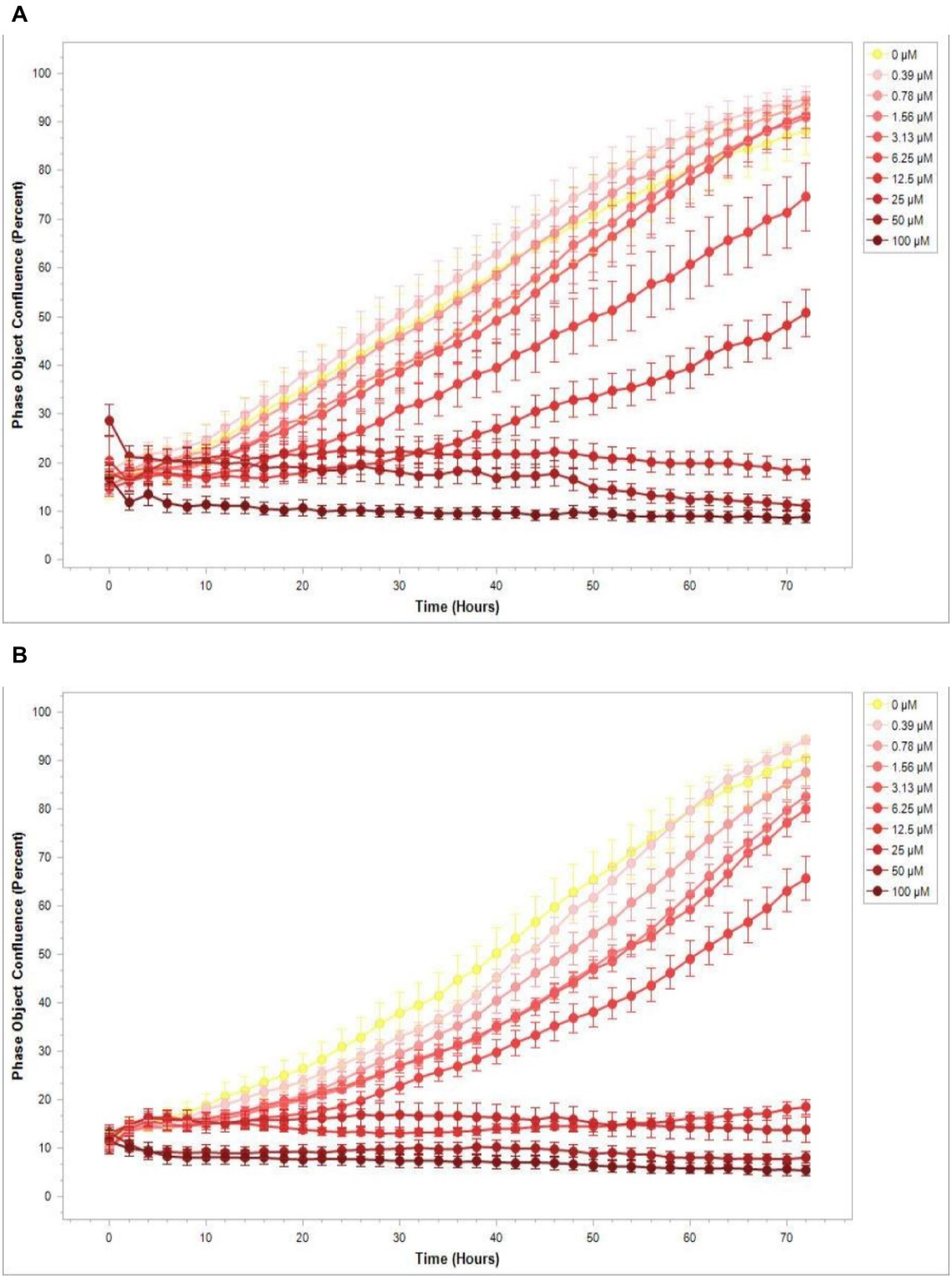


Figure 3.7. Effect of GL1a on prostate cancer cell proliferation. PC-3 cells were treated with vehicle (DMSO) or GL1a (0-100µM), in the presence or absence of serum. Cells were photographed every 2h for 72h using the Incucyte Live Cell Imaging System. (A) In the presence of serum, GL1a reduced proliferation of PC-3 cells in a time- and dose-dependent manner. (B) In the absence of serum, GL1a reduced proliferation at 0h, 24h, 48h, and 72h timepoints, with enhanced effects compared to in cells grown with serum. The yellow line represents the vehicle

control. Red lines represent GL1a treatments. Darker red colour indicates increasing doses of GL1a. Results are representative of 1 biological replicate and 3 technical replicates.

3.2.6. EFFECT OF GL1A ON APOPTOSIS IN PROSTATE CANCER CELLS

Flow cytometry was used to measure the effect of GL1a on cancer cell death. DU145 and PC-3 cells were selected for analysis as they represent androgen-independent cells typically resistant to existing treatments, and MTT results indicated that they were more sensitive to GL1a treatment than the androgen-dependent LNCaP cells. YO-PRO was used as a marker of early apoptosis. Propidium iodide (PI) was used as a marker of late apoptosis. Cells were treated for 48h with an IC_{50} dose of GL1a (10.5μM for PC-3, 12.3μM for DU145), determined by MTT assay. Cells were treated in the presence of serum, as the optimal culturing condition for these cell lines according to the supplier. GL1a caused no significant increase in early or late apoptosis and no significant reduction in the percentage of healthy cells in either cell line (**Figure 3.8**). GL1a did cause a small but significant increase in DNA fragmentation in PC-3 cells, indicating a possible cytotoxic effect (p=0.0264). Notably, GL1a significantly reduced the total number of events (i.e., the number of both live and dead cells) in both cell lines (p=0.0002 in DU145 cells, p=0.0012 in PC-3 cells), indicating a possible inhibitory effect on cell proliferation.

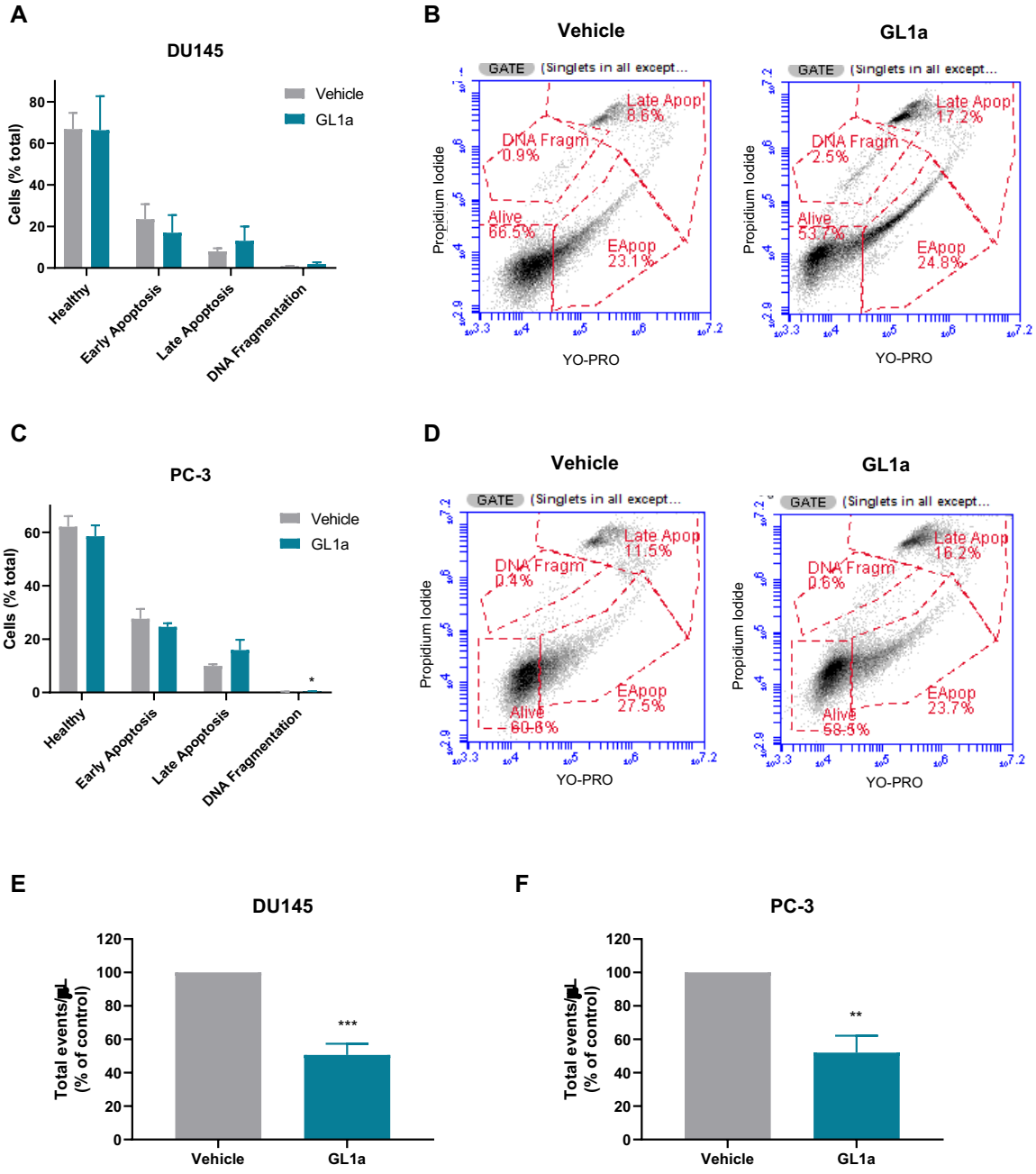


Figure 3.8. 48h GL1a treatment does not increase apoptosis in prostate cancer cells. DU145 and PC-3 cells were treated with an IC_{50} dose of GL1a (12.3µM for DU145, 10.5µM for PC-3) for 48h. YO-PRO was used as a marker of early apoptosis. Propidium iodide (PI) was used as a marker of late apoptosis. Samples were analysed using a BD Accuri C6 flow cytometer. GL1a caused no significant increase in early or late apoptosis in (A) DU145 or (C) PC-3 cells. (B) Representative plots of vehicle- and GL1a-treated DU145 cells. (D) Representative plots of vehicle- and GL1a-treated PC-3 cells. (E, F) GL1a significantly reduced the total number of events (live and dead cells) for both cell lines (Student's *t*-test, **p*<0.05 ***p*<0.01 ****p*<0.001). Results are representative of 3 biological replicates and 3 technical replicates.

3.2.7. EFFECT OF GL1A ON PROLIFERATION AND APOPTOSIS IN PC-3 CELLS

As PC-3 cells are the most aggressive of the prostate cancer cell lines tested, and were also most sensitive to GL1a treatment, these cells were selected for further investigation. Further flow cytometry analysis was conducted to assess the effects of GL1a on both cell proliferation and cell death. To measure proliferation, live cells were stained with CellTrace Violet, an extremely bright dye. As the cells divided, the dye was diluted and the reduction in fluorescence intensity was measured using a Beckman Coulter FC500 flow cytometer. Apoptosis was also measured using YO-PRO and PI staining to confirm the results obtained using the BD Accuri C6 flow cytometer. PC-3 cells were treated for 48h with an IC_{50} dose of GL1a (10.5μM). The CDK inhibitor palbociclib was included as a positive control for increased apoptosis and reduced proliferation. GL1a caused no significant increase in apoptosis (**Figure 3.9**) or reduction in cell proliferation (**Figure 3.10**) after 48h treatment. Palbociclib strongly induced apoptosis and inhibited cell proliferation. These results suggest that, at 48h, GL1a has no significant effect on apoptosis or cell proliferation in PC-3 cells.

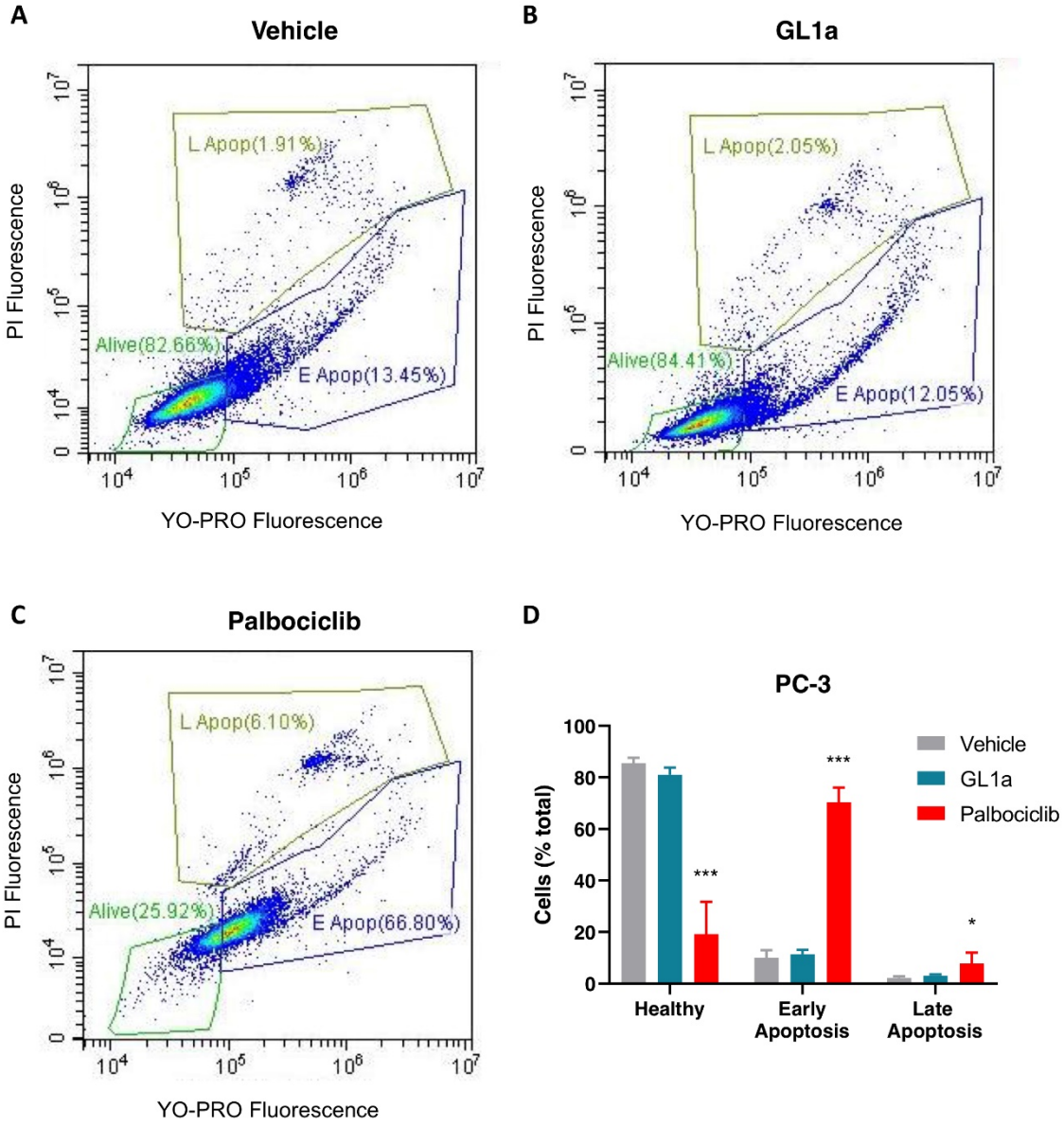


Figure 3.9. 48h GL1a treatment does not increase apoptosis in PC-3 cells. PC-3 cells were treated with an IC_{50} dose of GL1a for 48h. Palbociclib (10μM) was used as a positive control. Apoptosis was measured using flow cytometry. YO-PRO and propidium iodide (PI) were used as markers of early and late apoptosis, respectively. (A) Representative plot of YO-PRO and PI fluorescence in vehicle-treated PC-3 cells. (B) Representative plot of YO-PRO and PI fluorescence in GL1a-treated PC-3 cells. (C) Representative plot of YO-PRO and PI fluorescence in palbociclib-treated PC-3 cells. (D) GL1a had no significant effect on apoptosis. Results are representative of 3 biological replicates and 3 technical replicates.

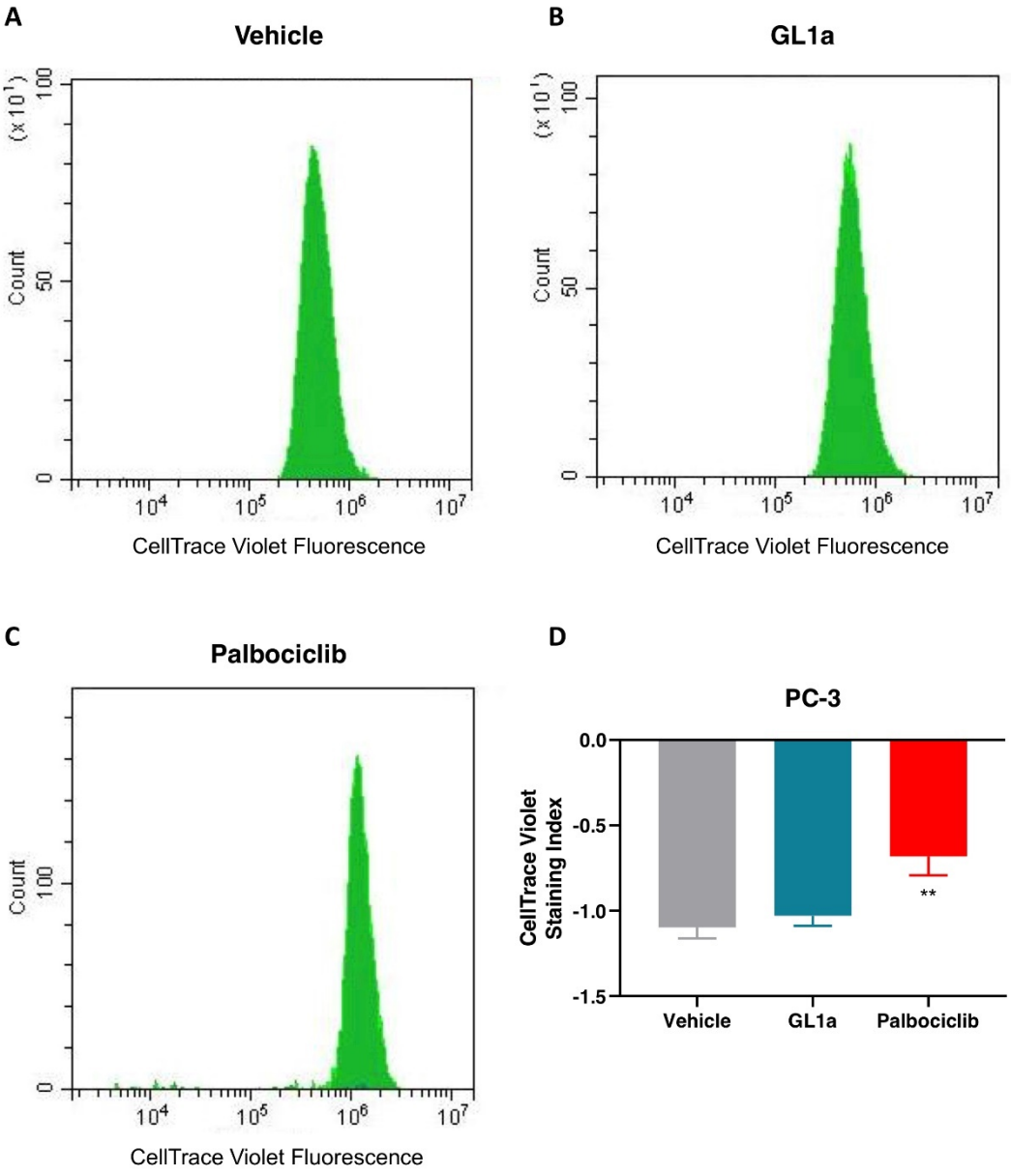


Figure 3.10. 48h GL1a treatment does not inhibit proliferation in PC-3 cells. PC-3 cells were treated with an IC_{50} dose of GL1a for 48h. Palbociclib (10μM) was used as a positive control. Cell proliferation was measured using flow cytometry. Live cells were stained with CellTrace Violet to assess cell proliferation. (A) Representative plot of CellTrace Violet fluorescence in vehicle-treated PC-3 cells. (B) Representative plot of CellTrace Violet fluorescence in GL1a-treated PC-3 cells. (C) Representative plot of CellTrace Violet fluorescence in palbociclib-treated PC-3 cells. (D) GL1a had no significant effect on cell proliferation. Results are representative of 3 biological replicates and 3 technical replicates.

3.2.8. IMAGING ANALYSIS PIPELINE

To gain a greater insight into the phenotypic effects of GL1a in prostate cells, fluorescence microscopy was used to assess the effect of various GL1a doses on cell proliferation and apoptosis in 2 prostate cancer cell lines (DU145, PC-3) and 1 non-cancerous prostate cell line (PWR-1E) at 24h and 72h timepoints. An image analysis pipeline was developed on the Harmony high-content imaging and analysis software to assess anti-proliferative and cytotoxic effects (**Figure 3.11**). Nuclei were selected and segmented based on Hoechst staining using the 'Find Nuclei' building block (Method B) in the Harmony software. The cytoplasm was selected based on Hoechst staining using the 'Find Cytoplasm' building block (Method A). The morphological properties of the nuclei (area, circularity) were also measured. Border objects were excluded from the analysis. Early apoptotic cells were selected based on YO-PRO staining. The positivity threshold was set based on the mean and sum YO-PRO values per cell. Late apoptotic cells were selected based on PI staining. Cells that were not positive for either YO-PRO or PI were classified as healthy cells. Representative images of vehicle- and GL1a-treated cells are shown in **Figure 3.12**.

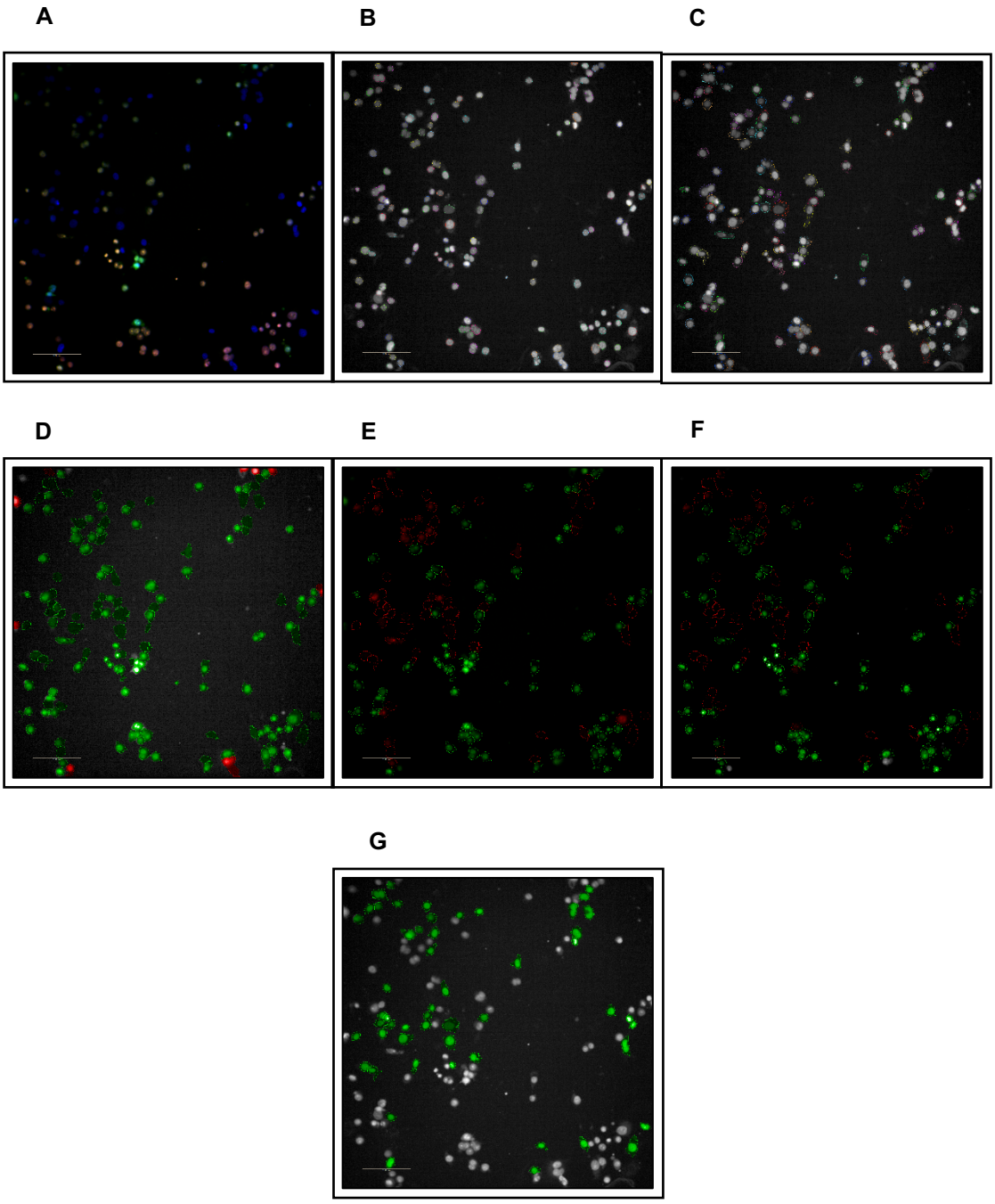


Figure 3.11. Image analysis pipeline for fluorescence microscopy experiments. (A) Single field-of-view of PWR-1E cells stained with Hoechst (blue), YO-PRO (green) and propidium iodide (PI) (red). (B) Selection of nuclei based on Hoechst staining. (C) Selection of cytoplasm based on Hoechst staining. (D) Exclusion of border objects (red). (E) Selection of YO-PRO-positive cells (green). (F) Selection of PI-positive cells (green). (G) Selection of healthy cells (green) based on lack of YO-PRO and PI staining. Original magnification x200. Scale bars = 100µm.

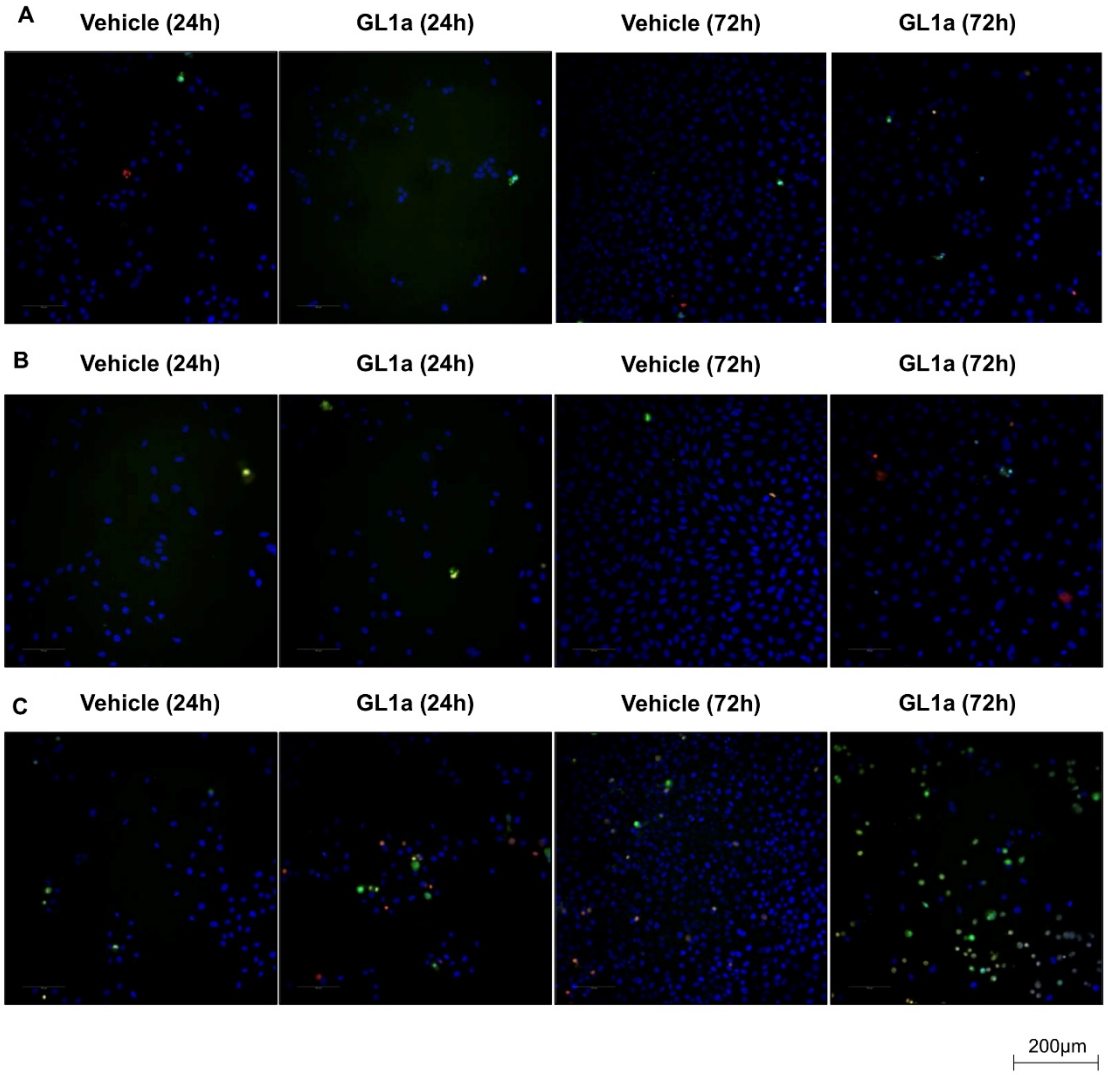


Figure 3.12. Representative images of GL1a-treated cells. (A) Representative images of DU145 cells treated with vehicle (DMSO) or GL1a (10µM) for 24h or 72h. (B) Representative images of PC-3 cells treated with vehicle (DMSO) or GL1a (10µM) for 24h or 72h. (C) Representative images of PWR-1E cells treated with vehicle (DMSO) or GL1a (1µM) for 24h or 72h. Nuclei are stained with Hoechst (blue). Early apoptotic cells are stained with YO-PRO (green). Late apoptotic cells are stained with PI (red). Original magnification x200.

3.2.9. FLUORESCENCE MICROSCOPY ANALYSIS OF GL1A EFFECTS

3.2.9.1. IN DU145 CELLS

To visualise the effects of GL1a treatment on prostate cancer cells, DU145 cells were treated with 1µM, 5µM, or 10µM GL1a. Cells were treated in the presence of serum, according to the supplier's recommendation. The kinase inhibitor staurosporine was

used as a positive control. Early apoptotic cells were stained using YO-PRO. Late apoptotic cells were stained using PI. Nuclei were stained using Hoechst. Live cells were imaged at 24h and 72h timepoints using the Opera Phenix high-content screening system. At 72h, GL1a significantly reduced cell confluence at doses of 5μM (p=0.02) or 10μM (p<0.001) (**Figure 3.13**). GL1a did not reduce nucleus area or circularity, both indicative of cell death, at either timepoint at any of the doses tested. GL1a did not significantly increase YO-PRO or PI positivity or reduce the fraction of healthy cells (**Figure 3.14**). Together, these results suggest that GL1a reduces DU145 cell viability primarily through reduced cell proliferation rather than through increased cell death.

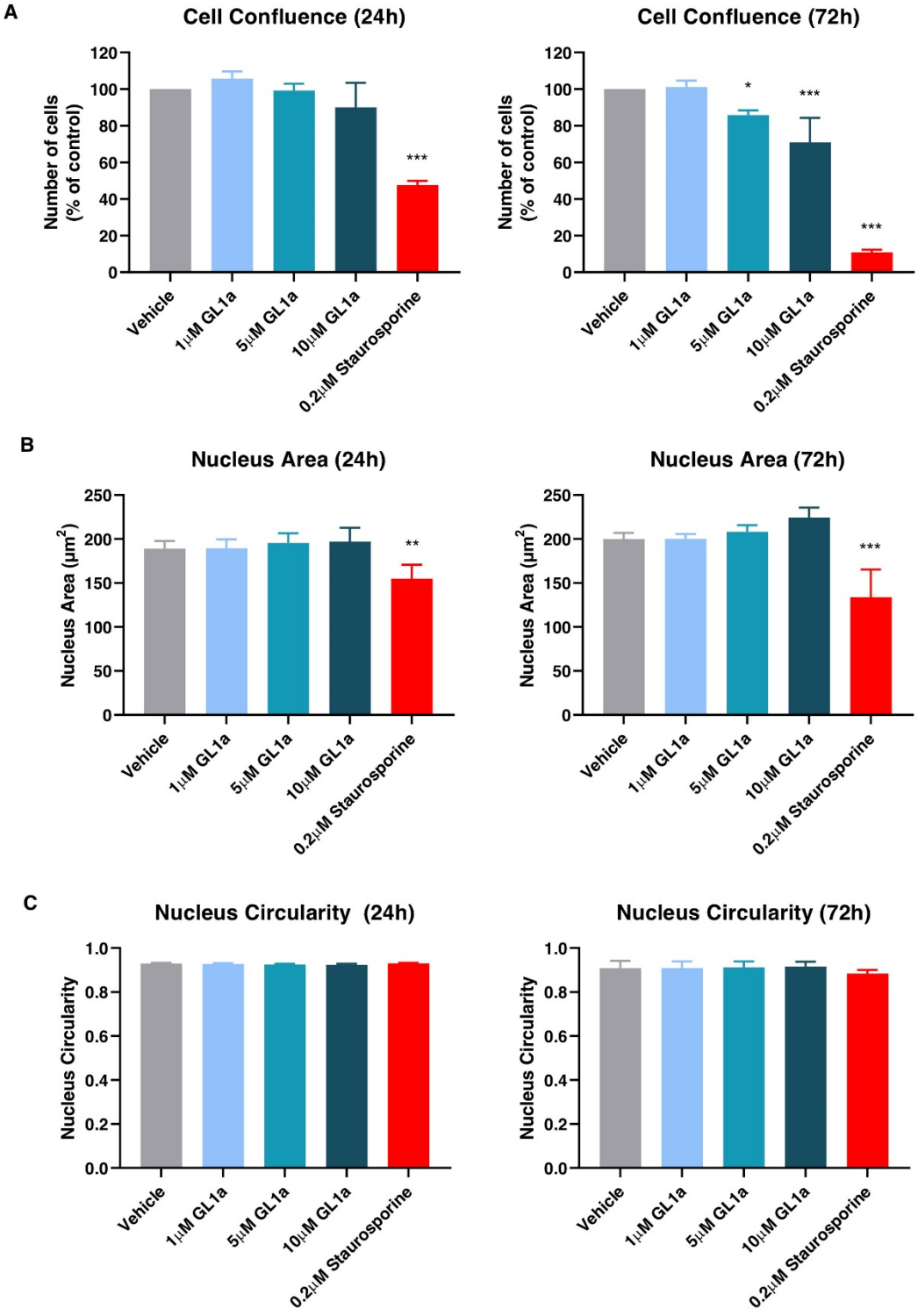


Figure 3.13. GL1a reduces proliferation of DU145 cells. DU145 cells were treated with vehicle (DMSO), GL1a (1μM, 5μM, or 10μM), or a positive control (staurosporine) for 24h or 72h. Nuclei were stained using Hoechst. Live cells were imaged using the Opera Phenix high-content screening system. (A) At 72h, 5μM or 10μM GL1a significantly reduced the confluence of the cells (Student's *t*-test, **p*<0.05 ****p*<0.001). (B) GL1a had no significant effect on nucleus area. (C) GL1a had no significant effect on nucleus circularity. Results are representative of 3 technical replicates and at least 3 biological replicates.

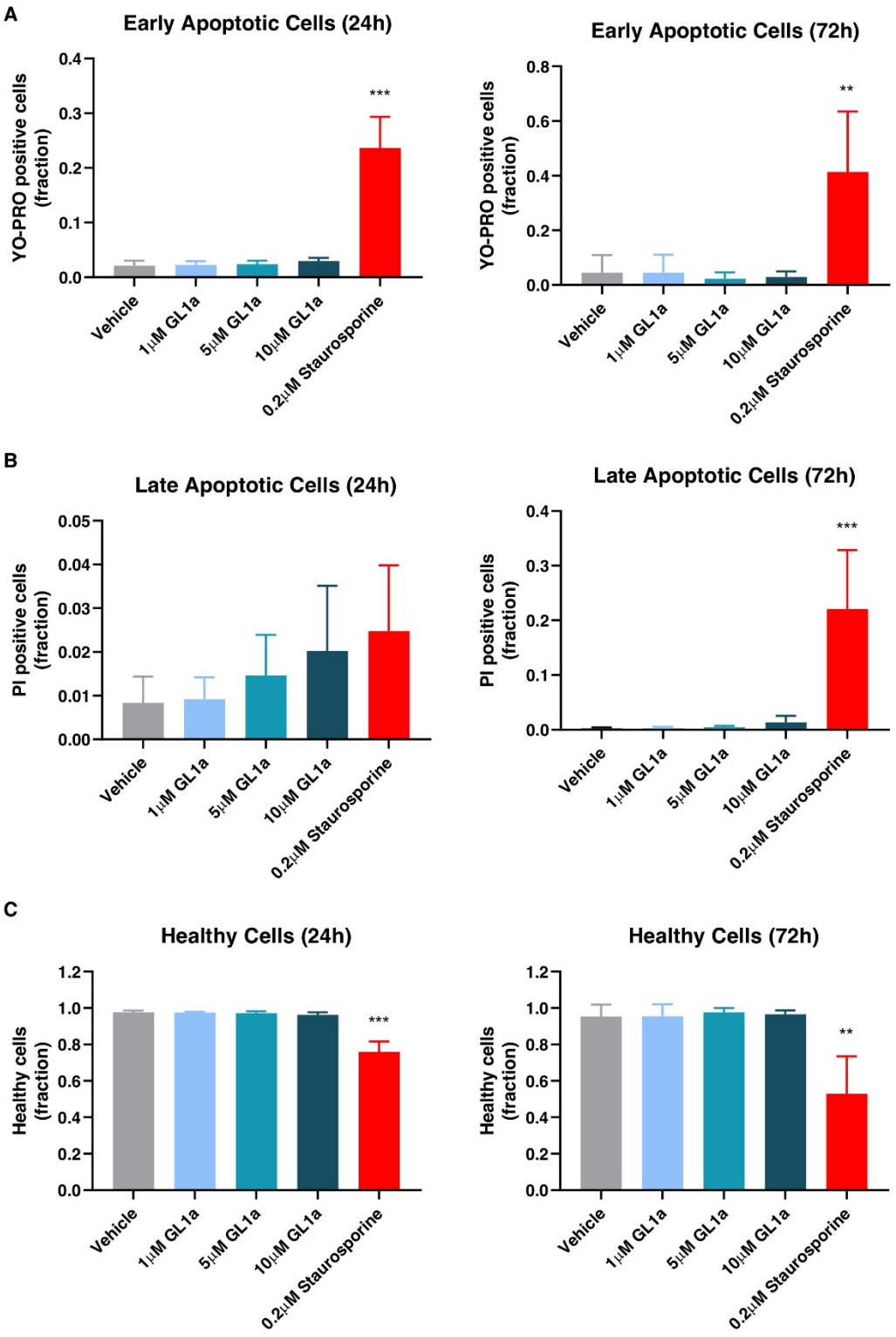


Figure 3.14. GL1a does not induce apoptosis in DU145 cells. DU145 cells were treated with vehicle (DMSO), GL1a (1μM, 5μM, or 10μM), or a positive control (staurosporine) for 24h or 72h. Nuclei were stained using Hoechst. YO-PRO was used to stain early apoptotic cells. PI was used to stain late apoptotic cells. Live cells were imaged using the Opera Phenix high-content screening system. (A) GL1a had no significant effect on the fraction of cells in early apoptosis. (B) GL1a had no significant effect on the fraction of cells in late apoptosis. (C) GL1a had no

significant effect on the fraction of healthy cells. Results are representative of 3 technical replicates and at least 3 biological replicates.

3.2.9.2. FLUORESCENCE MICROSCOPY ANALYSIS OF GL1A EFFECTS IN PC-3 CELLS

To visualise the effects of GL1a on PC-3 cell confluence and cell death, cells were treated with 1μM, 5μM, or 10μM GL1a, in the presence of serum. Live cells were imaged at 24h and 72h timepoints using the Opera Phenix high-content screening system. 10μM GL1a significantly reduced cell confluence at both 24h (p=0.010) and 72h (p<0.001) timepoints (**Figure 3.15**). GL1a caused no significant reduction in nucleus area or circularity. After 24h treatment, cells treated with the highest dose of GL1a showed increased YO-PRO positivity (p=0.0008) and PI positivity (p=0.0357), though this represented only a very small percentage of the total cell population (~2% of GL1a-treated cells compared to ~0.5% of vehicle-treated cells) (**Figure 3.16**). At 72h, GL1a had no significant pro-apoptotic effect. These results suggest that GL1a primarily inhibits PC-3 viability through reduced cell proliferation, though it may also produce a slight cytotoxic effect in these cells.

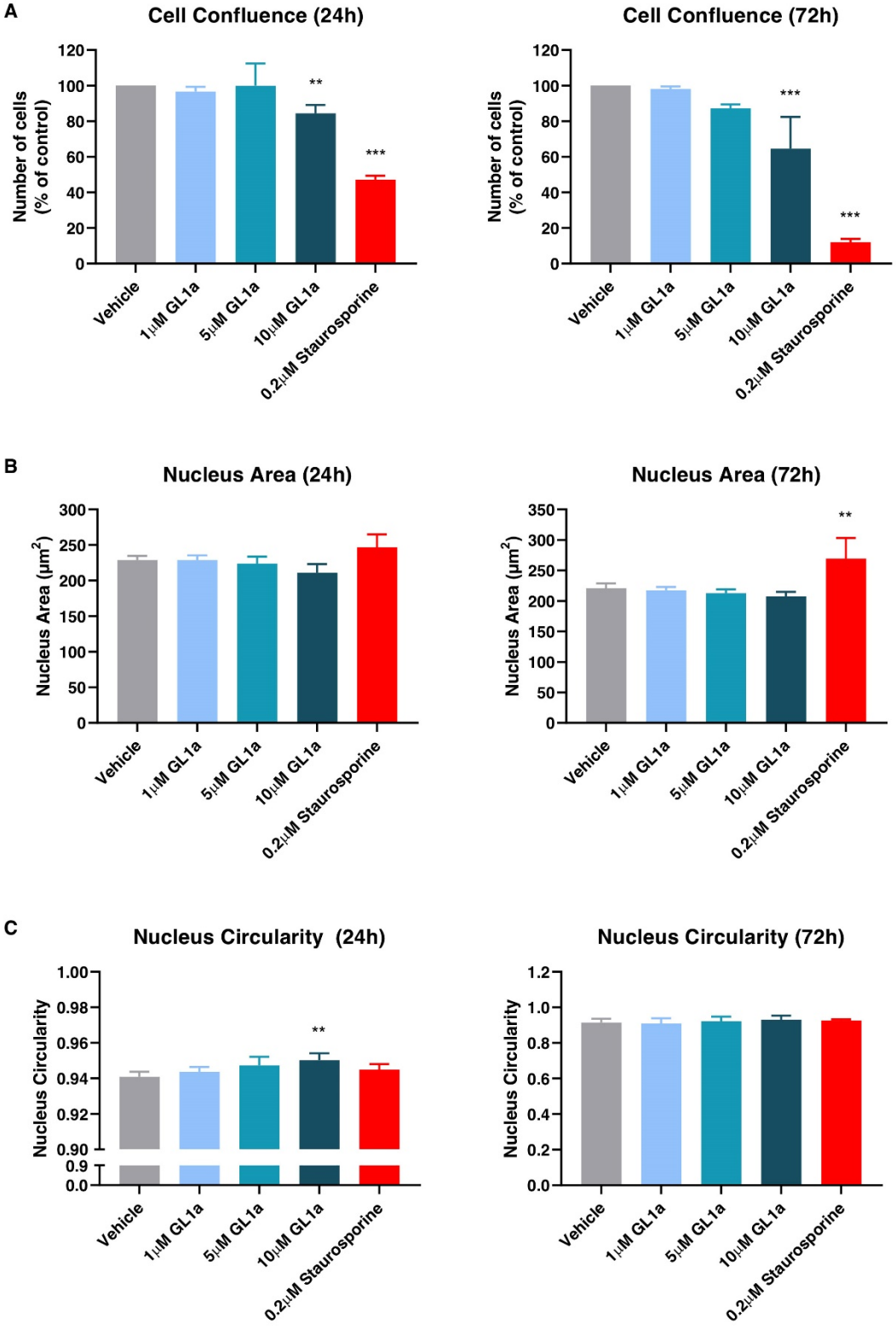


Figure 3.15. GL1a reduces proliferation of PC-3 cells. PC-3 cells were treated with vehicle (DMSO), GL1a (1μM, 5μM, or 10μM), or a positive control (staurosporine) for 24h or 72h. Nuclei were stained using Hoechst. Live cells were imaged using the Opera Phenix high-content screening system. (A) At 24h and 72h, 10μM GL1a significantly reduced the confluence of the cells (Student's *t*-test, $^{**}p<0.01$ $^{***}p<0.001$). (B) GL1a had no significant effect on nucleus area.

(C) 10μM GL1a significantly increased nucleus circularity (Student's *t*-test, $^{**}p<0.01$). Results are representative of 3 technical replicates and at least 3 biological replicates.

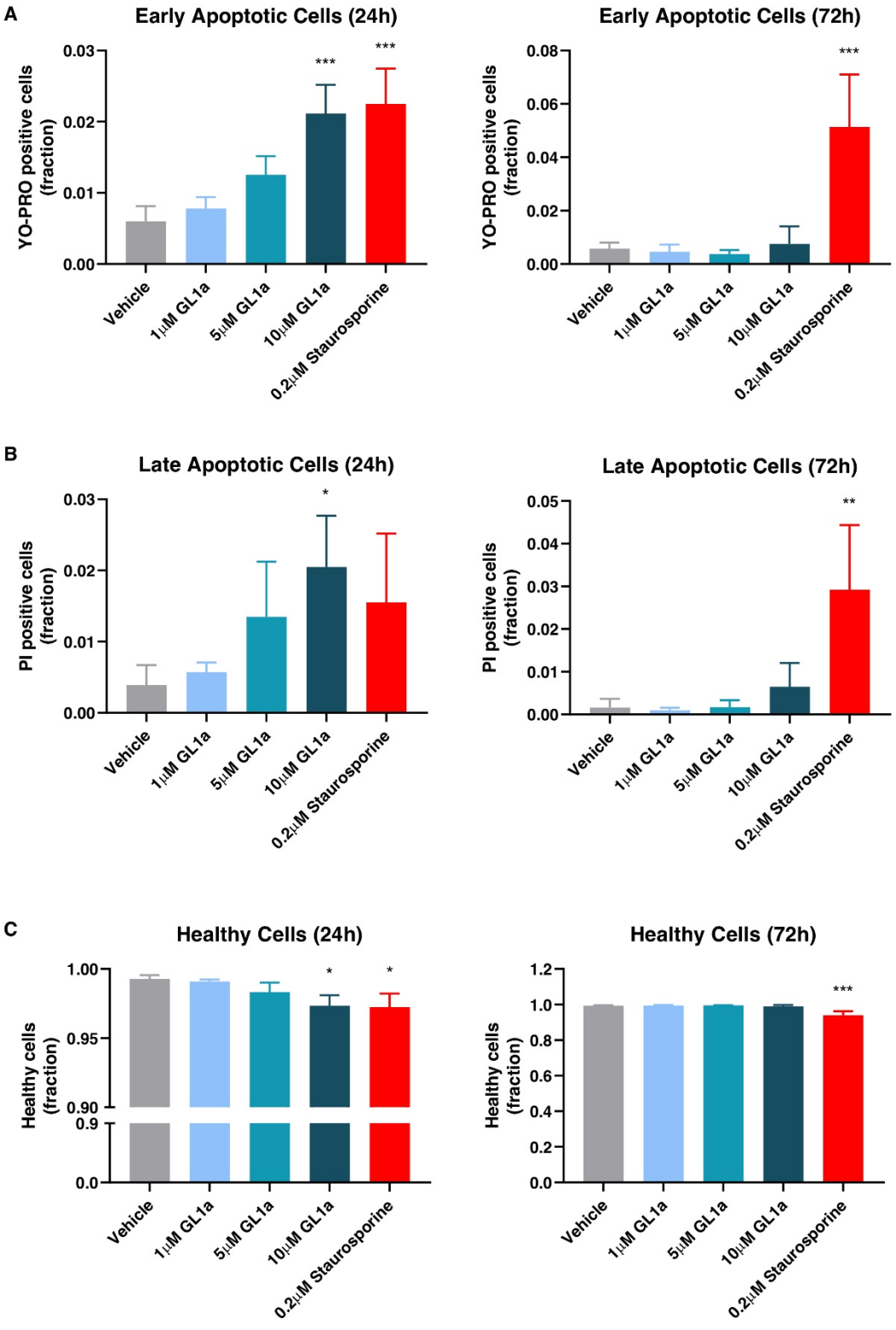


Figure 3.16. Effect of GL1a on apoptosis in PC-3 cells. PC-3 cells were treated with vehicle (DMSO), GL1a (1μM, 5μM, or 10μM), or a positive control (staurosporine) for 24h or 72h. Nuclei were stained using Hoechst. YO-PRO was used to stain early apoptotic cells. PI was used to stain late apoptotic cells. Live cells were imaged using the Opera Phenix high-content screening system. (A) At 24h, 10μM GL1a significantly increased the fraction of cells in early apoptosis, with

no significant effect after 72h (Student's *t*-test, ***p<0.001) (B) At 24h, GL1a significantly increased the fraction of cells in late apoptosis, with no significant effect after 72h (Student's *t*-test, *p<0.05). (C) At 24h, GL1a significantly reduced the fraction of healthy cells, with no significant effect after 72h (Student's *t*-test, *p<0.05). Results are representative of 3 technical replicates and at least 3 biological replicates.

3.2.9.3. FLUORESCENCE MICROSCOPY ANALYSIS OF GL1A EFFECTS IN PWR-1E CELLS

To determine whether GL1a reduces the viability of non-cancerous cells through increased cell death or reduced cell proliferation, PWR-1E cells were treated with 1μM, 2μM, or 10μM GL1a. Cells were treated in the absence of serum, according to the supplier's recommendation for this cell line, and visualised using fluorescence microscopy. The doses selected were slightly lower than those used for the other cell lines, as GL1a has enhanced effects under serum-free conditions (see **Figure 3.4**). Live cells were imaged at 24h and 72h timepoints using the Opera Phenix high-content screening system. GL1a significantly reduced cell confluence at all doses tested, with a strong inhibitory effect at 72h (**Figure 3.17**). GL1a also reduced nucleus area (p<0.001) and circularity (p<0.001), indicative of increased cell death. GL1a increased YO-PRO and PI positivity and reduced the fraction of healthy cells, particularly after 72h treatment (**Figure 3.18**). These results indicate that GL1a induces cell death through increased apoptosis in non-cancerous PWR-1E cells.

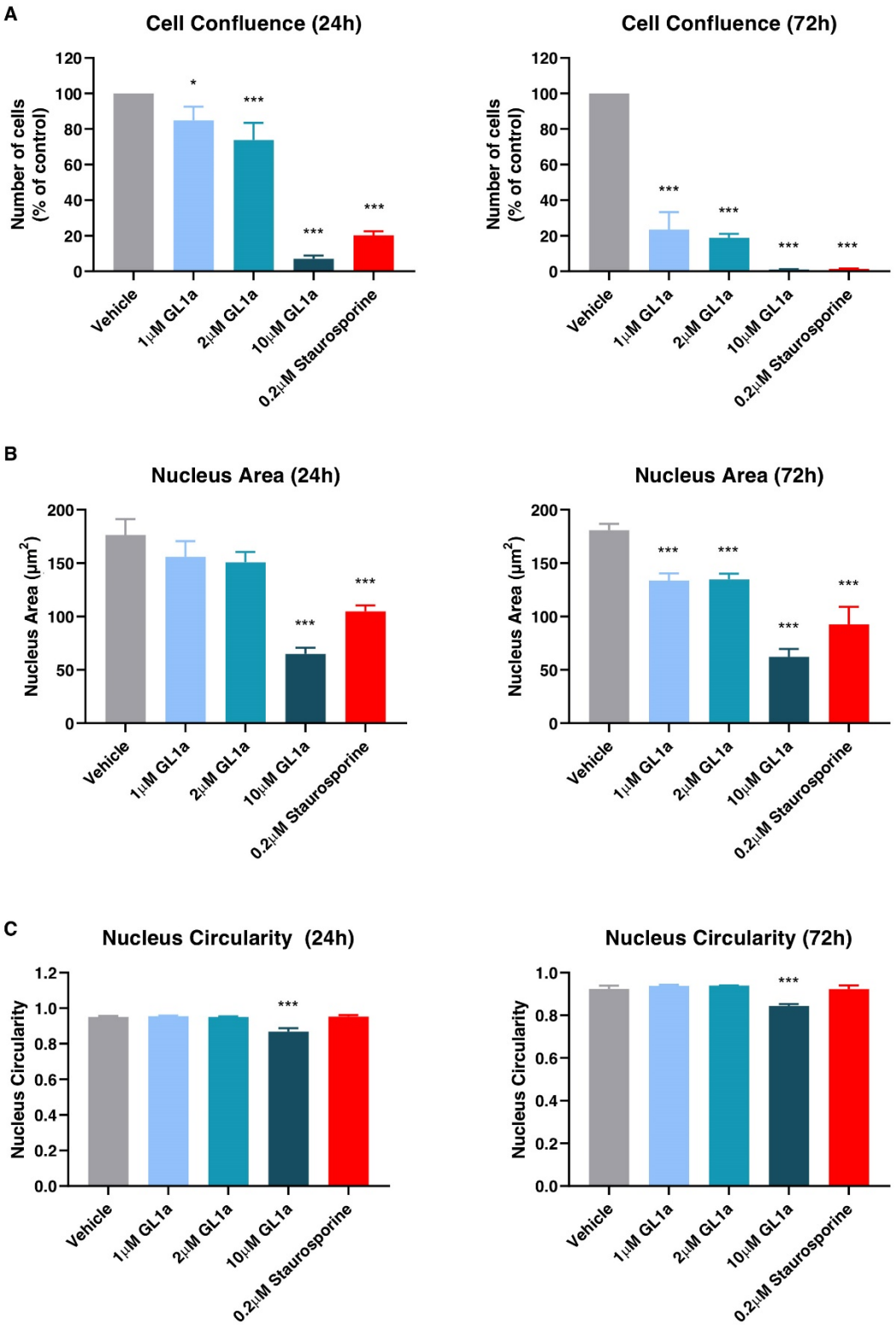


Figure 3.17. GL1a induces cytotoxicity in non-cancerous PWR-1E cells. PWR-1E cells were treated with vehicle (DMSO), GL1a (1μM, 2μM, or 10 μM), or a positive control (staurosporine) for 24h or 72h. Nuclei were stained using Hoechst. Live cells were imaged using the Opera Phenix high-content screening system. (A) At 24h and 72h, all GL1a doses tested significantly reduced the confluence of the cells (Student's *t*-test, **p*<0.05 ****p*<0.001). (B) GL1a significantly reduced nucleus area (Student's *t*-test, ****p*<0.001). (C) 10μM GL1a significantly reduced nucleus

circularity (Student's *t*-test, ***p<0.001). Results are representative of 3 technical replicates and at least 3 biological replicates.

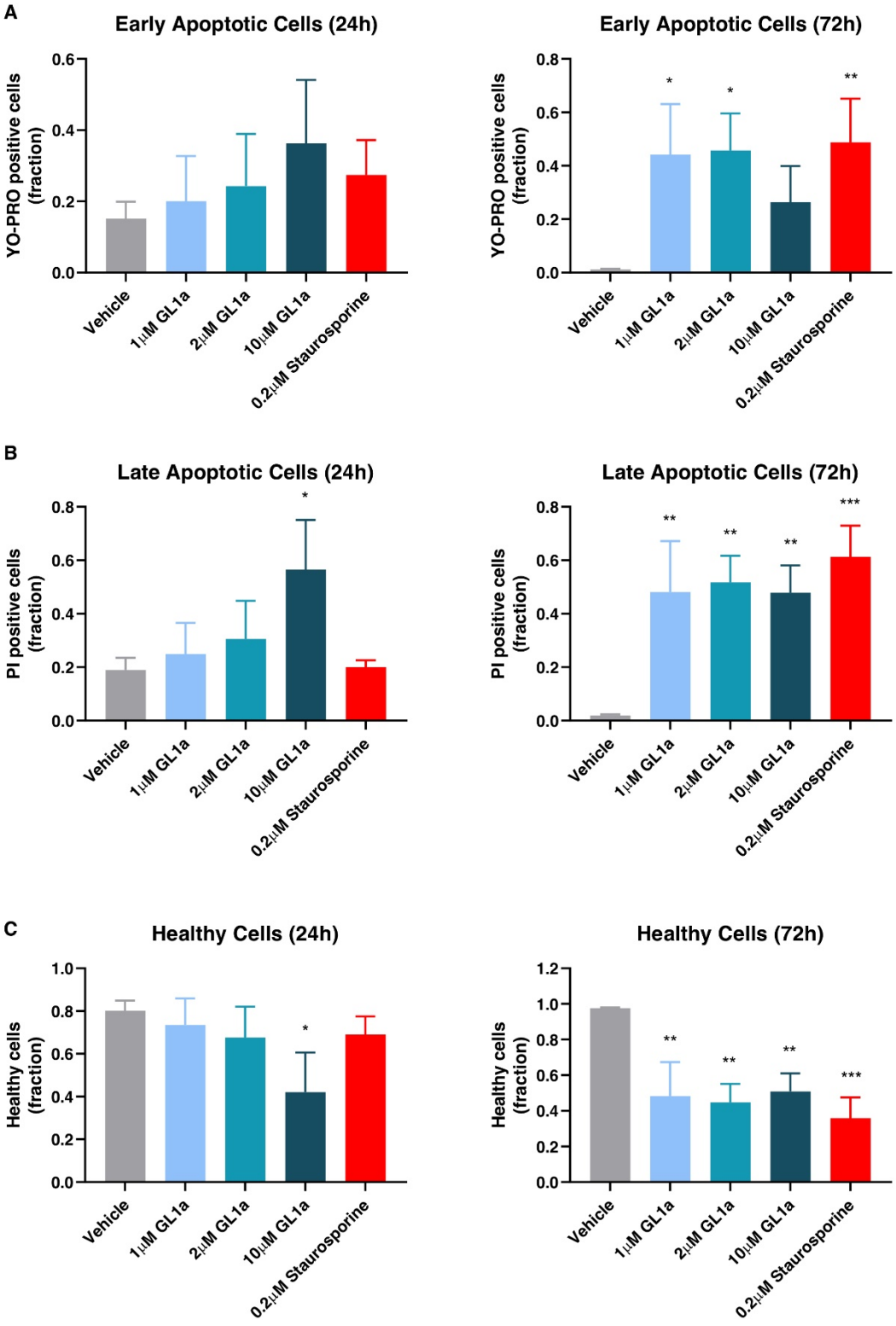


Figure 3.18. GL1a induces apoptosis in PWR-1E cells. PWR-1E cells were treated with vehicle (DMSO), GL1a (1μM, 2μM, or 10 μM), or a positive control (staurosporine) for 24h or 72h. Hoechst

was used to stain the nuclei. YO-PRO was used to stain early apoptotic cells. PI was used to stain late apoptotic cells. Live cells were imaged using the Opera Phenix high-content screening system. (A) At 72h, 1μM or 2μM GL1a significantly increased the fraction of cells in early apoptosis (Student's *t*-test, **p*<0.05) (B) GL1a significantly increased the fraction of cells in late apoptosis (Student's *t*-test, **p*<0.05 ***p*<0.01). (C) GL1a significantly reduced the fraction of healthy cells (Student's *t*-test, **p*<0.05 ***p*<0.01). Results are representative of 3 technical replicates and at least 3 biological replicates.

3.2.10. GL1A REDUCES PROSTATE CANCER CELL SURVIVAL

The clonogenic assay was used to determine the effect of GL1a on prostate cancer cell survival and colony-forming ability. DU145 and PC-3 cells were treated with IC_{50} doses of GL1a for 48h before detaching and reseeding the cells without treatment. After 7 days, colonies formed were stained and counted. In PC-3 cells, GL1a significantly reduced the number of colonies formed by approximately 30% (*p*=0.03) (**Figure 3.19**). GL1a also slightly reduced DU145 colony formation, though this was not statistically significant. These results indicate that the ability of PC-3 cells to survive and form colonies is reduced following GL1a treatment.

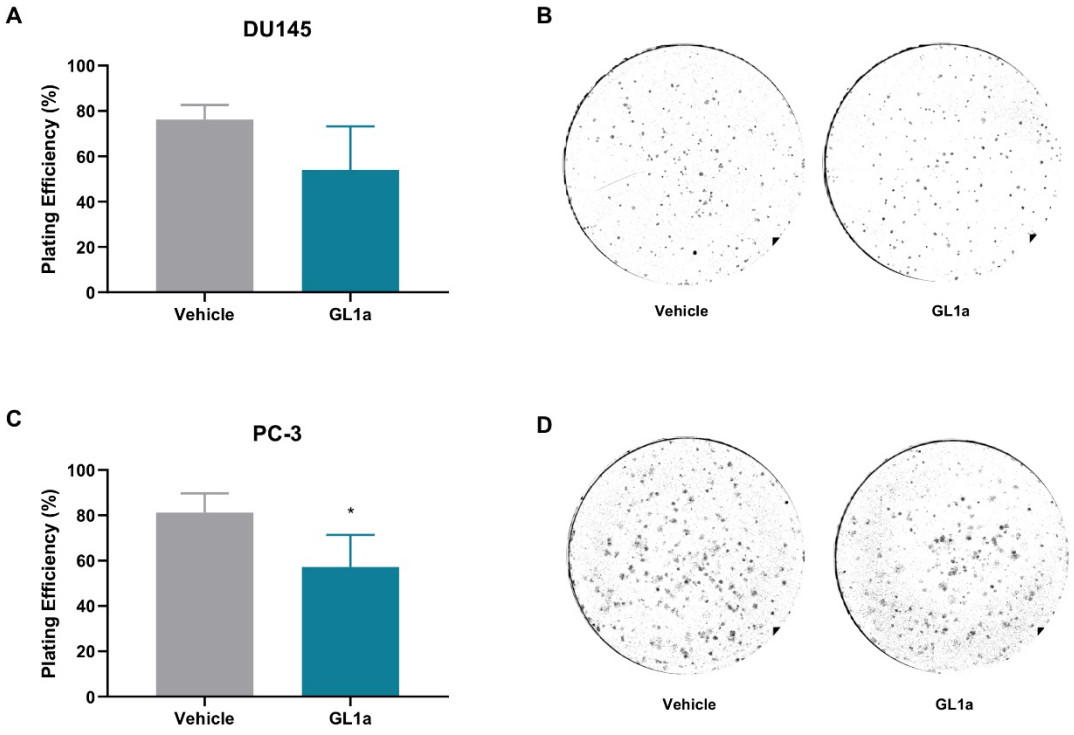


Figure 3.19. GL1a reduces the survival of PC-3 prostate cancer cells. DU145 and PC-3 cells were treated with an IC_{50} dose of GL1a for 48h, before reseeding at a low cell density without treatment. After 7 days, colonies were fixed and stained using glutaraldehyde and crystal violet. Colonies formed were photographed and quantified using ImageJ image analysis software. (A) GL1a had no significant effect on survival and colony-forming ability in DU145 cells. (B)

Representative images of colony formation in vehicle- and GL1a-treated DU145 cells. (C) GL1a significantly reduced the survival and colony-forming ability of PC-3 cells (Student's *t*-test, **p*<0.05). (D) Representative images of colony formation in vehicle- and GL1a-treated PC-3 cells. Results are representative of 3 biological replicates and 3 technical replicates.

3.2.11. EFFECT OF GL1A ON CELL MIGRATION

3.2.11.1. CELL DENSITY OPTIMISATION FOR SCRATCH WOUND-HEALING ASSAY

To determine the optimal cell seeding density and serum conditions for the scratch wound-healing assay, DU145, PC-3, and LNCaP cells were first seeded at increasing cell densities ($1.5x10^5$-$3x10^5$ cells per well for DU145 and PC-3, $3x10^5$-$6x10^5$ cells per well for LNCaP), in the presence or absence of serum. For DU145 and PC-3 cells, in the presence of serum, wells with cell densities of $2x10^5$-$3x10^5$ cells per well showed complete wound closure after 24h (**Figure 3.20**). In cells grown at a density of $1.5x10^5$ cells per well the scratch had completely closed after 48h. However, at this cell density, the scratch itself was not clearly visible. In LNCaP cells, wells with $4x10^5$-$6x10^5$ cells per well showed complete wound closure after 72h.

Serum deprivation inhibits cell proliferation and can remove confounding effects of proliferation on wound closure. In PC-3 cells grown without serum, the scratches had closed completely after 72h in cells grown at densities of $2x10^5$ or $3x10^5$ cells per well. In serum-deprived DU145 and LNCaP cells, the scratches did not close completely, even after 72h, though the scratch distances did reduce by ~80% and ~40%, respectively. We concluded that, in the absence of serum, cell migration should be measured at 72h. The optimal cell seeding densities for this assay were $2.5x10^5$ PC-3 cells per well, and $3x10^5$ DU145 or LNCaP cells per well.

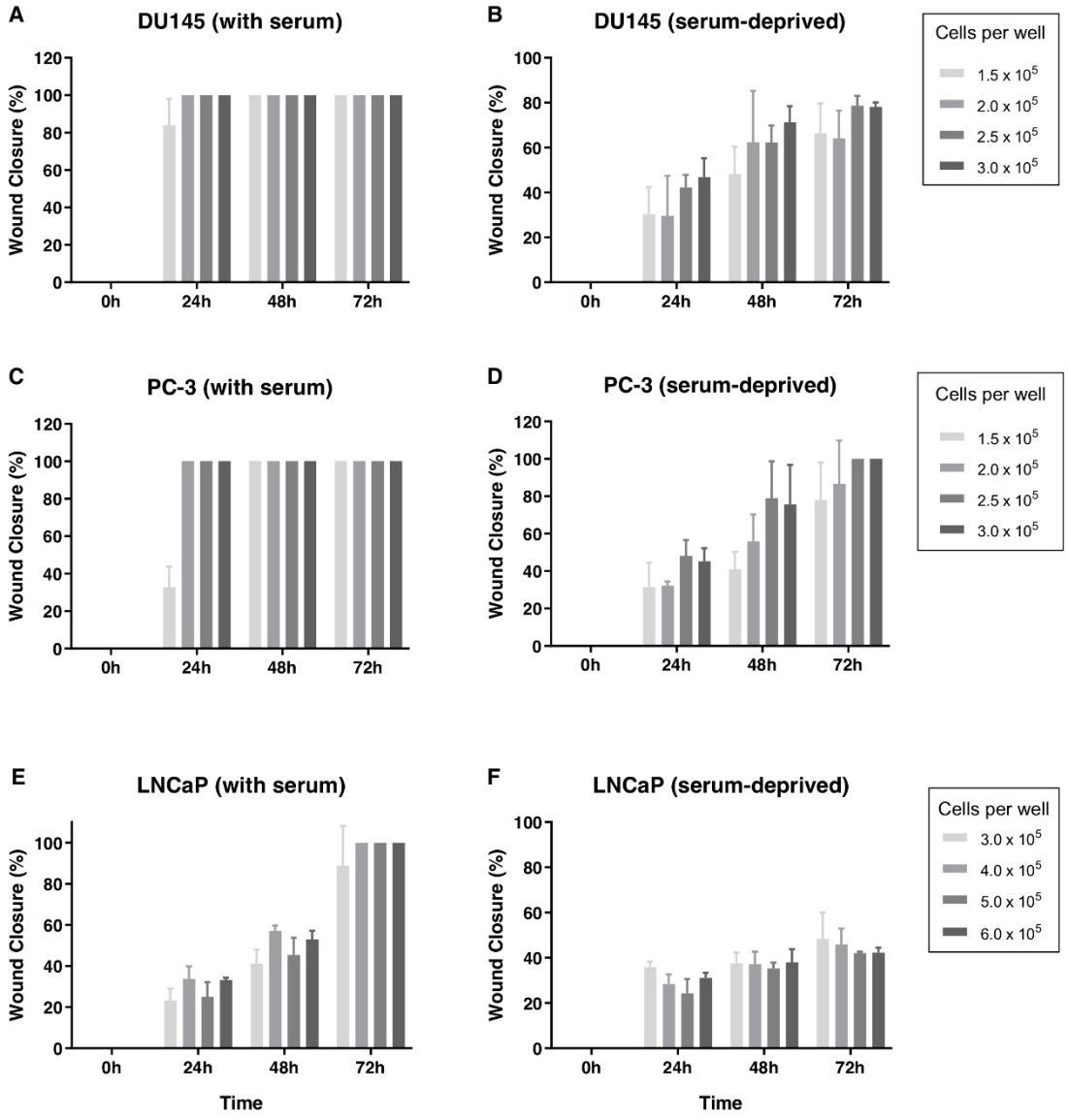


Figure 3.20. Optimisation of experimental conditions for the scratch wound-healing assay. DU145, PC-3, and LNCaP cells were seeded at varying cell densities. Scratches were made in cell monolayers and photographed at 0h, 24h, 48h, and 72h timepoints, in the presence or absence of serum. (A) In DU145 cells, in the presence of serum, wounds were completely closed at all timepoints, except after 24h at the lowest cell density tested. (B) In DU145 cells, in the absence of serum, wound closure was highest after 72h at cell densities of $2.5x10^5$ or $3.0x10^5$ cells per well. (C) In PC-3 cells, in the presence of serum, wounds were completely closed at all timepoints, except after 24h at the lowest cell density tested. (D) In PC-3 cells, in the absence of serum, wounds were completely closed after 72h at cell densities of $2.5x10^5$ or $3.0x10^5$ cells per well. (E) In LNCaP cells, in the presence of serum, wounds were completely closed after 72h at cell densities of $4.0x10^5$-$6.0x10^5$ cells per well. (F) In LNCaP cells, in the absence of serum, maximal wound closure was observed after 72h at a cell density of $3.0x10^5$ cells per well. Results are representative of 3 biological replicates and 3 technical replicates.

3.2.11.2. EFFECT OF GL1A ON PROSTATE CANCER CELL MIGRATION

The scratch wound-healing assay was used to measure the effect of GL1a on the migration of prostate cancer cells. DU145, PC-3, and LNCaP cells were seeded, serum-starved, scratched, and treated with vehicle (DMSO) or with the highest non-toxic dose of GL1a determined by MTT assay (400nM for DU145, 1.5μM for PC-3 and LNCaP) (see **Figure 3.4**). Under serum-deprived conditions, GL1a had no significant effect on wound closure at 72h in any of the cell lines tested (**Figure 3.21**). In LNCaP cells, only 40% wound closure was observed after 72h, suggesting that these cells have a low migratory capacity and may not be a valid model for testing this cancer hallmark. Further experiments were carried out using DU145 and PC-3 cells, which have a higher migratory capacity.

The scratch wound assay was repeated for DU145 and PC-3 cells to measure migration at 8h and 24h timepoints. Because these cells have a doubling time of approximately 24h, the shorter treatment time eliminated the need to serum-starve the cells to inhibit cell proliferation. Treating the cells in the presence of serum allowed for the use of higher GL1a doses without affecting cell viability. Cells were seeded, scratched, and treated with vehicle (DMSO) or with the highest non-toxic dose of GL1a determined by MTT assay (400nM for DU145, 6.25μM for PC-3). In PC-3 cells, GL1a appeared to cause a small reduction in cell migration at both timepoints, though this was not statistically significant. GL1a did not significantly alter the migration of DU145 cells (**Figure 3.22**). This suggests that GL1a does not inhibit prostate cancer cell migration.

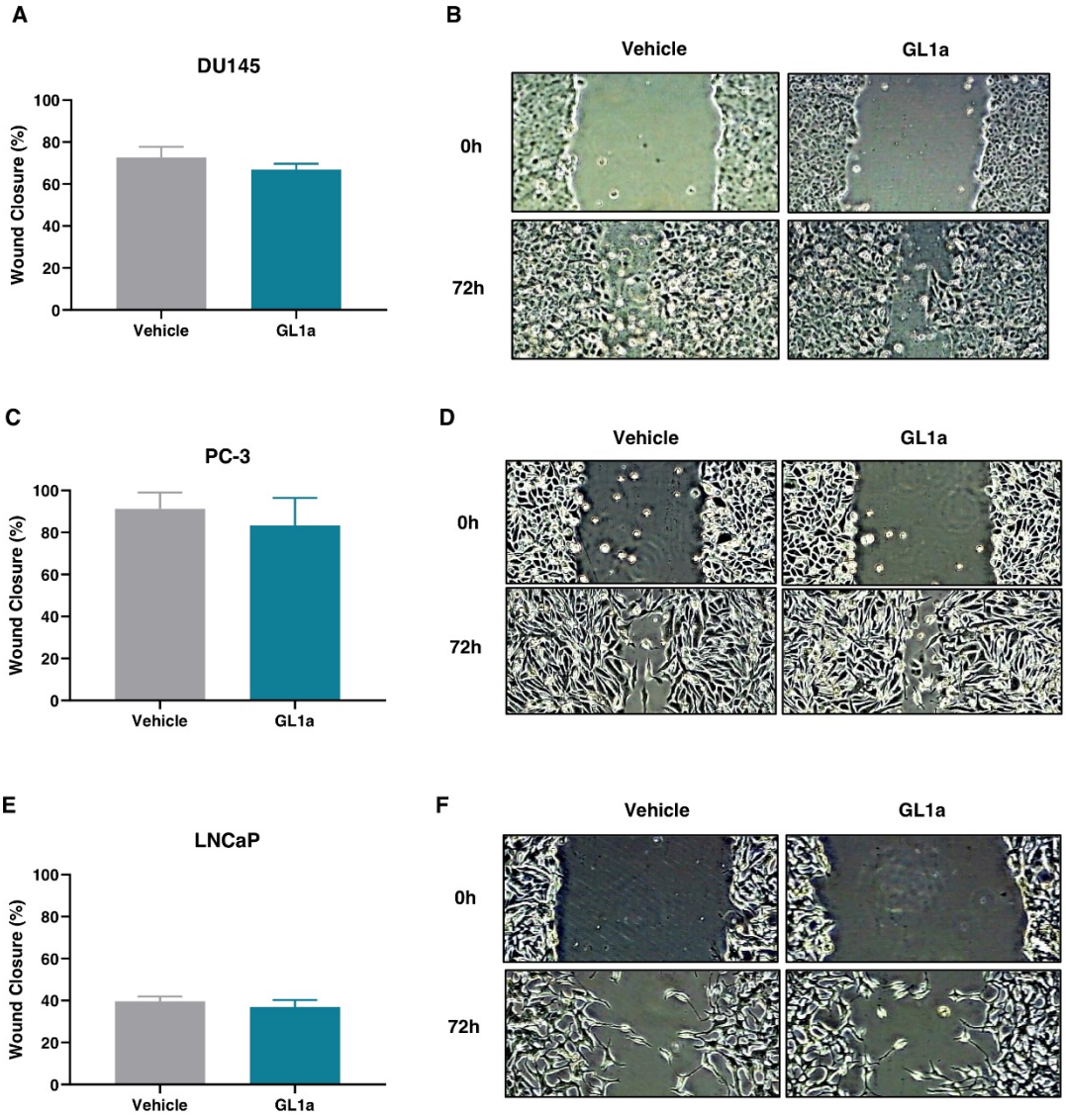


Figure 3.21. GL1a has no effect on prostate cancer cell migration at 72h under serum-free conditions. DU145, PC-3, and LNCaP cells were seeded in triplicate, serum-starved for 18h after adhesion, then scratched and treated with vehicle (DMSO) or with non-toxic doses of GL1a (400nM for DU145, 1.5μM for PC-3 and LNCaP). The scratches were photographed at 0h and 72h. GL1a had no significant effect on wound closure in (A) DU145, (C) PC-3, or (E) LNCaP cells (Student's *t*-test, **p*<0.05). Representative images of scratches in (B) DU145, (D) PC-3, and (F) LNCaP cells. Results are representative of 3 biological replicates. Original magnification x40.

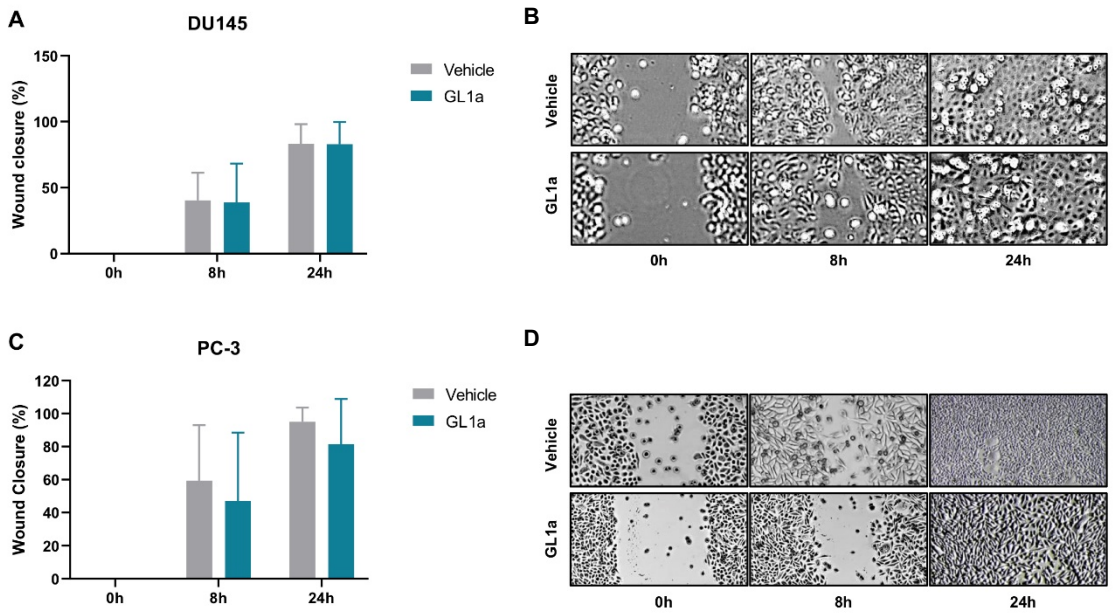


Figure 3.22. GL1a has no effect on prostate cancer cell migration at 8h or 24h, in the presence of serum. DU145 and PC-3 cells were seeded in triplicate, then scratched and treated with vehicle (DMSO) or a non-toxic dose of GL1a (400nM for DU145, 6.25μM for PC-3). The scratches were photographed at 0h, 8h, and 24h timepoints. GL1a had no significant effect on wound closure in (A) DU145 or (C) PC-3 cells (Student's *t*-test, **p*<0.05). Representative images of scratches in (B) DU145 and (D) PC-3 cells. Results are representative of 3 biological replicates. Original magnification x40.

3.2.12. GL1A REDUCES INVASIVENESS OF PROSTATE CANCER CELLS

The transwell Matrigel assay was used to investigate the effect of GL1a on cell invasion. DU145 and PC-3 cells were seeded in Matrigel-coated inserts in serum-free media. The wells beneath were filled with media containing 10% FBS to provide a chemotactic gradient. Cells were treated with the highest non-toxic dose of GL1a as determined by MTT assay (400nM for DU145, 1.5μM for PC-3). After 48h GL1a treatment, invasive cells were stained and counted. GL1a significantly reduced the invasiveness of PC-3 cells by approximately 30% (*p*=0.0003) (**Figure 3.23**). GL1a had no significant effect on DU145 cell invasion. These results indicate that GL1a inhibits the invasiveness of highly metastatic PC-3 cells.

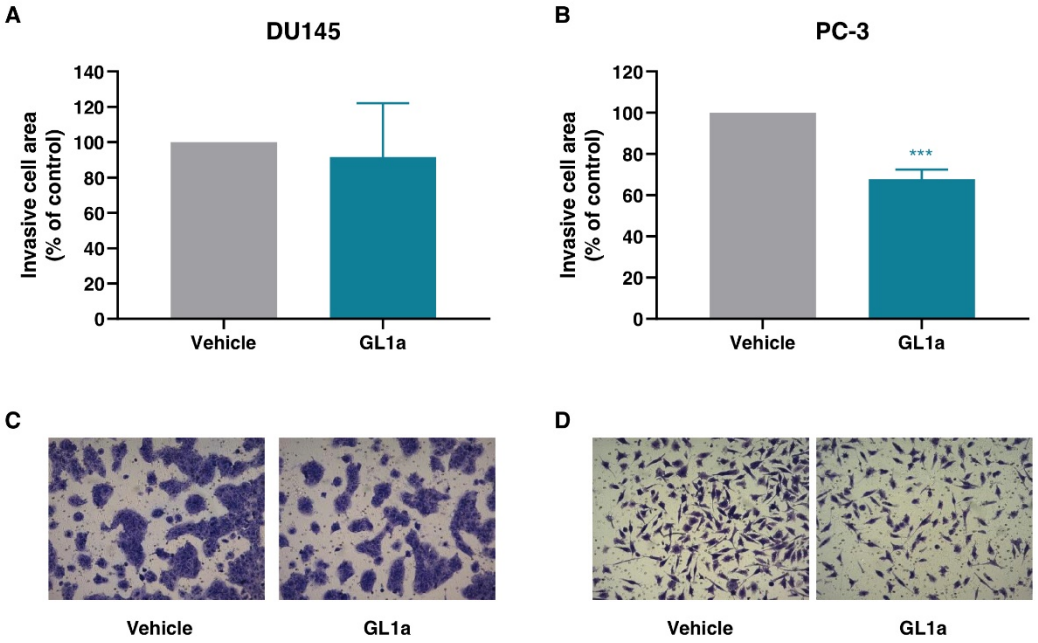


Figure 3.23. GL1a reduces the invasiveness of PC-3 prostate cancer cells. DU145 and PC-3 cells were seeded in serum-free media in Matrigel-coated inserts and treated with vehicle (DMSO) or a non-toxic dose of GL1a (400nM for DU145, 1.5μM for PC-3). Complete media was added to the wells beneath the inserts to promote invasion. After 48h, invasive cells were stained with crystal violet and photographed. The invasive cell area was quantified using ImageJ image analysis software. (A) GL1a had no significant effect on invasion in DU145 cells (Student's *t*-test, **p*<0.05). (B) GL1a significantly reduced the invasiveness of PC-3 cells (Student's *t*-test, ****p*<0.001). (C) Representative images of invasive DU145 cells. (D) Representative images of invasive PC-3 cells. Results are representative of 3 biological replicates and 3 technical replicates. Original magnification x100.

3.2.13. GL4A REDUCES PROSTATE CANCER CELL VIABILITY

The MTT assay was used to measure the effect of an additional phytocannabinoid, GL4a, on prostate cancer cell viability. PC-3 cells were treated with GL4a (0-100μM) for 72h, in the presence or absence of serum. GL4a significantly reduced cell viability under both conditions, with IC_{50} values of 14.64μM (with serum) and ~1.557μM (serum-deprived) (**Figure 3.24**). Like GL1a, GL4a reduced prostate cancer cell viability, with enhanced effects under serum-deprived conditions.

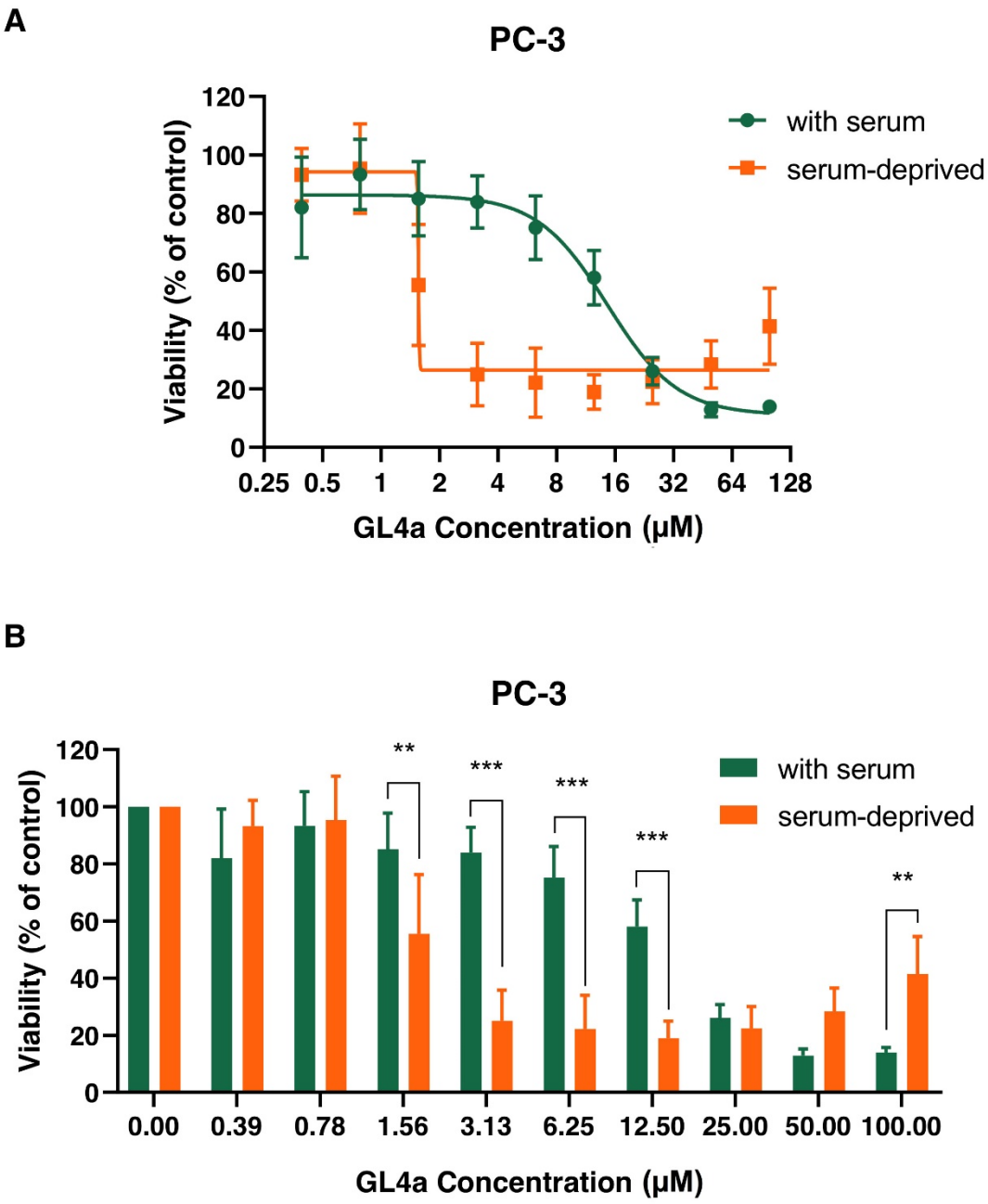


Figure 3.24. GL4a reduces the viability of PC-3 prostate cancer cells. PC-3 cells were treated with GL4a (0-100μM) for 72h in the presence or absence of serum. Cell viability was measured using the MTT assay. (A) GL4a reduced the viability of PC-3 cells. (B) Effects of GL1a were enhanced in serum-deprived cells (2-way ANOVA, $^{**}p<0.01$ $^{***}p<0.001$). Results are representative of 3 biological replicates and 3 technical replicates.

3.2.14. EFFECT OF GL4A ON APOPTOSIS IN PROSTATE CANCER CELLS

Having shown that GL4a reduced PC-3 cell viability, flow cytometry was used to determine whether this was caused by increased induction of apoptosis. YO-PRO was used as a marker of early apoptosis. PI was used as a marker of late apoptosis. PC-3 cells were treated for 48h with an IC_{50} dose of GL4a (14.64μM), determined by MTT assay. The results indicate that GL4a may slightly increase the percentage of cells in early apoptosis and reduce the percentage of healthy cells, but these effects did not reach statistical significance (**Figure 3.25**). 48h treatment with GL4a appears to reduce PC-3 cell viability primarily through a mechanism other than increased apoptosis.

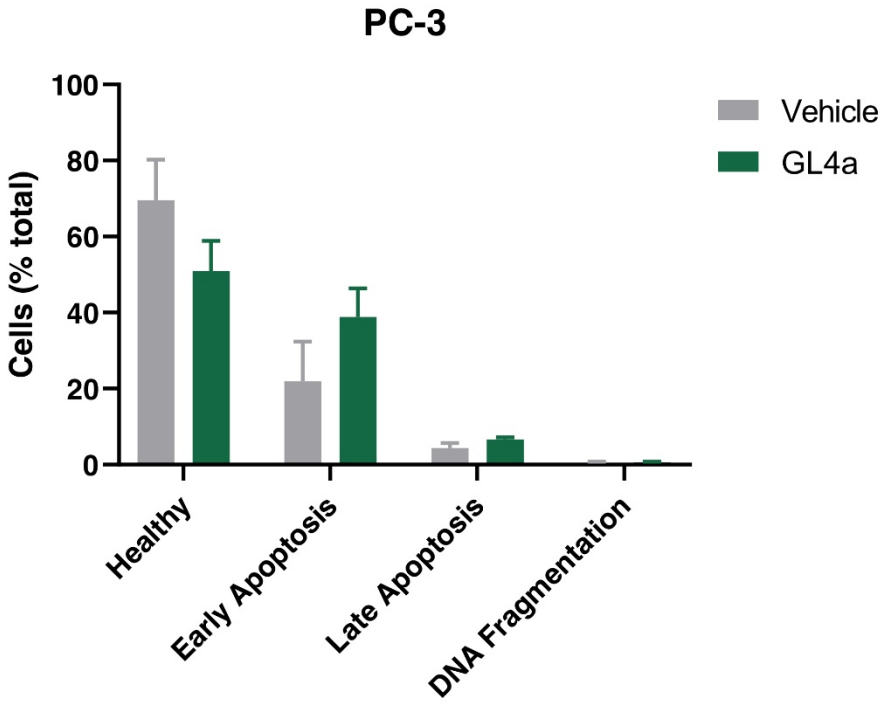
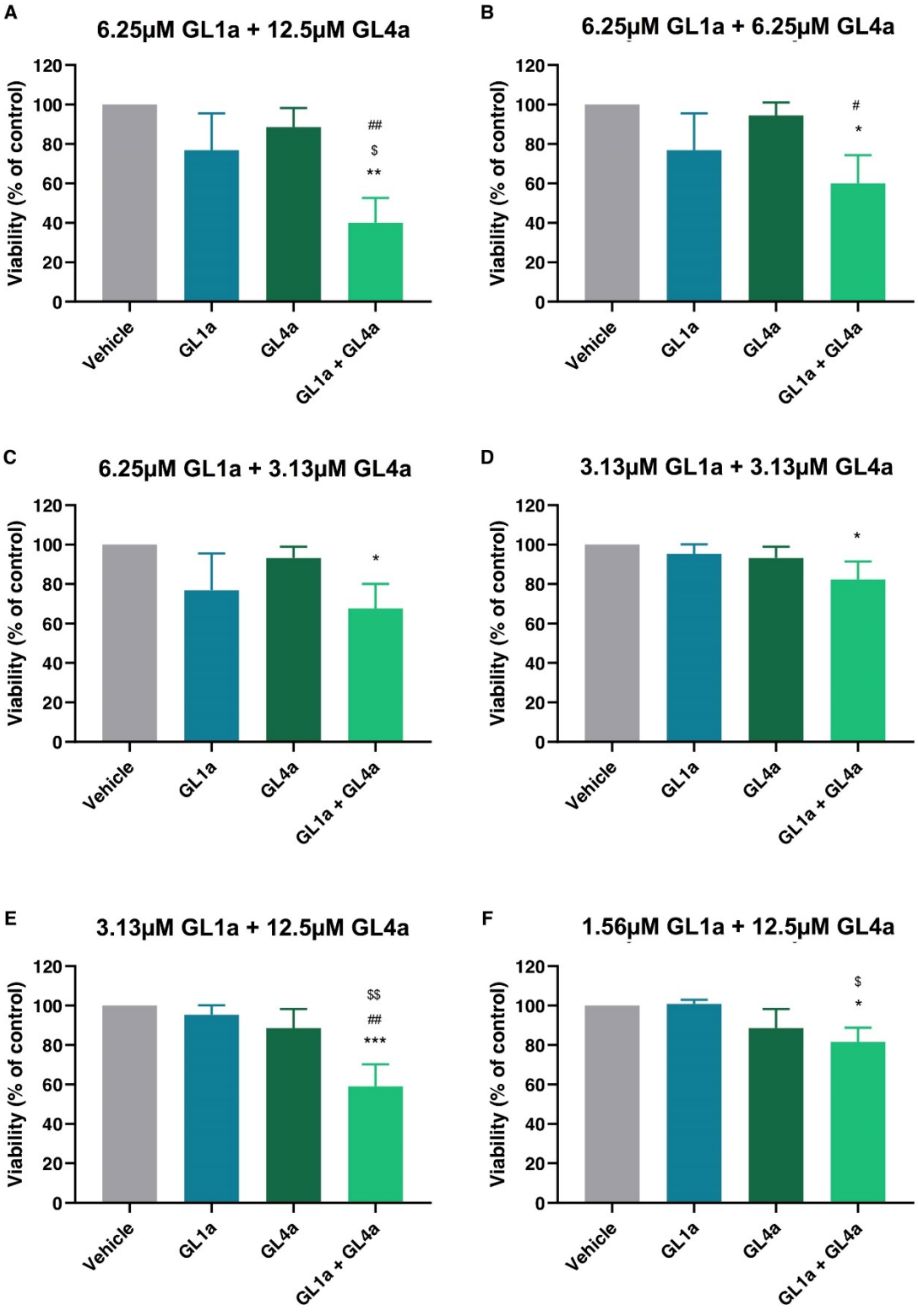


Figure 3.25. 48h GL4a treatment does not increase apoptosis in PC-3 cells. PC-3 cells were treated with an IC_{50} dose of GL4a for 48h. Apoptosis was measured using flow cytometry. YO-PRO was used as a marker of early apoptosis. PI was used as a marker of late apoptosis. GL4a had no significant effect on apoptosis (Student's *t*-test, **p*<0.05). Results are representative of 3 biological replicates and 3 technical replicates.

3.2.15. EFFECTS OF CANNABINOID COMBINATION TREATMENTS ON PROSTATE CANCER CELL VIABILITY

Several existing studies have shown that mixtures of cannabinoids can produce enhanced therapeutic effects compared to individual isolated compounds[137]. The

synergistic activity between various cannabis components has been termed the 'entourage effect'[137]. To determine whether cannabinoid combination treatments produced enhanced effects on prostate cancer cell viability, PC-3 cells were treated with various doses of GL1a and GL4a, alone or in combination. Cell viability was measured using the MTT assay. **Figure 3.26** shows the dose combinations that produced significantly enhanced effects on viability compared to treatment with the individual compounds at the same doses. At various dose combinations, the combination treatment produced enhanced effects compared to treatment with either compound alone. For example, combining 3.13μM or 6.25μM GL1a with 12.5μM GL4a reduced viability by 50-60%, while the individual compounds each produced a less than 30% reduction in viability. Additionally, low doses of cannabinoids that produced no effect on viability when administered alone significantly reduced viability when administered in combination. For example, combining 1.56μM GL1a with 6.25μM GL4a produced a small but significant ~20% reduction in cell viability, an effect not seen with either compound individually. Effects of additional dose combinations are shown in **Appendix Figure A7.2.** Compusyn analysis software was used to determine the combination index (CI) value for each combination. CI values less than 0.9 indicate synergy. CI values of 0.9-1.1 indicate an additive effect. CI values greater than 1.1 indicate antagonism. The results indicate some synergistic activity of cannabinoid compounds, particularly at the lowest doses tested, though a high degree of variability was observed between biological replicates. These results suggest that combination cannabinoid treatments may produce enhanced anti-cancer effects in prostate cancer.


A
6.25µM GL1a + 12.5µM GL4a
Viability (% of control)
120
100
80
60
40
20
0
##
$
**
Vehicle
GL1a
GL4a
GL1a + GL4a
B
6.25µM GL1a + 6.25µM GL4a
Viability (% of control)
#
*
Vehicle
GL1a
GL4a
GL1a + GL4a
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6.25µM GL1a + 3.13µM GL4a
Viability (% of control)
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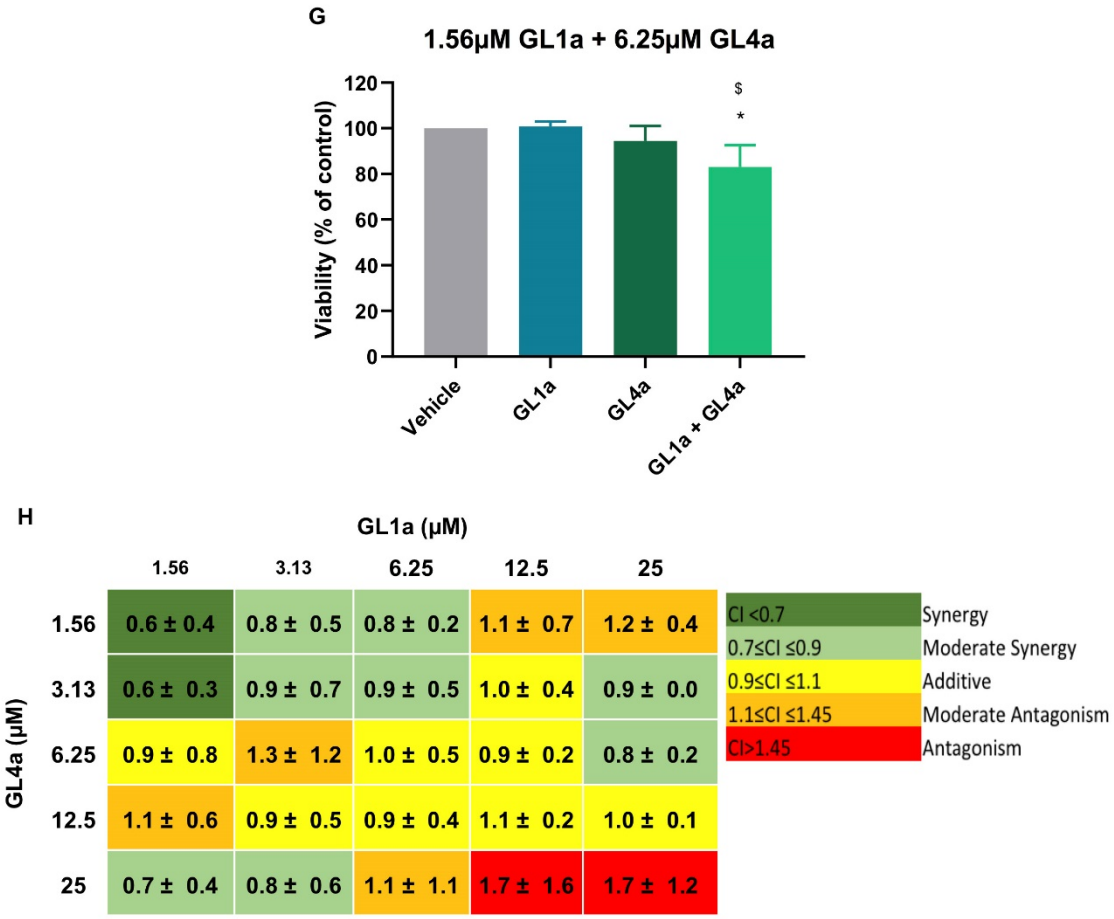


Figure 3.26. Effects of cannabinoid combination treatments on prostate cancer cell viability. PC-3 cells were treated for 72h with various doses of GL1a and GL4a, alone or in combination. Cell viability was measured using the MTT assay. Synergistic effects were determined using Compusyn analysis software. (A) Treatment with 6.25μM GL1a + 12.5μM GL4a reduced viability compared to vehicle or to treatment with either compound alone. (B) Treatment with 6.25μM GL1a + 6.25μM GL4a reduced viability compared to treatment with vehicle or with GL4a alone. (C) Treatment with 6.25μM GL1a + 3.13μM GL4a significantly reduced cell viability. (D) Treatment with 3.13μM + 3.13μM GL4a significantly reduced cell viability. (E) Treatment with 3.13μM GL1a + 12.5μM GL4a reduced cell viability compared to vehicle or to treatment with either compound alone. (F) Treatment with 1.56μM GL1a + 12.5μM GL4a reduced cell viability compared to treatment with vehicle or with GL1a alone. (G) Treatment with 1.56μM GL1a + 6.25μM GL4a reduced cell viability compared to treatment with vehicle or with GL1a alone. (H) Combination index (CI) values for cannabinoid combination treatments. Values shown are CI values +/- standard deviation. CI values <0.9 indicate synergy. CI values of 0.9-1.1 indicate an additive effect. CI values >1.1 indicate antagonism. *p<0.05 **p<0.01 ***p<0.001 compared to vehicle alone. $$p$<0.05 $$$p$<0.01 compared to GL1a alone. #p<0.05 ##p<0.01 compared to GL4a alone. Results are representative of 3 biological replicates and 3 technical replicates.

3.3. DISCUSSION

The aim of this chapter was to investigate the phenotypic effects of the phytocannabinoid compounds GL1a and GL4a in prostate cancer cell lines. Substantial preclinical evidence indicates that cannabinoids have anti-proliferative, cytotoxic, and anti-metastatic effects in a range of cancer types. However, few studies have investigated the chemotherapeutic potential of cannabinoids in prostate cancer.

Inhibition of cancer cell viability is a useful indicator that a drug has anti-cancer potential. Treatment of prostate cancer cells with GL1a reduced cell viability in a dose-dependent manner. Notably, the androgen-independent cell lines DU145 and PC-3 were more sensitive to GL1a treatment than the androgen-dependent LNCaP model. The IC_{50} values were lower than those previously reported in these cell lines[155], possibly due to minor differences in plant compound extraction methods, culture media composition, and cell seeding densities. GL4a also reduced PC-3 cell viability, with IC_{50} values similar to those observed with GL1a treatment. Interestingly, under serum deprivation conditions, increased viability was observed in cells treated with the highest dose of cannabinoids, above the dose range that would likely be used in treatment. The increased MTT absorbance could be due to interactions of the cannabinoids with the MTT reagent. Cannabinoids are known to have antioxidant activity, and some evidence suggests that antioxidants themselves can reduce MTT in a dose-dependent manner in the absence of cells[249]. However, we cannot rule out the possibility that, at high doses, GL1a activates additional targets with opposing effects. Therefore, care should be taken when identifying safe and effective doses for future *in vivo* and clinical studies.

The inhibition of viability by both GL1a and GL4a was enhanced under serum deprivation conditions, in agreement with previous studies of cannabinoids in cancer[155,248]. This is possibly due to binding of the cannabinoids to high molecular weight serum proteins, effectively reducing their availability in the presence of serum[155]. There is no clear consensus on which conditions are more biologically relevant. However, serum deprivation can induce cellular stress, reduce cell viability, and alter cell cycle progression[250-252]. To eliminate these confounding factors, subsequent experiments were conducted in the presence of serum. The inhibition of prostate cancer cell viability by GL1a and GL4a indicates their potential as anti-cancer agents. Our next goal was to determine whether the reduction in cell viability was associated with induction of cell death or inhibition of cell proliferation.

Resistance to cell death represents one of the fundamental hallmarks of cancer. Thus, agents that can induce cancer cell death possess chemotherapeutic potential. Many studies have shown that cannabinoids induce cell death through increased apoptosis in cancer cells[50,54,119,154,187,188]. To determine whether cannabinoids induce apoptosis in prostate cancer cells, we used flow cytometry to measure markers of early and late apoptosis in GL1a-treated and GL4a-treated cells. After 48h treatment, the cannabinoids produced no significant pro-apoptotic effect in DU145 or PC-3 cells. There was a small but significant increase in DNA fragmentation in GL1a-treated PC-3 cells, which may indicate some cytotoxicity, though this occurred in only a very small percentage of the total cell population. These findings were supported by fluorescence microscopy analysis showing no increase in apoptosis in GL1a-treated cells after 72h treatment. A slight increase in apoptosis was observed after 24h treatment in PC-3 cells, indicating some cytotoxicity, though again this was limited to a very small proportion of the total cell population. Previous studies of phytocannabinoids in prostate cancer did not specifically measure apoptotic cells but did report a small increase in caspase-3/7 activity in LNCaP cells following CBD treatment with serum[155]. In agreement with the current study, CBD did not induce apoptosis in DU145 or PC-3 cells in the presence of serum. CBD treatment did increase caspase activity and TUNEL positivity in DU145 and PC-3 cells, but only under serum deprivation conditions. The addition of bovine serum albumen to the cells strongly inhibited the pro-apoptotic effects of CBD in serum-deprived LNCaP cells, suggesting that serum proteins may reduce the availability and cytotoxicity of cannabinoids in these cells[155]. Notably, in the current study GL1a did significantly reduce the total number of events (representative of both live and dead cells) in samples from both DU145 and PC-3 cells. The reduction in cell numbers without increased apoptosis could indicate inhibition of cell proliferation by GL1a.

Uncontrolled cell proliferation is the most fundamental hallmark of cancer cells. Substantial evidence supports a role for cannabinoids in inhibiting cancer cell proliferation in a range of cancer types, including breast cancer, pancreatic cancer, gastric cancer, and glioma[52-54,178]. Further flow cytometry analysis was conducted to assess the pro-apoptotic and/or anti-proliferative potential of GL1a. We confirmed that GL1a treatment of PC-3 cells did not increase apoptosis or reduce the percentage of healthy cells after 48h. Interestingly, the GL1a treatment produced no significant reduction in cell proliferation. This was unexpected, given the 50% reduction in viability observed after 72h GL1a treatment at this dose. We suspect that the lack of an anti-proliferative or cytotoxic effect observed with this technique may be due to the shorter

treatment time used. Therefore, we also employed fluorescence microscopy analysis to enable a deeper investigation of the dose- and time-dependent phenotypic effects of GL1a in both prostate cancer and non-cancerous cells. GL1a reduced the confluence of DU145 cells after 72h, indicating reduced cell proliferation. Reduced proliferation was also observed in PC-3 cells at the highest dose tested, at both timepoints. These results support our initial observations that GL1a may reduce prostate cancer cell viability primarily through inhibition of cell proliferation, rather than through induction of apoptosis. Furthermore, a preliminary investigation using the Incucyte live cell imaging system showed a time- and dose-dependent reduction in PC-3 cell proliferation during a 72h GL1a treatment. It is important to note that the Incucyte results are representative of a single biological replicate. However, the observations provide some evidence that GL1a alters prostate cancer cell proliferation. The above observations form the basis of the work presented in Chapter 4, where we assessed the effect of GL1a on the expression of proteins that regulate cell proliferation. De Petrocellis *et al*. reported previously that CBD induced cell cycle arrest in DU145 cells under serum-free conditions, though they did not directly assess whether this translated to a reduction in cell proliferation[155]. In future studies, it would be useful to confirm the anti-proliferative effects of GL1a by measuring a specific cell proliferation marker, for example Ki67. Additionally, our results from the clonogenic assay suggest that the ability of PC-3 cells to survive and proliferate is reduced following GL1a treatment. Cannabinoids have previously been shown to reduce clonogenic survival following CBD treatment in colorectal cancer and gastric cancer[54,188]. These findings further support the potential of GL1a as an anti-cancer agent in prostate cancer.

Activation of cell invasion and metastasis is another crucial step in cancer progression. Agents that can inhibit metastasis could play an important role in cancer treatment. Some evidence indicates that cannabinoids inhibit the migration of cancer cells[52,175,202,253]. Here, GL1a had no significant effect on prostate cancer cell migration at any of the timepoints tested. However, GL1a strongly reduced the invasiveness of PC-3 cells. These results suggest that GL1a does not affect the motility of prostate cancer cells but reduces their ability to degrade the extracellular matrix and invade other tissues, a key step in prostate cancer progression. CBD reduces cell invasion in various cancer types, including lung cancer, glioblastoma, and breast cancer[52,197,198,200]. To our knowledge, these findings provide the first evidence that cannabinoids may have anti-metastatic potential in prostate cancer.

One problem associated with existing anti-cancer treatments is a lack of cancer cell specificity, leading to unwanted toxicity. A key finding from this chapter was the effect that the cannabinoids exerted on non-cancerous prostate epithelial cells. To determine whether the reduction of cell viability by GL1a was specific to cancer cells, effects of GL1a on cell viability were measured in three non-cancerous cell lines. Notably, GL1a reduced the viability of all three cell lines tested, with strongest effects in the non-cancerous prostate cell lines PWR-1E and RWPE-1. Furthermore, fluorescence microscopy analysis in PWR-1E cells revealed that an IC_{50} dose of GL1a substantially increased the percentage of cells in early and late apoptosis and reduced nucleus area (indicative of a cytotoxic effect), effects which were accompanied by a reduction in cell confluence. This suggests that GL1a has a strong cytotoxic effect in this cell line. It should be noted that these cells are routinely grown in serum-free media, so enhanced cannabinoid effects would be expected. It would be useful to assess the phenotypic effects of GL1a in other non-cancerous cell lines, particularly in cells grown with serum, for a clearer comparison to the effects seen in the prostate cancer cells.

However, even comparing cells grown in the absence of serum, GL1a IC_{50} doses were lower in non-cancerous cells than in cancer cells. These findings contrast with previous studies reporting that CBD reduced cell viability in cell line models of colon, breast, and gastric cancers, and HNSCC, without affecting the viability of corresponding non-cancerous cell lines[175,203,224,254]. Furthermore, Sharma *et al.* reported that CBD inhibited the viability of prostate cancer cells, with no significant effect observed in the prostate epithelial cell lines BPH-1 and PNT1B[226]. The reported cancer-specific effects of CBD were associated with over-expression of cannabinoid receptors (CB_1 and CB_2) in the cancer cell lines tested, compared to the non-cancerous cells. Notably, both cell lines used in that study were grown with serum, which is likely to reduce the efficacy of CBD. Furthermore, all the above experiments were conducted following a shorter 24h CBD treatment, and it is possible that a reduction in viability would be seen with longer treatments. However, ongoing research in our laboratory has demonstrated that GL1a reduces the viability of non-cancerous prostate cell lines even with shorter 24h treatments. In agreement with the current study, Deng *et al.* reported that 72h CBD treatment reduced viability at similar potencies in glioblastoma cells and in non-cancerous neural progenitor cells[255]. The pharmacology of cannabinoids is complex, with cannabinoids capable of targeting a wide range of receptors. The activity of CBD in specific cell lines is likely affected by variations in the expression levels of different cannabinoid targets. A deeper investigation into receptor targets of GL1a and effects of

GL1a on intracellular signalling pathways may provide a greater insight into potential off-target effects.

Assessing truly normal cells in prostate cancer is difficult as primary culture of prostate epithelial cells is technically challenging. The immortalised cell lines used in this study are artificially transformed to proliferate indefinitely, and thus are not necessarily representative of healthy, normal cells. For example, immortalisation is known to alter cellular properties including differentiation, DNA damage response, and chromosome structure[256,257]. In fact, the doses used in the current study are well within the range that has been reported safe and well-tolerated *in vivo.* Several studies report that CBD doses up to 1,500 mg/day are safe and well-tolerated in humans[258]. Moreover, cannabis-based medicines are currently approved for the treatment of various medical conditions (such as epilepsy and multiple sclerosis) and display low levels of toxicity, suggesting that the reduced viability observed in these specific immortalised cell lines may not necessarily translate to off-target toxicity *in vivo*. Effects observed in artificially immortalised cells may not necessarily rule out the use of GL1a in cancer treatment, though further research into the cancer-specificity of GL1a is warranted.

It is important to note that the above phenotypic screening was conducted using artificial 2D cell line models. Assessment of the effects of cannabinoids in more physiologically relevant models, for example, 3D cell culture models or animal models, will determine the likelihood of the observed anti-cancer effects translating to therapeutic benefits in a clinical setting.

In conclusion, the plant-derived cannabinoid GL1a reduces the viability and proliferation of prostate cancer cells. Furthermore, GL1a reduces the survival and invasiveness of aggressive PC-3 prostate cancer cells. Combining GL1a with other cannabinoids such as GL4a may produce enhanced anti-cancer activity. However, GL1a also reduces viability and induces apoptosis in non-cancerous cell lines, and further investigation into its cancer-specificity is warranted. Together, these findings suggest that cannabinoids may have potential as chemotherapeutic agents in prostate cancer. Investigation of the mechanisms underlying the observed phenotypic effects will provide a greater insight into the anti-cancer activity of these compounds.

CHAPTER 4

Mechanisms of action of cannabinoids in prostate cancer

4.1. RATIONALE

Identifying drug targets and mechanisms of action is an important step in evaluating the potential of a drug as an anti-cancer agent. Understanding how a drug produces its phenotypic effects can provide an insight into whether that compound could be particularly effective against specific cancer types and subtypes, and determine which patients are likely to benefit from a particular drug treatment. Additionally, investigating drug targets and mechanisms can help to determine whether a compound has the potential to add value to existing treatments. Furthermore, from a drug safety perspective, understanding a drug's mechanism(s) of action can provide an indication of likely off-target effects that could cause toxicity.

Deciphering cannabinoid mechanisms of action is challenging. As naturally occurring compounds, the pharmacology of phytocannabinoids is complex[259]. Cannabinoids display activity at a wide variety of molecular targets throughout the human body and can thus modulate many physiological processes[260]. Even within the context of cancer, existing research shows that phytocannabinoids can target many different receptors to produce phenotypic effects, including cannabinoid receptors, TRP ion channels, PPARγ, and GPR55[53,114,139,140,153,179,213]. Modulation of these targets by cannabinoids produces downstream effects on signalling pathways involved in cancer cell proliferation, cell death, and metastasis. However, few studies have investigated the effects of phytocannabinoids in prostate cancer and the anti-cancer mechanisms of cannabinoids in prostate cancer are not well-understood.

Having shown that cannabinoids produce phenotypic effects in prostate cancer cells, including reduced cell viability, proliferation, survival, and invasiveness (see **Chapter 3**), the aim of this chapter was to elucidate the mechanisms by which cannabinoids produce these effects.

This was done by investigating:

- The receptor targets of GL1a in prostate cancer cells
- The involvement of oxidative stress in GL1a anti-cancer effects
- The effects of GL1a on the phosphorylation of proteins involved in key cancer-related signalling pathways
- The effects of GL1a on expression levels of proteins involved in cell cycle progression and cell invasion

4.2. RESULTS

4.2.1. IDENTIFICATION OF RECEPTOR TARGETS OF GL1A IN PROSTATE CANCER CELLS

4.2.1.1. EXPRESSION OF CANNABINOID TARGETS IN PROSTATE CANCER CELLS

Previous studies have demonstrated receptor expression of CB_1, CB_2, TRPV1, TRPV2, and GPR55 in prostate cancer cells[134,155,225,261]. We attempted to reproduce these findings using qPCR and Western blot. mRNA expression of *TRPV1* and *TRPV2* was similar to that reported previously by De Petrocellis *et al.*[155], with both receptors expressed in DU145, PC-3, LNCaP, and PWR-1E cells. *TRPV1* expression was highest in DU145 cells, while *TRPV2* expression was highest in PC-3 cells. Expression of both receptors was lowest in LNCaP cells (**Figure 4.1**). No mRNA expression of *CNR1* (CB_1), *CNR2* (CB_2), or *GPR55* was detected in the prostate cell lines, in contrast to previous studies reporting expression of the above receptors at both the mRNA and protein level in prostate cancer cell lines. However, we were unable to source a robust positive control cell line known to express these receptors. Therefore, we cannot rule out that the lack of expression detected was due to problems with the primers and probes used in the experiment. Future studies should incorporate a strong positive control to confirm that these targets are not expressed at an mRNA level. Additionally, Western blot was used to measure the protein expression of the above targets in prostate cancer cells. While each target was expressed to some extent, the antibodies used showed high levels of non-specific binding, making it difficult to draw definitive conclusions from these experiments (**Figure 4.2**). However, our results support previous findings that CB_1, CB_2, TRPV1, TRPV2, and GPR55 are expressed by prostate cancer cells and lend further support to inconclusive findings by qPCR.

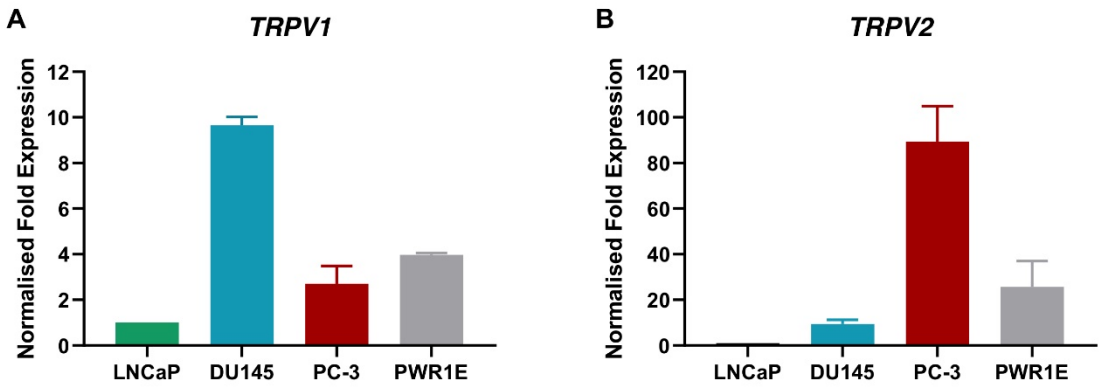


Figure 4.1. mRNA expression of *TRPV1* and *TRPV2* in prostate cell lines. (A) mRNA expression of *TRPV1* in prostate cell lines. (B) mRNA expression of *TRPV2* in prostate cell lines. Ct values for each cell line were normalised to the Ct values of the corresponding housekeeping genes (*ACTB, UBC*), and fold changes are shown relative to the cell line showing the lowest mRNA expression for each gene.

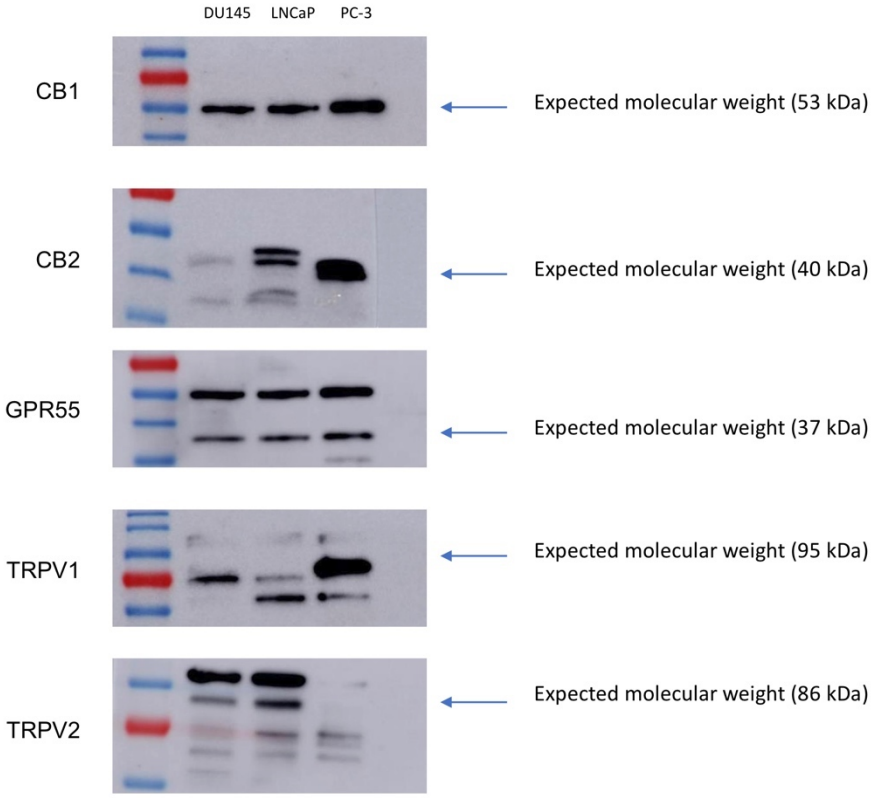


Figure 4.2. Representative blots showing protein expression of cannabinoid receptors in prostate cancer cell lines. High levels of non-specific binding were observed.

4.2.1.2. INVOLVEMENT OF THE CB_1 RECEPTOR IN GL1A EFFECTS

To determine the involvement of the CB_1 cannabinoid receptor in the anti-viability effects of GL1a, prostate cancer cells were pre-treated with a CB_1 receptor antagonist before assessing the effect of GL1a on cell viability. Based on previous studies of cannabinoid receptor activity in cancer, the selective inverse agonist SR141716 was chosen as the most potent and widely used CB_1 antagonist. The antagonists and agonists used in these experiments are summarised in **Table 4.1.** Cells were treated with SR141716 (10nM, 100nM, or 1μM) for 1h prior to 72h treatment with an IC_{50} dose of GL1a (12.3μM for DU145, 10.5μM for PC-3, 18.0μM for LNCaP). Cell viability was measured using the MTT assay. The results displayed are for the tested dose closest to the drug's *K*i value for that target. Higher doses, similar to those used previously in the literature, were also tested (**Appendix Figure A7.3**). The antagonist alone had no effect on cell viability. No significant difference in viability was observed in cells pre-treated with the antagonist compared to cells treated with GL1a alone, in any of the cell lines (**Figure 4.3**). These results suggest that blocking the CB_1 receptor using a selective antagonist does not inhibit the activity of GL1a.

Table 4.1. Summary of the antagonists and agonists used in this study, including supplier, pharmacological activity, and doses tested.

NAME	TARGET	ACTIVITY	DOSES	SUPPLIER	CAT NO.
SR141716	CB_1	Selective inverse agonist	10nM, 100nM, 1μM	Sigma-Aldrich	SML0800
SR144528	CB_2	Selective inverse agonist	10nM, 100nM, 1μM	Sigma-Aldrich	SML1899
CAPSAZEPINE	TRPV channels	Selective antagonist	1μM, 5μM, 10μM	Sigma-Aldrich	C191
LPI	GPR55	Endogenous agonist	3μM	Sigma-Aldrich	L7635

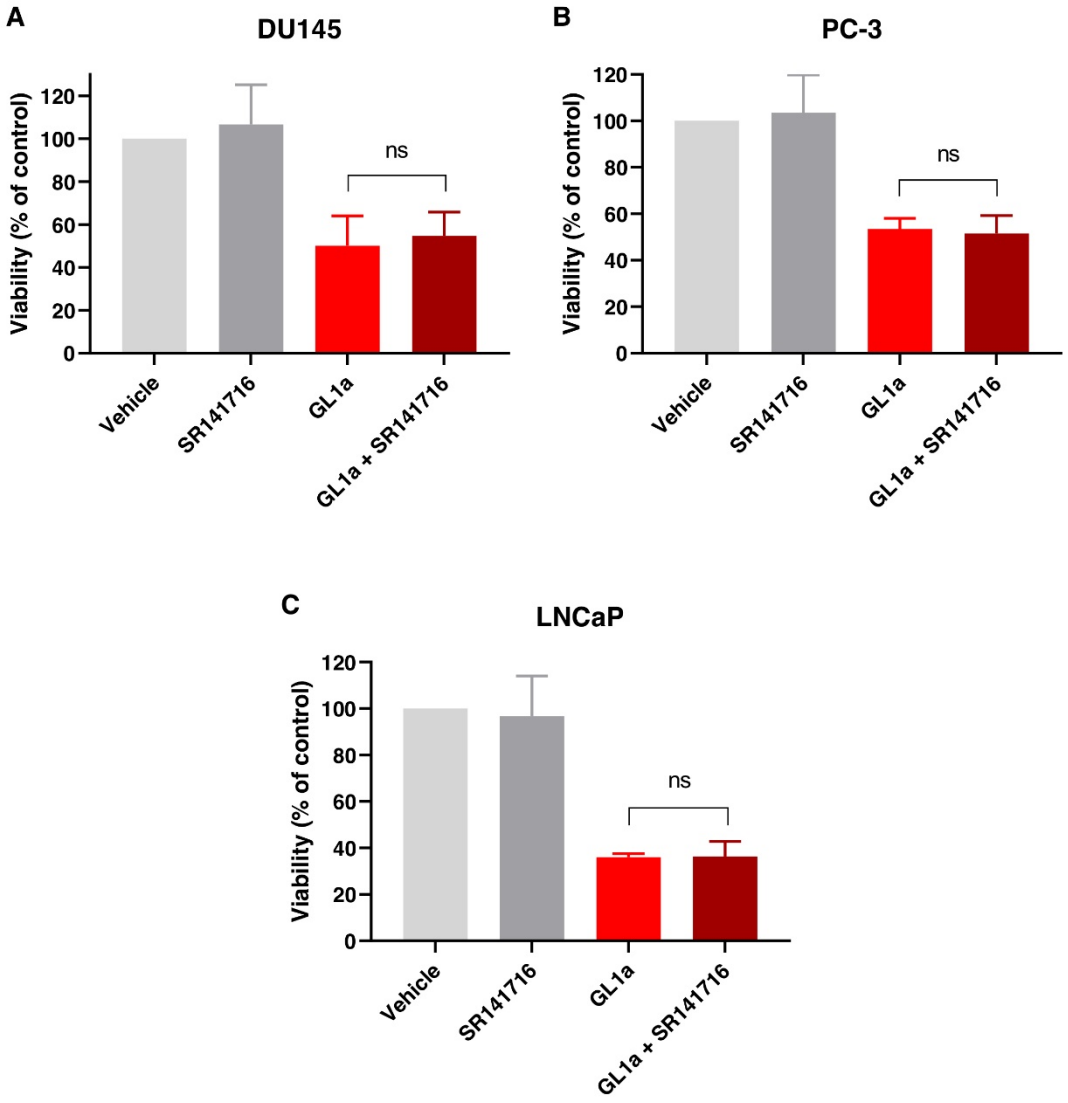


Figure 4.3. The effect of GL1a on cell viability is not blocked by a CB_1 receptor antagonist. Prostate cancer cells were pre-treated with the CB_1 antagonist SR141716 (10nM) for 1h before treatment with an IC_{50} dose of GL1a for 72h. Cell viability was measured using the MTT assay. (A) SR141716 did not alter the effect of GL1a on viability of DU145 cells. (B) SR141716 did not alter the effect of GL1a on viability of PC-3 cells. (C) SR141716 did not alter the effect of GL1a on viability of LNCaP cells (1-way ANOVA, *p<0.05). Results are representative of 3 biological replicates and 3 technical replicates.

4.2.1.3. INVOLVEMENT OF THE CB_2 RECEPTOR IN GL1A EFFECTS

To investigate the role of the CB_2 cannabinoid receptor in the anti-viability effects of GL1a, cells were pre-treated with the selective CB_2 receptor inverse agonist SR144528. SR144528 was selected as the most potent and widely used CB_2 antagonist from previous cancer studies. Cells were treated with SR144528 (10nM, 100nM, or 1μM) for 1h prior to 72h treatment with an IC_{50} dose of GL1a. Cell viability was measured using

the MTT assay. The antagonist alone did not affect cell viability. Pre-treatment with the CB_2 antagonist did not significantly alter the GL1a-induced reduction in viability in any of the cell lines (**Figure 4.4, Appendix Figure A7.4**).

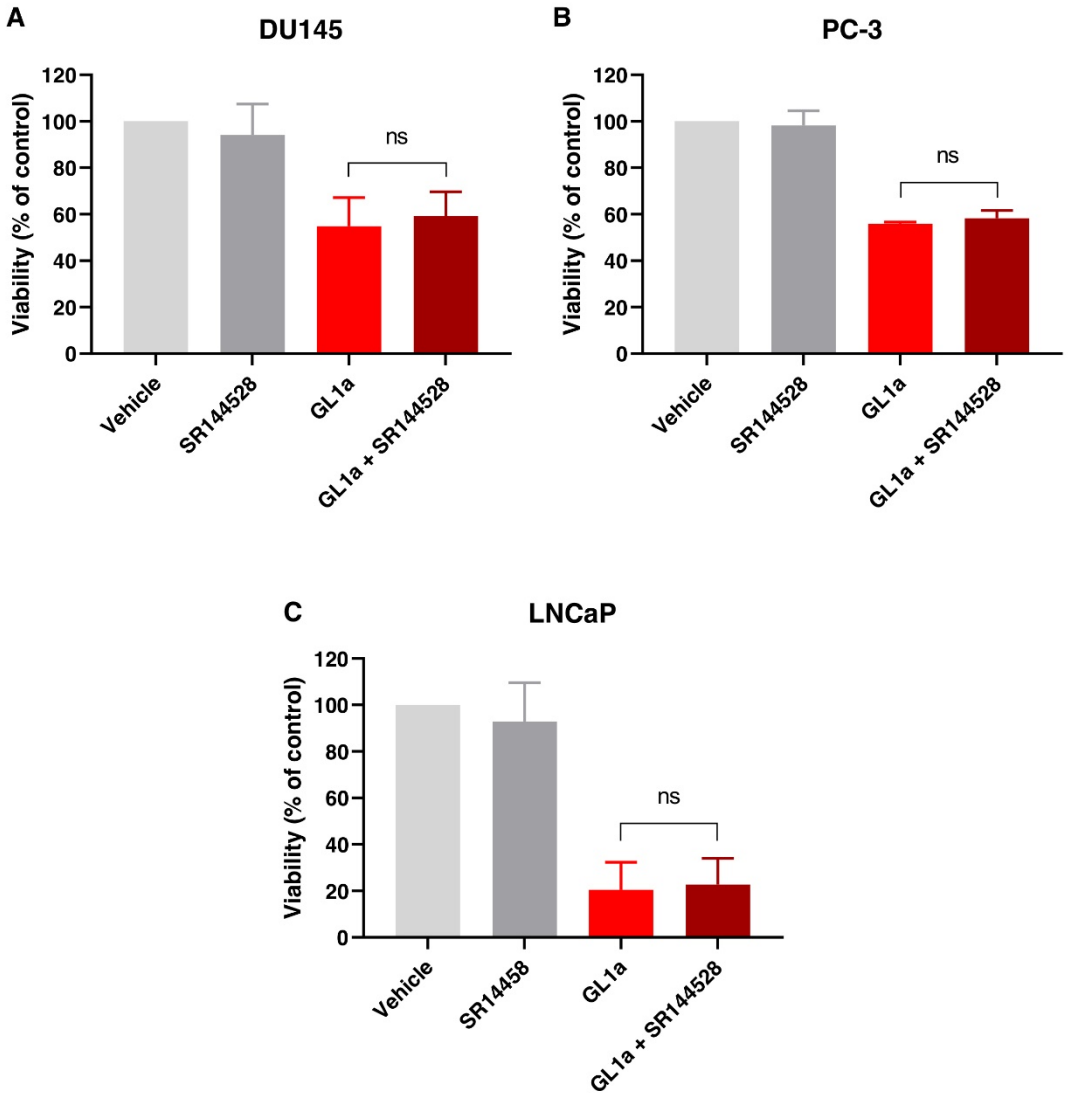


Figure 4.4. The effect of GL1a on cell viability is not blocked by a CB_2 receptor antagonist. Prostate cancer cells were pre-treated with the CB_2 antagonist SR144528 (10nM) for 1h before treatment with an IC_{50} dose of GL1a for 72h. Cell viability was measured using the MTT assay. (A) SR144528 did not alter the effect of GL1a on viability of DU145 cells. (B) SR144528 did not alter the effect of GL1a on viability of PC-3 cells. (C) SR144528 did not alter the effect of GL1a on viability of LNCaP cells (1-way ANOVA, *p<0.05). Results are representative of 3 biological replicates and 3 technical replicates.

4.2.1.4. INVOLVEMENT OF TRPV ION CHANNELS IN GL1A EFFECTS

To investigate the involvement of TRPV ion channels in GL1a anti-viability effects, cells were pre-treated with a TRPV channel antagonist before assessing the effect of GL1a on cell viability. Capsazepine was selected as a widely used antagonist which can block the activity of all TRPV channels. Cells were treated with capsazepine (1μM, 5μM, or 10μM) for 1h before treating with an IC_{50} dose of GL1a for 72h. The MTT assay was used to measure viability. The antagonist alone did not significantly affect the viability of DU145 or LNCaP cells. However, capsazepine alone significantly reduced the viability of PC-3 cells at doses of 5μM or 10μM (**Appendix Figure A7.5**). No significant difference was observed between cells pre-treated with the antagonist and those treated with GL1a alone (**Figure 4.5, Appendix Figure A7.5**). These findings suggest blocking TRPV ion channels using a selective antagonist does not inhibit the activity of GL1a in these cells.

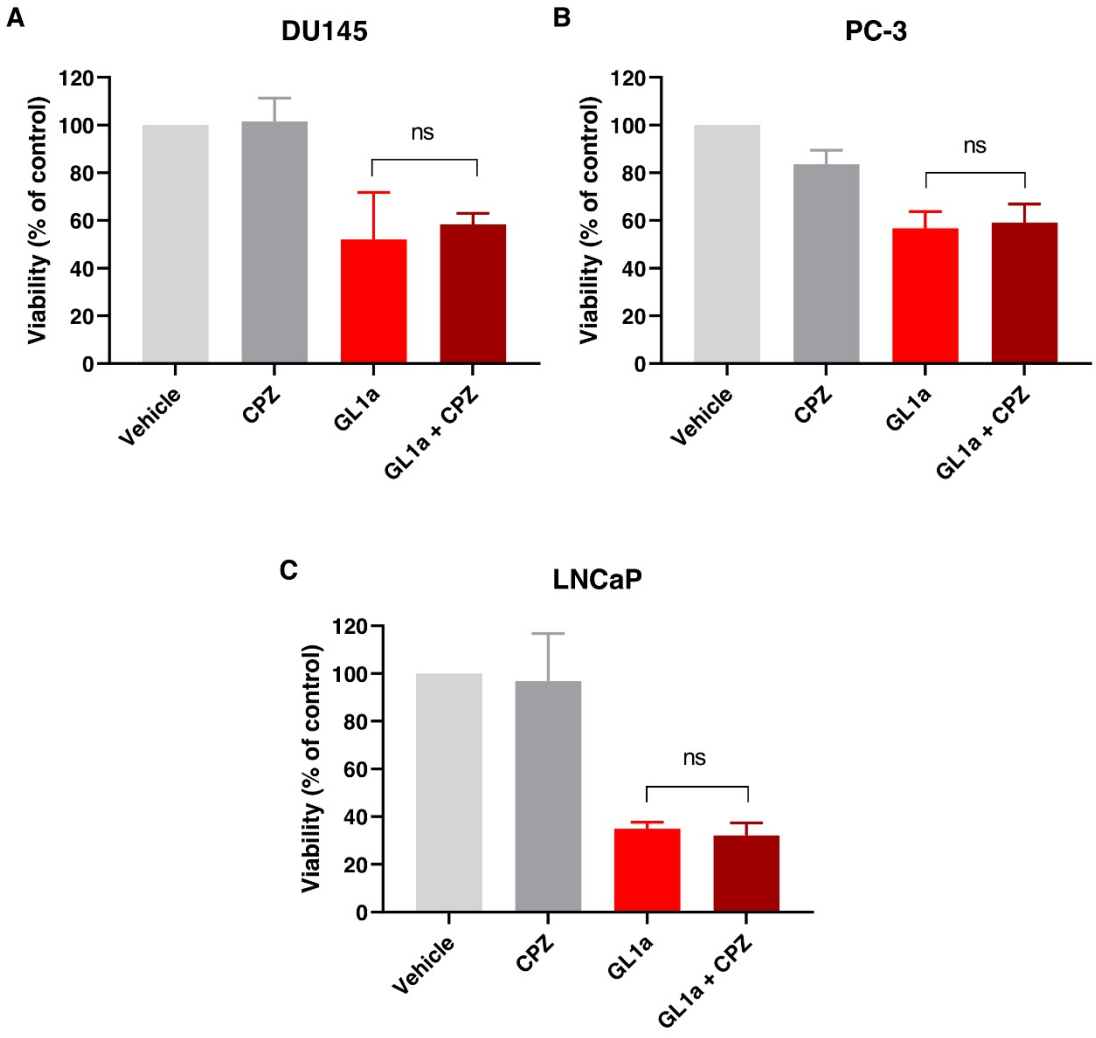


Figure 4.5. The effect of GL1a on cell viability is not blocked by a TRPV channel antagonist. Prostate cancer cells were pre-treated with the TRPV channel antagonist capsazepine (1μM for PC-3, 3μM for DU145 and LNCaP) for 1h before treatment with an IC_{50} dose of GL1a for 72h. Cell viability was measured using the MTT assay. (A) Capsazepine did not alter the effect of GL1a on viability of DU145 cells. (B) Capsazepine did not alter the effect of GL1a on viability of PC-3 cells. (C) Capsazepine did not alter the effect of GL1a on viability of LNCaP cells (1-way ANOVA, *p<0.05). Results are representative of 3 biological replicates and 3 technical replicates.

4.2.1.5. INVOLVEMENT OF GPR55 IN GL1A EFFECTS

To determine whether the effects of GL1a occurred through antagonism of the putative cannabinoid receptor GPR55, DU145 and PC-3 cells were pre-treated with a GPR55 agonist. Lysophosphatidylinositol (LPI) was selected, as the endogenous ligand at this receptor. Cells were treated with LPI (3μM) for 1h before 72h treatment with an IC_{50} dose of GL1a. Cell viability was measured using the MTT assay. The agonist alone did not significantly affect cell viability. The effects of GL1a on cell viability were not altered by

pre-treatment with the GPR55 agonist (**Figure 4.6**), suggesting that GL1a does not reduce viability through antagonism of GPR55 in prostate cancer cells.

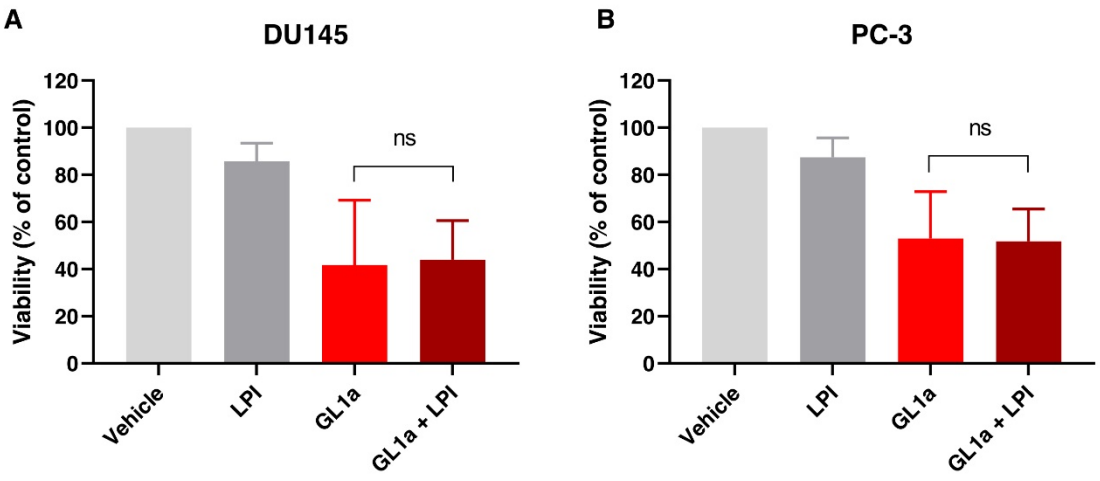


Figure 4.6. The effect of GL1a on cell viability is not blocked by a GPR55 receptor agonist. Prostate cancer cells were pre-treated with the GPR55 endogenous ligand lysophosphatidylinositol (LPI) (3μM) for 1h before treatment with an IC_{50} dose of GL1a for 72h. Cell viability was measured using the MTT assay. (A) LPI did not alter the effect of GL1a on viability of DU145 cells. (B) LPI did not alter the effect of GL1a on viability of PC-3 cells (1-way ANOVA, *p<0.05). Results are representative of 3 biological replicates and 3 technical replicates.

4.2.2. INVOLVEMENT OF OXIDATIVE STRESS IN GL1A EFFECTS ON CELL VIABILITY

Cannabinoids commonly induce cancer cell death by increasing oxidative stress[54,119,187]. To determine whether increased oxidative stress was necessary for the GL1a-induced reduction in viability, DU145 and PC-3 cells were pre-treated with the antioxidant NAC (10mM) for 1h prior to 72h GL1a treatment. The MTT assay was used to measure viability. The antioxidant alone had no significant effect on cell viability. There was no difference in viability between cells pre-treated with NAC and those treated with GL1a alone in either cell line (**Figure 4.7**). Neutralising oxidative stress using an antioxidant did not block the effect of GL1a, suggesting that the GL1a-induced reduction in viability does not require increased oxidative stress.

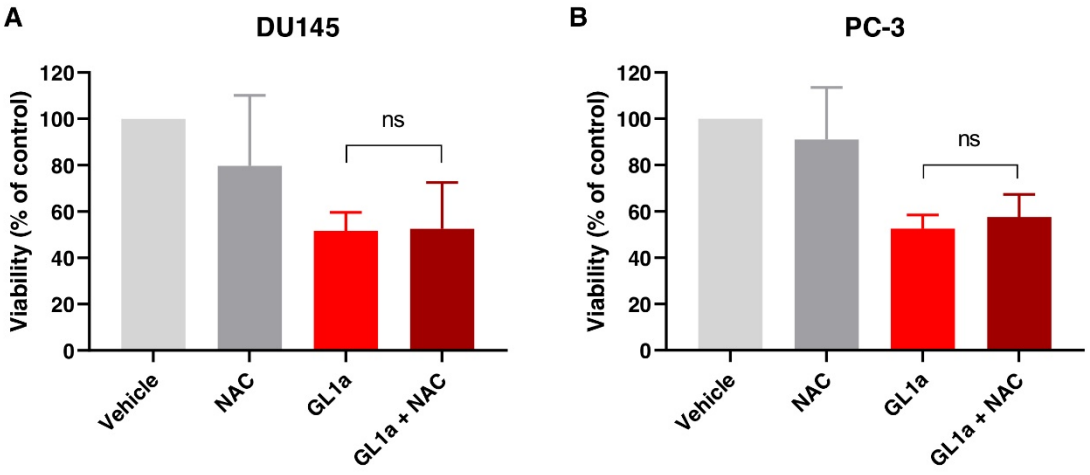


Figure 4.7. The effect of GL1a on cell viability is not blocked by an antioxidant. Prostate cancer cells were pre-treated with the antioxidant N-acetylcysteine (NAC) (10mM) for 1h before treatment with an IC_{50} dose of GL1a for 72h. Cell viability was measured using the MTT assay. (A) NAC did not alter the effect of GL1a on viability of DU145 cells. (B) NAC did not alter the effect of GL1a on viability of PC-3 cells (1-way ANOVA, $*p<0.05$). Results are representative of 3 biological replicates and 3 technical replicates.

4.2.3. ROLE OF AUTOPHAGY IN GL1A EFFECTS ON CELL VIABILITY

To investigate whether GL1a reduces cell viability through increased induction of autophagy, cells were pre-treated with the autophagy inhibitor chloroquine (10μM) for 1h prior to 72h GL1a treatment. Cell viability was measured using the MTT assay. No significant difference in the GL1a-induced reduction in viability was observed when cells were pre-treated with chloroquine (**Figure 4.8**). Moreover, in the presence of chloroquine, the anti-viability effect of GL1a was maintained, with a significantly greater reduction in viability observed with chloroquine + GL1a treatment compared to with chloroquine alone. However, treatment with chloroquine alone significantly reduced the viability of both cell lines, which suggests that autophagy may play a protective role in these cells. The results indicate that GL1a does not reduce prostate cancer cell viability through increased autophagy.

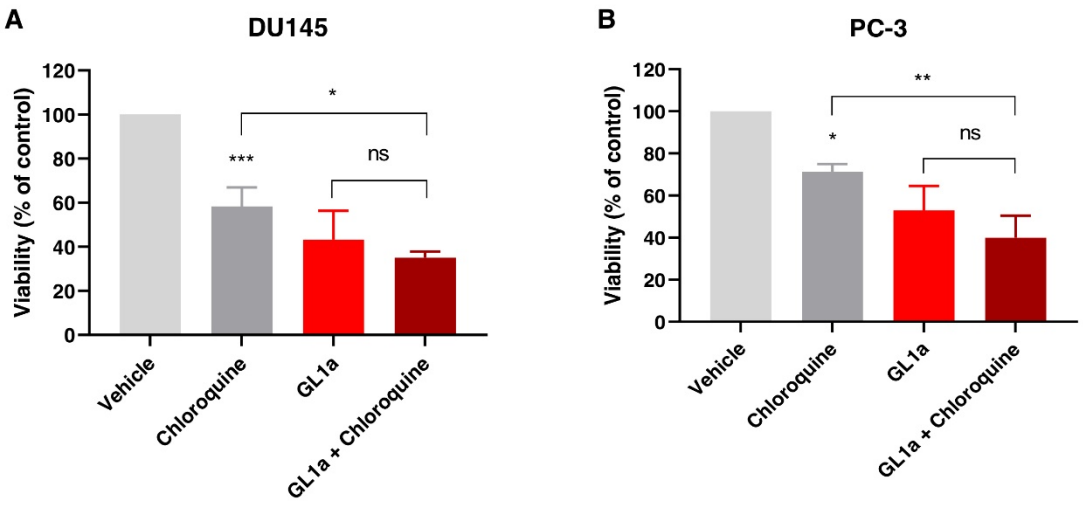


Figure 4.8. The effect of GL1a on cell viability is not blocked by an autophagy inhibitor. Prostate cancer cells were pre-treated with the autophagy inhibitor chloroquine (10μM) for 1h before treatment with an IC_{50} dose of GL1a for 72h. Cell viability was measured using the MTT assay. (A) Chloroquine alone significantly reduced DU145 cell viability. Chloroquine did not alter the effect of GL1a on viability of DU145 cells. (B) Chloroquine alone significantly reduced PC-3 cell viability. Chloroquine did not alter the effect of GL1a on viability of PC-3 cells (1-way ANOVA, *p<0.05 ***p<0.001 compared to the vehicle control). Results are representative of 3 biological replicates and 3 technical replicates.

4.2.4. GL1A MODULATES THE EXPRESSION OF CELL CYCLE PROTEINS

Based on our findings from **Chapter 3, Section 3.2.9**, showing that GL1a reduced prostate cancer cell proliferation, we decided to investigate whether this occurred through modulation of the expression of proteins that drive cell cycle progression. Western blot was used to measure the effect of GL1a on the expression of proteins involved in both the G1/S phase and G2/M phase transitions of the cell cycle (**Figure 4.9**).

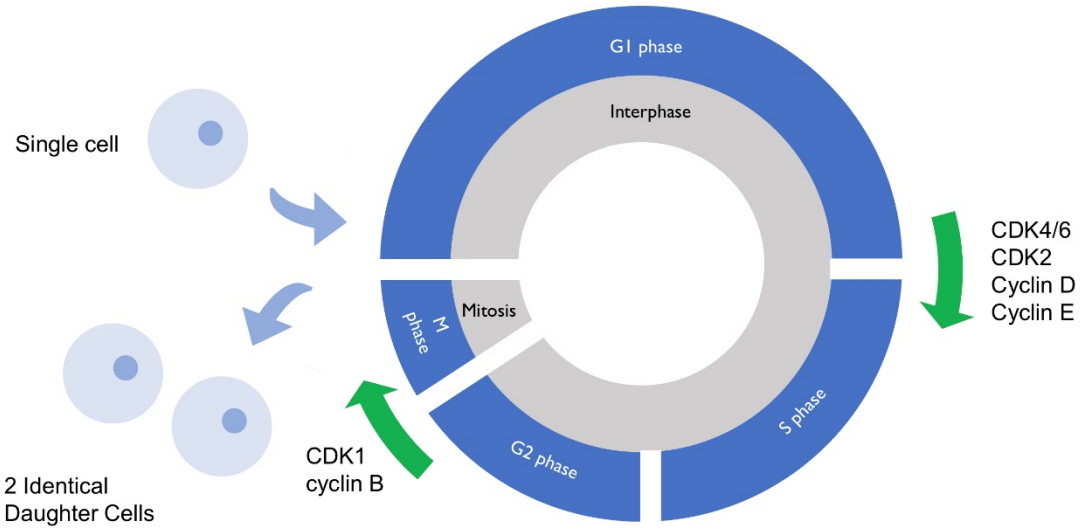
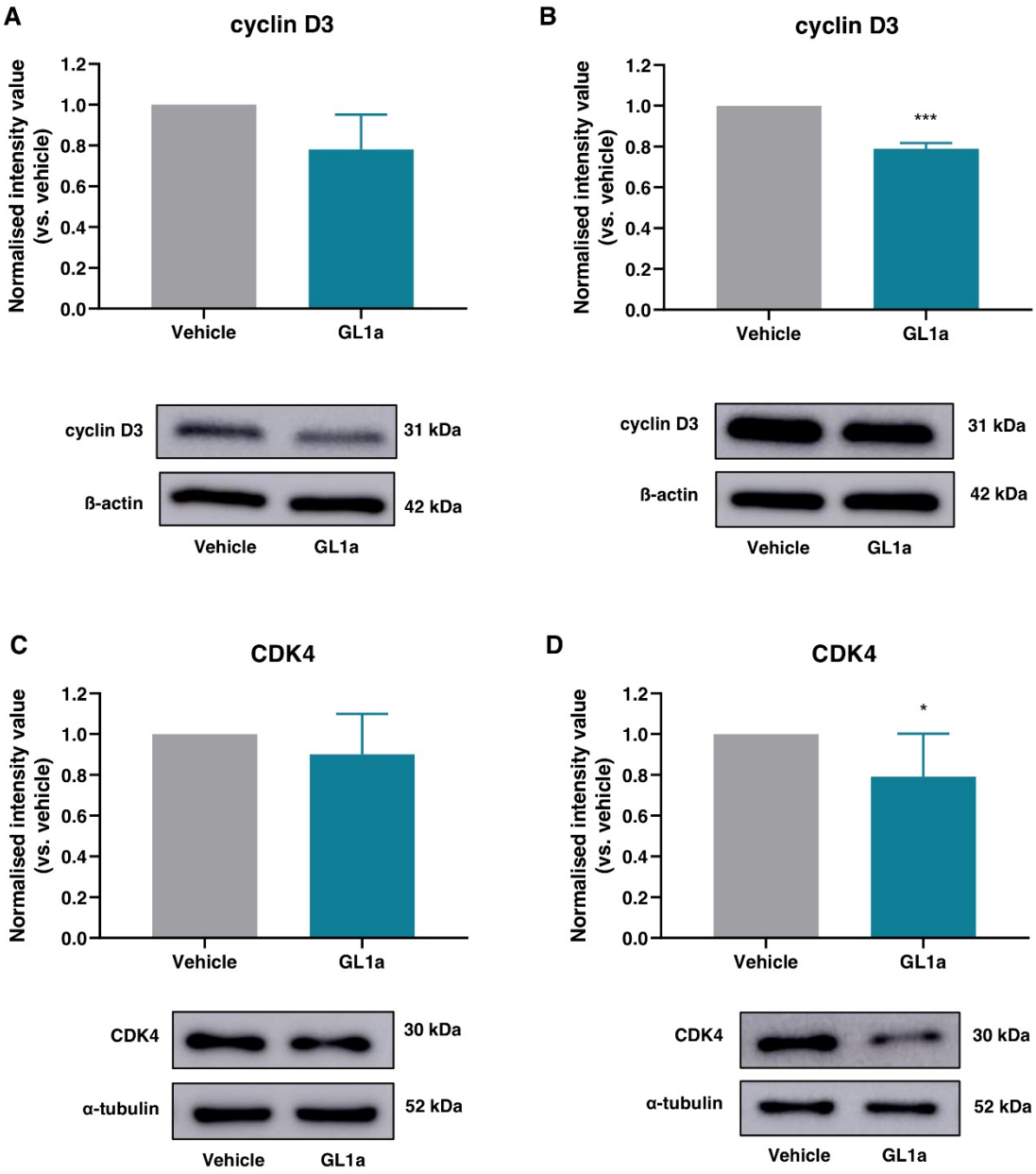


Figure 4.9. Proteins involved in cell cycle regulation. CDK4/6, CDK2, cyclin D, and cyclin E drive the G1/S phase transition. CDK1 and cyclin B drive the G2/M phase transition.

4.2.4.1. GL1A REDUCES EXPRESSION OF G1/S PHASE CELL CYCLE PROTEINS IN PROSTATE CANCER CELLS

To determine whether GL1a induces G1/S phase cell cycle arrest in prostate cancer cells, the protein expression of G1/S phase cell cycle proteins was measured using Western blot. The cells were treated with vehicle (DMSO) alone or with an IC_{50} dose of GL1a (12.3μM for DU145 cells, 10.5μM for PC-3 cells) for 48h. GL1a significantly reduced the expression of cyclin D3 in PC-3 cells (p=0.0002) (**Figure 4.10**). In DU145 cells, GL1a reduced cyclin D3 expression by approximately 20%, though this was not statistically significant. In DU145 cells, GL1a significantly reduced the expression of the cyclin-dependent kinases CDK2 (p=0.049) and CDK4 (p=0.04). Additionally, GL1a significantly reduced CDK2 expression in PC-3 cells (p=0.04). GL1a had no significant effect on the expression of cyclin E1 in either cell line. These results suggest that GL1a may induce G1/S phase cell cycle arrest in prostate cancer cells by reducing the expression of key proteins that drive the G1/S phase transition.


A
cyclin D3
Normalised intensity value (vs. vehicle)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
GL1a
cyclin D3
31 kDa
ß-actin
42 kDa
Vehicle
GL1a
B
cyclin D3
Normalised intensity value (vs. vehicle)
***
Vehicle
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cyclin D3
31 kDa
ß-actin
42 kDa
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CDK4
Normalised intensity value (vs. vehicle)
Vehicle
GL1a
CDK4
30 kDa
α-tubulin
52 kDa
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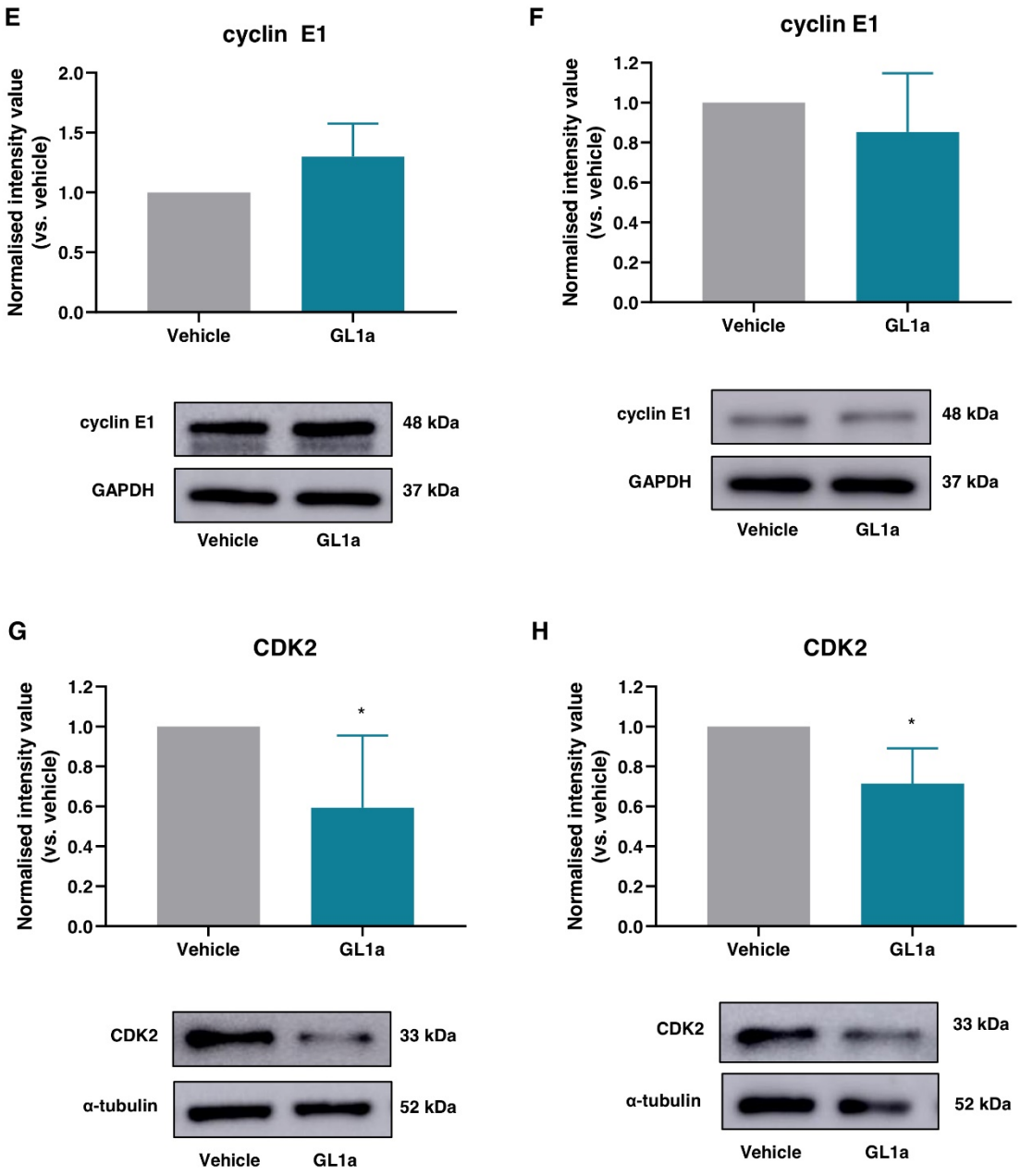


Figure 4.10. GL1a alters the expression of G1/S phase cell cycle proteins in prostate cancer cells. DU145 and PC-3 cells were treated with vehicle (DMSO) or an IC_{50} dose of GL1a (12.3μM for DU145, 10.5μM for PC-3) for 48h. Protein expression was measured using Western blot. (A) GL1a had no significant effect on cyclin D3 expression in DU145 cells. (B) GL1a significantly reduced cyclin D3 expression in PC-3 cells (Student's *t*-test, ***p<0.001). (C) GL1a significantly reduced CDK4 expression in DU145 cells (Student's *t*-test, *p<0.05). (D) GL1a had no significant effect on CDK4 expression in PC-3 cells. (E, F) GL1a had no significant effect on cyclin E1 expression in DU145 or PC-3 cells. (G, H) GL1a significantly reduced CDK2 expression in DU145 and PC-3 cells (Student's *t*-test, *p<0.05). Results are representative of at least 3 biological replicates.

4.2.4.2. GL1A REDUCES EXPRESSION OF G2/M PHASE CELL CYCLE PROTEINS IN PROSTATE CANCER CELLS

To assess whether GL1a induced cell cycle arrest at the G2/M checkpoint, Western blot was used to measure the expression of proteins that drive the G2/M phase transition. DU145 and PC-3 cells were treated with an IC_{50} dose of GL1a for 48h. GL1a significantly reduced CDK1 expression in DU145 ($p<0.0001$) and PC-3 ($p=0.02$) cells, with a particularly strong effect in DU145 cells (**Figure 4.11**). GL1a had no significant effect on CDK7 expression in either cell line. These results provide evidence that GL1a induces cell cycle arrest through downregulation of CDK1.

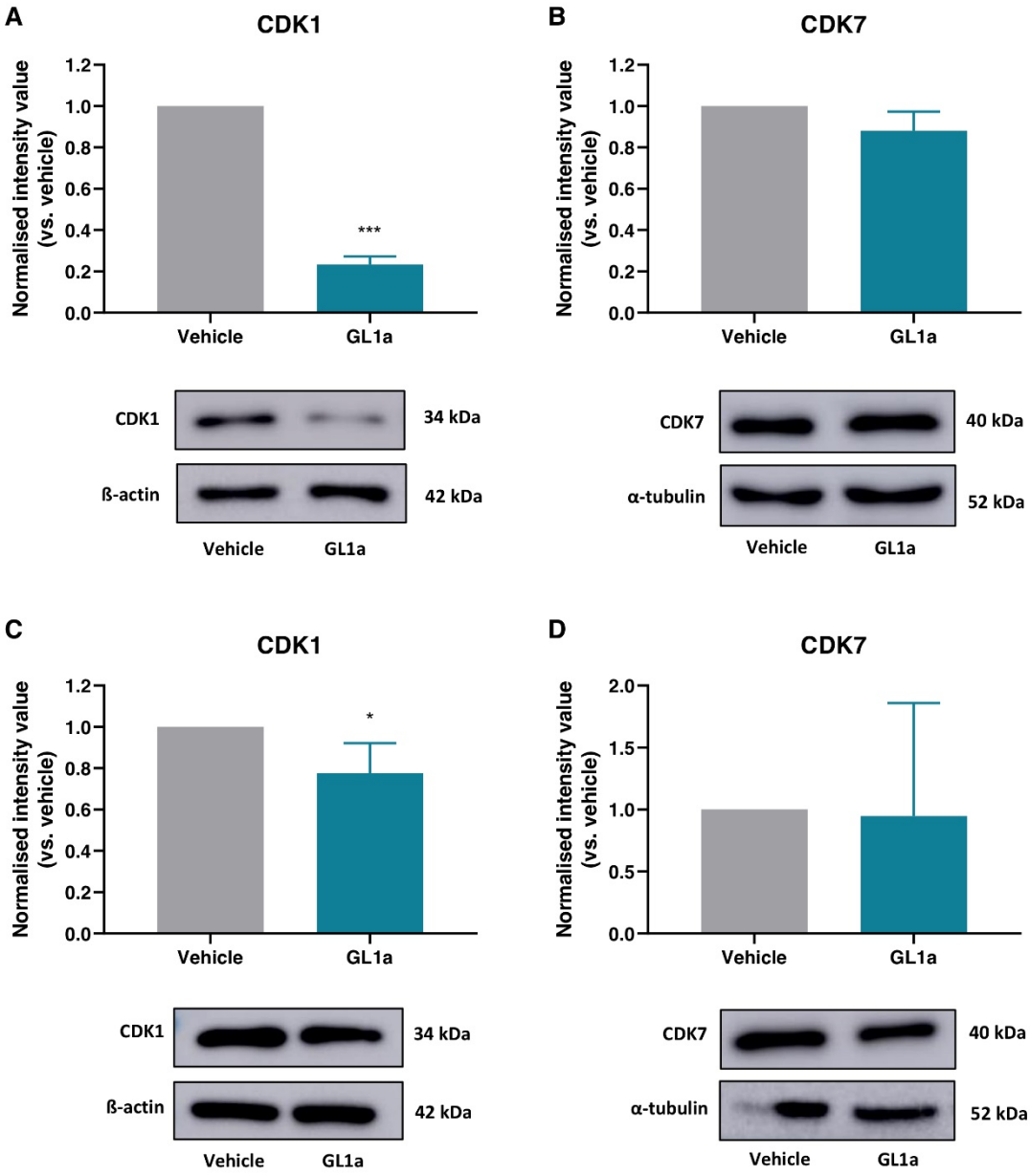


Figure 4.11. GL1a alters the expression of G2/M phase cell cycle proteins in prostate cancer cells. DU145 and PC-3 cells were treated with vehicle (DMSO) or an IC_{50} dose of GL1a (12.3μM for DU145, 10.5μM for PC-3) for 48h. Protein expression was measured using Western blot. (A) GL1a significantly reduced CDK1 expression in DU145 cells (Student's *t*-test, ***p<0.001). (B) GL1a had no significant effect on CDK7 expression in DU145 cells. (C) GL1a significantly reduced CDK1 expression in PC-3 cells (Student's *t*-test, *p<0.05). (D) GL1a had no significant effect on CDK7 expression in PC-3 cells. Results are representative of at least 3 biological replicates.

4.2.5. GL1A REDUCES AKT PHOSPHORYLATION IN DU145 CELLS

Western blot was used to investigate whether the GL1a-induced downregulation of cell cycle proteins occurred through reduced phosphorylation and activation of the protein kinases AKT and ERK. DU145 and PC-3 cells were treated with an IC_{50} dose of GL1a for 48h. GL1a reduced AKT phosphorylation by approximately 40% in DU145 cells (p=0.0006) (**Figure 4.12**). GL1a had no significant effect on AKT phosphorylation in PC-3 cells, though a high degree of variability was observed between biological replicates, making the results difficult to interpret. GL1a had no significant effect on ERK phosphorylation in DU145 cells. No phosphorylated ERK was detected in PC-3 cells. Together, these results suggest that inhibition of AKT phosphorylation by GL1a in DU145 cells may contribute to the observed reduction in the expression of cell cycle proteins.

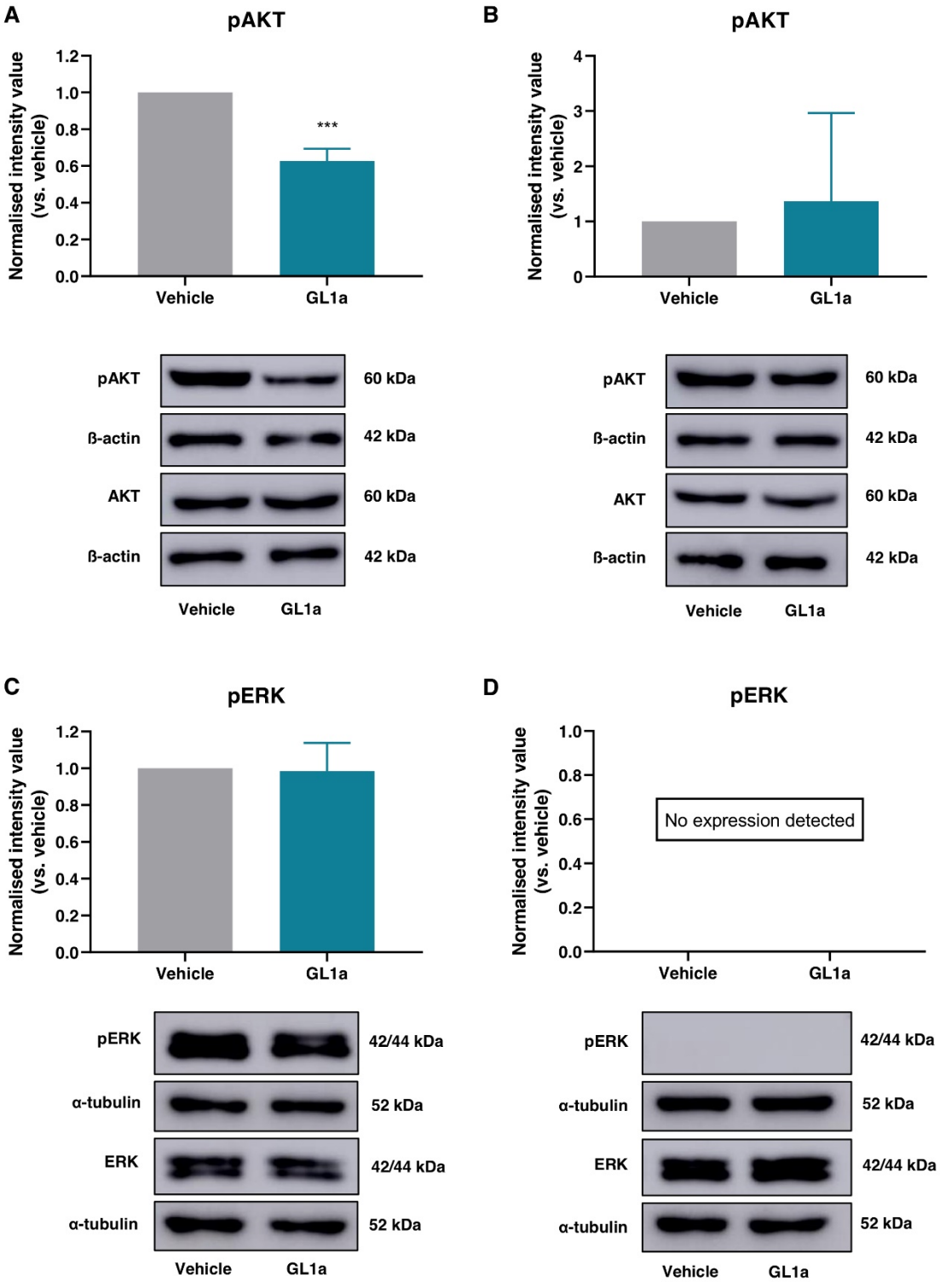


Figure 4.12. GL1a alters protein phosphorylation in prostate cancer cells. DU145 and PC-3 cells were treated with vehicle (DMSO) or an IC_{50} dose of GL1a (12.3μM for DU145, 10.5μM for PC-3) for 48h. Protein expression was measured using Western blot. (A) GL1a significantly reduced AKT phosphorylation in DU145 cells (Student's *t*-test, ***$p<0.001$). (B) GL1a had no significant effect on AKT phosphorylation in PC-3 cells. (C) GL1a had no significant effect on ERK phosphorylation in DU145 cells. (D) No phosphorylated ERK was detected in PC-3 cells. Results are representative of at least 3 biological replicates.

4.2.6. EFFECT OF GL1A ON EXPRESSION OF CELL INVASION PROTEINS

To investigate the mechanisms underlying the observed GL1a-induced reduction in cell invasion, Western blot was used to measure the expression of the invasion-associated proteins E-cadherin and vimentin. E-cadherin is associated with the non-invasive epithelial phenotype. Vimentin is associated with the invasive mesenchymal phenotype. PC-3 cells were treated with a non-toxic dose of GL1a (1.5μM) for 48h, in the absence of serum. An approximately 2-fold increase in E-cadherin expression was observed following GL1a treatment, though this did not reach statistical significance due to the high degree of variability between biological replicates (**Figure 4.13**). GL1a had no significant effect on the expression of vimentin. The high variability makes it difficult to draw a definitive conclusion from this experiment. However, these findings suggest that the GL1a-induced reduction in PC-3 invasiveness may be associated with increased expression of E-cadherin.

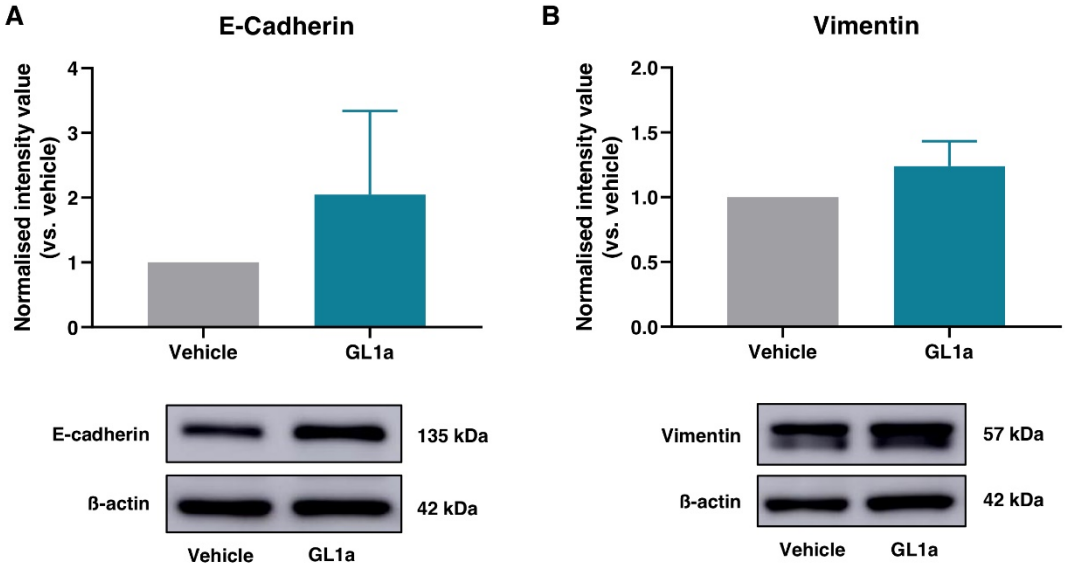


Figure 4.13. GL1a does not significantly alter expression of cell invasion proteins. PC-3 cells were treated with vehicle (DMSO) or a non-toxic dose of GL1a (1.5μM) for 48h, in the absence of serum. Protein expression was measured using Western blot. (A) GL1a had no significant effect on E-cadherin expression. (B) GL1a had no significant effect on vimentin expression (Student's *t*-test, **p*<0.05). Results are representative of at least 3 biological replicates.

4.2.7. EFFECT OF GL1A ON SECRETION OF MATRIX METALLOPROTEASES

To determine whether GL1a reduced cell invasion by inhibiting the secretion of matrix metalloproteases (MMPs), an ELISA-based assay was used to measure the levels of various MMPs in cell culture supernatants from PC-3 cells treated with a non-toxic dose of GL1a for 48h. GL1a did not significantly alter the concentrations of MMP-1, MMP-3, or MMP-9 in the supernatant (**Figure 4.14**), suggesting that GL1a does not inhibit PC-3 cell invasion through reduced secretion of these MMPs.

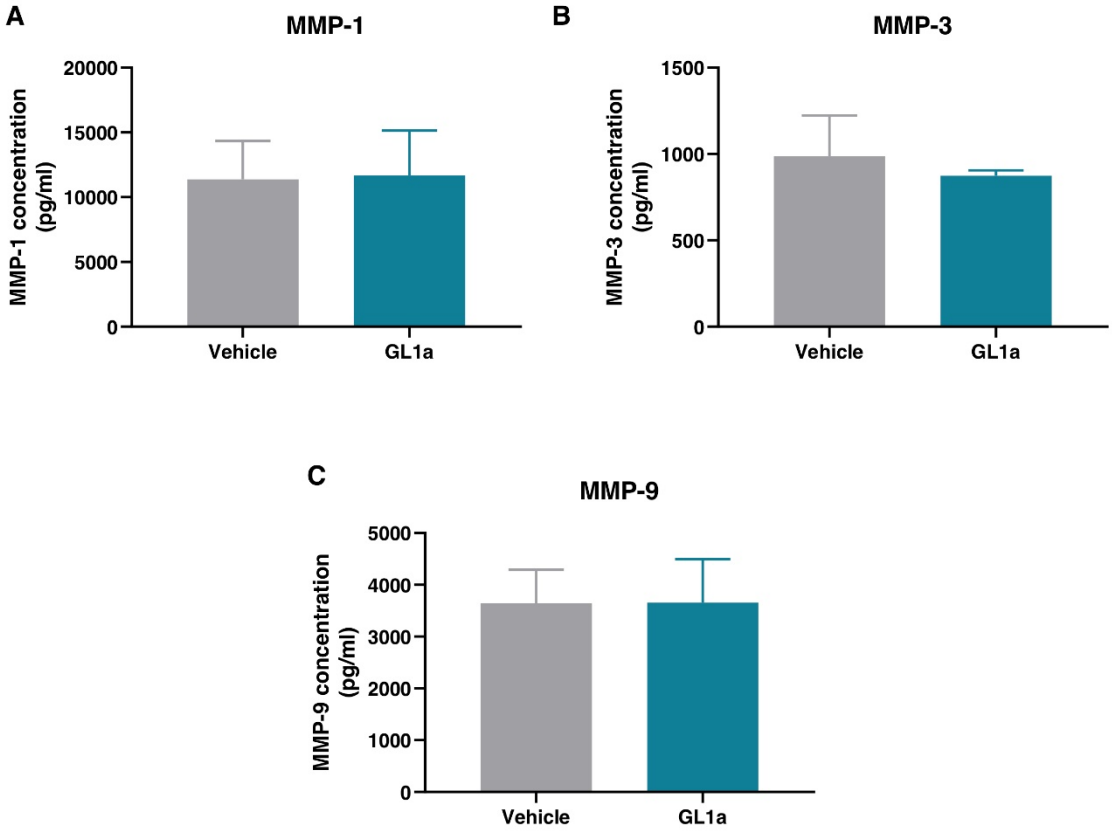


Figure 4.14. GL1a does not alter the secretion of matrix metalloproteases MMP-1, MMP-3, or MMP-9. PC-3 cells were treated with vehicle (DMSO) or a non-toxic dose of GL1a (1.5μM), in the absence of serum. After 48h treatment, supernatants were collected and MMP levels were measured using the Human MMP 3-Plex Ultra-Sensitive ELISA kit. (A) GL1a did not alter the secretion of MMP-1. (B) GL1a did not alter the secretion of MMP-3. (C) GL1a did not alter the secretion of MMP-9 (Student's *t*-test, **p*<0.01). Results are representative of 3 biological replicates and 3 technical replicates.

4.3. DISCUSSION

While cannabinoid mechanisms have been extensively studied in certain cancer types, for example, glioblastoma, the mechanisms driving the phenotypic effects of cannabinoids in prostate cancer are not well understood. The aim of this chapter was to investigate the mechanisms of action underlying the observed phenotypic effects of GL1a in prostate cancer cells. Specifically, we aimed to identify the receptor targets of GL1a in prostate cancer cells, investigate the involvement of oxidative stress and autophagy in GL1a-induced cytotoxicity, and determine the effects of GL1a on the expression of proteins that drive cancer cell proliferation and invasion.

One of the major challenges in elucidating cannabinoid mechanisms of action is identifying the receptor targets mediating cannabinoid phenotypic effects. Cannabinoids play roles in many physiological processes and can modulate the activity of a wide range of receptors and targets. Common targets of cannabinoids in cancer cells include the major cannabinoid receptors, CB_1 and CB_2, the transient receptor potential ion channels TRPV1 and TRPV2, and the putative novel cannabinoid receptor GPR55. All the above targets have previously been demonstrated to be present in prostate cancer cells[134,155,225,261]. However, these observations have not been independently verified by others. We were able to detect expression of each of these targets at either the mRNA or protein level in the prostate cell lines. However, blocking the interaction of GL1a with the above targets using selective antagonists or agonists did not alter the effect of GL1a on prostate cancer cell viability, indicating that the GL1a-induced reduction in viability does not require increased activation of cannabinoid receptors or TRPV ion channels, or antagonism of GPR55. This supports previous findings that antagonists of CB_1, CB_2, or TRPV1 did not attenuate the effects of CBD on cell viability or caspase activity in prostate cancer cells under serum-deprived conditions[155]. However, cannabinoids can modulate the activity of cannabinoid receptors through mechanisms other than direct activation or antagonism. For example, CBD can act as a negative allosteric modulator of CB_1, altering receptor conformation and ligand-binding activity[148,149]. Some studies report that CBD acts as an inverse agonist at CB_2, while others identify CBD as a partial CB_2 agonist[147,149]**.** Knocking down these targets using siRNA could provide an insight into whether GL1a is indirectly modulating the activity of these receptors. Alternatively, GL1a may reduce viability of prostate cancer cells primarily by interacting with one of the many other cannabinoid targets. Previous studies have shown that cannabinoids can alter cancer-related processes through interactions with the transcription factor PPARγ, the TRP channels TRPM8 and TRPA1,

serotonin receptors, and steroid receptors, among many other targets[139,142,154,190]. Measuring the expression levels of various known cannabinoid targets in each prostate cancer cell line may provide an insight into the likely targets of GL1a in these cells. Together, the results indicate that GL1a reduces prostate cancer cell viability independently of cannabinoid receptor activation, TRPV channel activation, or GPR55 antagonism. While various antagonist doses were tested in this study, including doses close to the K_i values for these antagonists at their respective targets, and doses used previously in the literature, we cannot definitively confirm that these receptors were antagonised by the antagonists at the doses used. Future studies should incorporate selective agonists of these targets to confirm successful receptor blockade and truly assess whether the effects on viability occur independently of these targets.

Apart from modulation of cannabinoid receptors, one of the most frequently reported methods by which cannabinoids inhibit cell viability is through stimulation of ROS production, leading to increased oxidative stress. Cannabinoids reduce cancer cell viability through increased ROS production in several cancer types[54,119,189]. In gastric cancer cells, CBD stimulated ROS production leading to increased apoptosis[54]. In glioma and breast cancer cells, CBD increased ROS production, and the effects of CBD on ROS production, cell viability, and apoptosis were blocked by co-treatment with an antioxidant[119,189]**.** In the current study, pre- and co-treatment of DU145 and PC-3 cells with an antioxidant did not alter the effect of GL1a on cell viability, indicating that, under these conditions, GL1a-induced reduction in prostate cancer cell viability is not dependent on increased oxidative stress. Our results agree with a previous prostate cancer study reporting that CBD enhances ROS production in LNCaP, but not in DU145 or PC-3 cells[155]. In that study, CBD treatment reduced the expression of the AR in LNCaP cells. Some evidence indicates that AR modulation increases ROS production[262-264], which may explain why the same pro-oxidant effects are not seen in the androgen-independent cell lines.

Altered autophagy signalling is a common feature of cancer cells. The role of autophagy in cancer is complex, and depending on the context, autophagy can either promote cancer cell survival or induce cancer cell death[265,266]. Autophagy can protect cancer cells by counteracting the metabolic stress induced by nutrient deprivation or hypoxia[265]. However, autophagy can also contribute to cell death in combination with other death-promoting signals[265]. Several previous studies report that the psychoactive phytocannabinoid THC induces cancer cell death by stimulating increased autophagy[175,182,192,193]. Some evidence also indicates that CBD can induce

autophagy and modulate the cross-talk between apoptosis and autophagy[172,175,189-191]. In the current study, inhibition of autophagy did not alter the effect of GL1a on prostate cancer cell viability, suggesting that GL1a does not induce prostate cancer cell death through increased autophagy. However, treatment of the cells with the autophagy inhibitor alone did reduce cell viability, suggesting that autophagy may play a protective role in prostate cancer cells. This contrasts with a study in HNSCC showing that autophagy inhibition by chloroquine attenuated the effects of CBD on cancer cell viability, indicating that CBD promotes cell death through autophagy in HNSCC[175]. To investigate this further, it would be useful to measure the effects of GL1a on specific markers of autophagy, such as LC3 or beclin, to determine whether GL1a alters autophagy signalling in these cells. However, the above results indicate that GL1a does not reduce prostate cancer cell viability through increased autophagy.

Inhibition of cell cycle progression leads to reduced proliferation of cancer cells. Having shown in Chapter 3 that GL1a reduces the proliferation of prostate cancer cells, Western blot analysis was conducted to determine whether this was accompanied by altered expression of cell cycle proteins. Increased cell proliferation in cancer is associated with increased activity of cyclins and CDKs which drive cell cycle progression[267]. Previous studies show that cannabinoids reduce the expression of cyclins and CDKs in cancer cells. In multiple myeloma and pancreatic cancer, for example, inhibition of cell proliferation by CBD was accompanied by reduced expression of cyclin D[50,53]. In gastric cancer, CBD inhibited the formation of the CDK2/cyclin E complex which drives cell cycle progression[54]. Here, we show that GL1a reduces the expression of CDK2, CDK4, and cyclin D3, key proteins that drive the G1/S phase cell cycle transition. Our results complement previous findings that CBD increases the expression of the CDK inhibitors p21 and $p27^{kip}$ in prostate cancer cells[155]. Inhibition of the G1/S phase transition is a useful therapeutic strategy in cancer, and several inhibitors of G1/S phase CDKs have been approved for cancer treatment[268]. Additionally, GL1a reduced the expression of CDK1, a key regulator of cell cycle progression primarily involved in the G2/M phase transition. THC has been previously shown to inhibit breast cancer cell proliferation by reducing the expression of CDK1 (16818634). One recent study showed that a synthetic CBD analogue inhibited CDK1 mRNA expression in a model of cardiac fibrosis[269]. However, to our knowledge, this study provides the first evidence that non-intoxicating cannabinoids reduce CDK1 expression in cancer. CDK1 is considered the master cell cycle regulator which, when activated, facilitates the initiation of mitosis[267]. CDK1, unlike other CDKs, is essential for driving cell cycle progression in mammalian

cells[270]. Notably, CDK1 inhibition of non-cancerous cells can reduce their survival, suggesting that CDK1 inhibition may produce toxic effects in immortalised non-cancerous cells[271]. This may explain the observed cytotoxic effects of GL1a in non-cancerous PWR-1E and RWPE-1 prostate cells. Due to toxicity, CDK1 inhibitors have not yet been FDA approved for cancer treatment. However, several *in vivo* studies report that CDK1 inhibition reduces tumour growth in mice without adverse effects[272,273]. Furthermore, some evidence suggests that CDK1 inhibition may be a particularly effective therapeutic approach in specific cancer types[274]. Together, these results indicate that GL1a inhibits prostate cancer cell proliferation by downregulating proteins that drive cell cycle progression through both the G1/S and G2/M checkpoints. The above results provide clear evidence that GL1a modulates the expression of cell cycle proteins in prostate cancer cells, supporting our findings that GL1a inhibits prostate cancer cell proliferation.

Modulation of cell cycle regulators in cancer commonly occurs through downregulation of the ERK and AKT signalling pathways[275,276]. Phosphorylation and activation of the protein kinase AKT promotes cancer cell proliferation, survival, and invasiveness[276-278]. AKT hyperphosphorylation is a common feature of prostate cancer, with increased AKT activity observed in 50% of prostate cancers[48]. Phosphorylation of ERK also plays a major role in cell cycle regulation in cancer[275]. Previous studies show that CBD reduces ERK and AKT phosphorylation in glioma, multiple myeloma, leukaemia, pancreatic cancer, and breast cancer, accompanied by reduced cell proliferation, migration, and invasion, and increased apoptosis[50-53,119,185]. Here, we show that GL1a reduces AKT phosphorylation in DU145 cells, which likely contributes to the reduced expression of cell cycle proteins, reduced cell proliferation, and reduced viability observed in these cells. No change in AKT phosphorylation was observed in GL1a-treated PC-3 cells, though the high degree of variability between biological replicates makes the results difficult to interpret. These results are surprising, given that DU145 cells express the AKT pathway inhibitor PTEN, and thus have lower baseline AKT activity than PC-3 cells[279,280]. However, some previous research has shown that DU145 cells can be more sensitive than PC-3 cells to treatment with AKT inhibitors[281]. No change in ERK phosphorylation was observed in DU145 cells following GL1a treatment, and phosphorylated ERK was not detected in PC-3 cells. To our knowledge, no previous studies have investigated the effects of cannabinoids on ERK and AKT phosphorylation in prostate cancer. Our results suggest that, in DU145 cells, GL1a may reduce cell viability by inhibiting the phosphorylation and activation of AKT, leading to

downregulation of the cell cycle proteins CDK1, CDK2, and CDK4, induction of cell cycle arrest, and inhibition of cell proliferation.

Apart from reducing cell proliferation, we showed in Chapter 3 that GL1a inhibits PC-3 cell invasion. Our next aim was to investigate the mechanisms underlying the observed anti-invasive effect. Previous studies have shown that cannabinoids reduce cell invasion in several cancer types, including breast cancer, lung cancer, glioblastoma, and HNSCC[52,175,199,200,203,204]. In breast cancer, inhibition of cell invasion by CBD was accompanied by reduced secretion of the matrix metalloproteases MMP-2 and MMP-9[52]. Another study reported that CBD reverted the invasive mesenchymal phenotype of breast cancer cells, accompanied by increased expression of the adherens junction protein E-cadherin, which is associated with a non-invasive epithelial phenotype[197]. In lung cancer cells, CBD-induced inhibition of the epithelial-mesenchymal transition was accompanied by reduced expression of the mesenchymal marker vimentin[174]. Here, we found no change in the secretion of MMP-1, MMP-3, or MMP-9 by PC-3 cells following GL1a treatment. GL1a induced a two-fold increase in E-cadherin expression (not-significant), suggesting that GL1a may promote a non-invasive epithelial phenotype in prostate cancer cells. No effect on vimentin expression was observed. Additionally, it is possible that GL1a alters the secretion of other matrix metalloproteases, or the expression of other proteins involved in cell invasion. For example, several studies in other cancer types show that CBD reduces cell invasion by inhibiting the pro-metastatic protein Id-1[178,200,282]. Id-1 is over-expressed in prostate cancer[283,284] and promotes cell invasion[285]**,** and as such may be a useful target to investigate in future studies. Furthermore, assessing whether the effects of cannabinoids on cell invasion are blocked by cannabinoid receptor antagonists or agonists may provide a deeper understanding of the anti-invasive mechanisms of GL1a in prostate cancer cells. Our findings suggest that GL1a may inhibit PC-3 cell invasiveness by increasing the expression of E-cadherin, though further research is needed to confirm this. Overall, GL1a reduces the invasiveness of highly metastatic prostate cancer cells, though the underlying mechanisms require further investigation.

Together, our results indicate that GL1a reduces cell proliferation by reducing the expression of proteins that drive cell cycle progression, including CDK2, CDK4, cyclin D3, and CDK1. GL1a may reduce the expression of these proteins by inhibiting the phosphorylation and activation of the protein kinase AKT. GL1a also reduces the invasiveness of PC-3 cells, possibly involving increased expression of the epithelial marker E-cadherin. The effects of GL1a in prostate cancer cells appear to occur

independently of cannabinoid receptors, TRP ion channels, GPR55 antagonism, increased autophagy, or increased oxidative stress. Further investigation is needed to identify the receptor targets mediating the effects of GL1a in prostate cancer cells.

CHAPTER 5
Chemopreventive potential of cannabinoids in prostate cancer

5.1. RATIONALE

The usefulness of chemotherapy alone as an anti-cancer strategy is limited by treatment-associated toxicity and the development of drug resistance[93]. Chemoprevention refers to the use of agents to reduce or delay the development or progression of cancer. The World Health Organisation estimates that 30-50% cancers may be preventable[94]. Therefore, identifying agents that can inhibit carcinogenesis and cancer progression has great potential to reduce cancer incidence and mortality.

Oxidative stress is a major driving force in the development of many cancers, including prostate cancer[21]. As such, antioxidants like selenium, vitamin E, lycopene, and sulforaphane have been investigated as chemopreventive agents in prostate cancer, with some promising results[25,109,286,287] (**see Chapter 1, Section 1.1.9**). However, no chemopreventive agents for prostate cancer have yet been granted FDA approval. Some evidence in non-cancer models suggests that, while cannabinoids can increase oxidative stress at high doses, the opposite effect is observed at low doses, with cannabinoids producing antioxidant and cytoprotective effects[288]. However, very few studies have investigated whether cannabinoids have antioxidant or chemopreventive properties in cancer. Determining whether low-dose cannabinoids display antioxidant activity in prostate cells may provide an insight into their chemopreventive potential.

Hydrogen peroxide (H_2O_2) is a reactive oxygen species commonly used to induce oxidative stress *in vitro*. The ability of a drug compound to protect cells from H_2O_2-induced oxidative damage can indicate chemopreventive potential[289,290]. The aim of this chapter was to assess the antioxidant and cytoprotective effects of cannabinoids against H_2O_2 damage in non-cancerous prostate cells.

This was done by:

- Identifying low doses of cannabinoids most likely to have cytoprotective effects
- Investigating the ability of cannabinoids to protect non-cancerous cells against H_2O_2-induced cytotoxicity
- Investigating the ability of cannabinoids to protect non-cancerous cells against H_2O_2-induced oxidative stress

5.2. RESULTS

5.2.1. EFFECT OF LOW-DOSE CANNABINOIDS ON VIABILITY OF NON-CANCEROUS PROSTATE CELLS

To determine the optimal cannabinoid concentrations for investigation of antioxidant and chemopreventive effects, non-cancerous PWR-1E cells were treated with various low doses of GL1a, GL1b, and GL4a (0-2000nM). After 72h treatment, cell viability was measured using the MTT assay. GL1a and GL1b caused no significant reduction in PWR-1E cell viability at doses in the nanomolar range (1-way ANOVA, *p<0.05) (**Figure 5.1**). GL4a caused some reduction in viability even at low doses, with a significant reduction observed at a dose of 31.3nM (p=0.008). As GL1a and GL1b appeared to produce less cytotoxicity, these compounds were selected for further investigation.

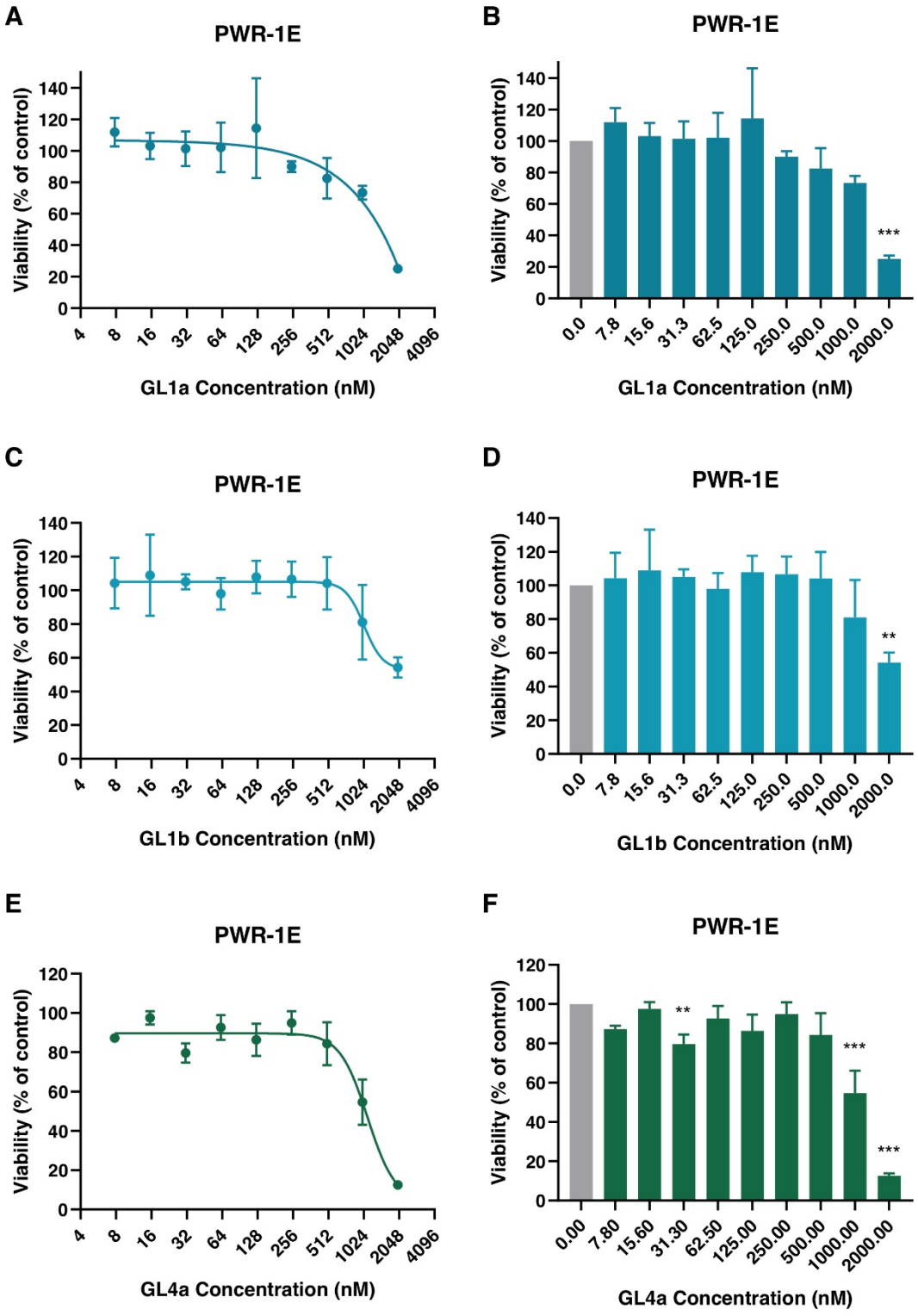


Figure 5.1. Effect of low-dose cannabinoids on PWR-1E cell viability. PWR-1E cells were treated with low doses of cannabinoids (0-2000nM) for 72h. Cell viability was measured using the MTT assay. (A) Dose-response curve for inhibition of PWR-1E cell viability by GL1a. (B) GL1a caused no significant reduction in PWR-1E cell viability at doses up to 1000nM (1-way ANOVA, *p<0.05). (C) Dose-response curve for inhibition of PWR-1E cell viability by GL1b. (D) GL1b caused no significant reduction in PWR-1E cell viability at doses up to 1000nM (1-way ANOVA,

*p<0.05). (E) Dose-response curve for inhibition of PWR-1E cell viability by GL4a. (F) GL4a significantly reduced cell viability at a dose of 31.3nM, as well as at doses ≥1000nM (1-way ANOVA, **p<0.01 ***p<0.001). Data are reported as the mean ± SD calculated from 3 independent experiments.

5.2.2. IDENTIFICATION OF CYTOTOXIC DOSES OF HYDROGEN PEROXIDE IN PROSTATE CELLS

To identify cytotoxic doses of H_2O_2 in PWR-1E cells, cells were treated with various doses (0-2000µM) for 24h. To replicate the final experimental conditions, cells were treated 48h after seeding. Cell viability was measured using the MTT assay. H_2O_2 significantly reduced cell viability at doses ≥15.6µM. The IC_{50} and IC_{25} doses for inhibition of cell viability by H_2O_2 were 138.2µM and 74.5µM, respectively (**Figure 5.2**). These doses were used to induce cytotoxicity and oxidative stress in the subsequent experiments.

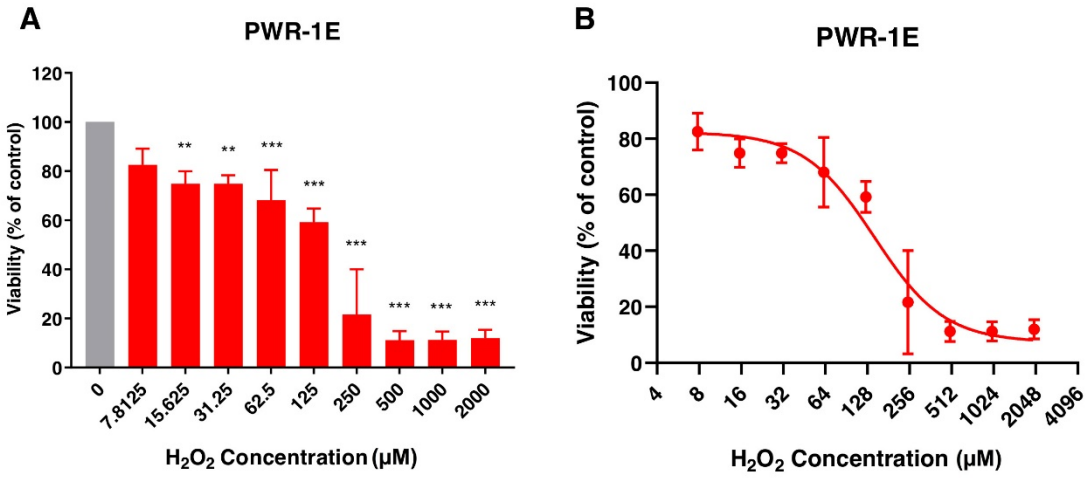


Figure 5.2. Inhibition of PWR-1E cell viability by hydrogen peroxide. PWR-1E cells were seeded in triplicate. After 48h, cells were treated with various doses (0-2000µM) of hydrogen peroxide (H_2O_2) for 24h. Cell viability was measured using the MTT assay. (A) H_2O_2 doses ≥15.6µM significantly reduced cell viability (1-way ANOVA, **p<0.01 ***p<0.001). (B) The IC_{50} and IC_{25} doses for inhibition of PWR-1E cell viability were 138.2µM and 74.5µM, respectively. Data are reported as the mean ± SD calculated from 3 independent experiments.

5.2.3. PROTECTIVE EFFECTS OF GL1B AGAINST HYDROGEN PEROXIDE-INDUCED CYTOTOXICITY

To assess the cytoprotective potential of GL1b, cells were pre-treated with various low doses of GL1b (125nM, 250nM, 500nM) for 24h in the absence of serum, before a further 24h co-treatment with an IC_{50} dose of H_2O_2 (138.2μM) (**Figure 5.3**). Cell viability was measured using the MTT assay. No difference in viability was observed between cells pre-treated with GL1b and those treated with H_2O_2 alone (**Figure 5.4**).

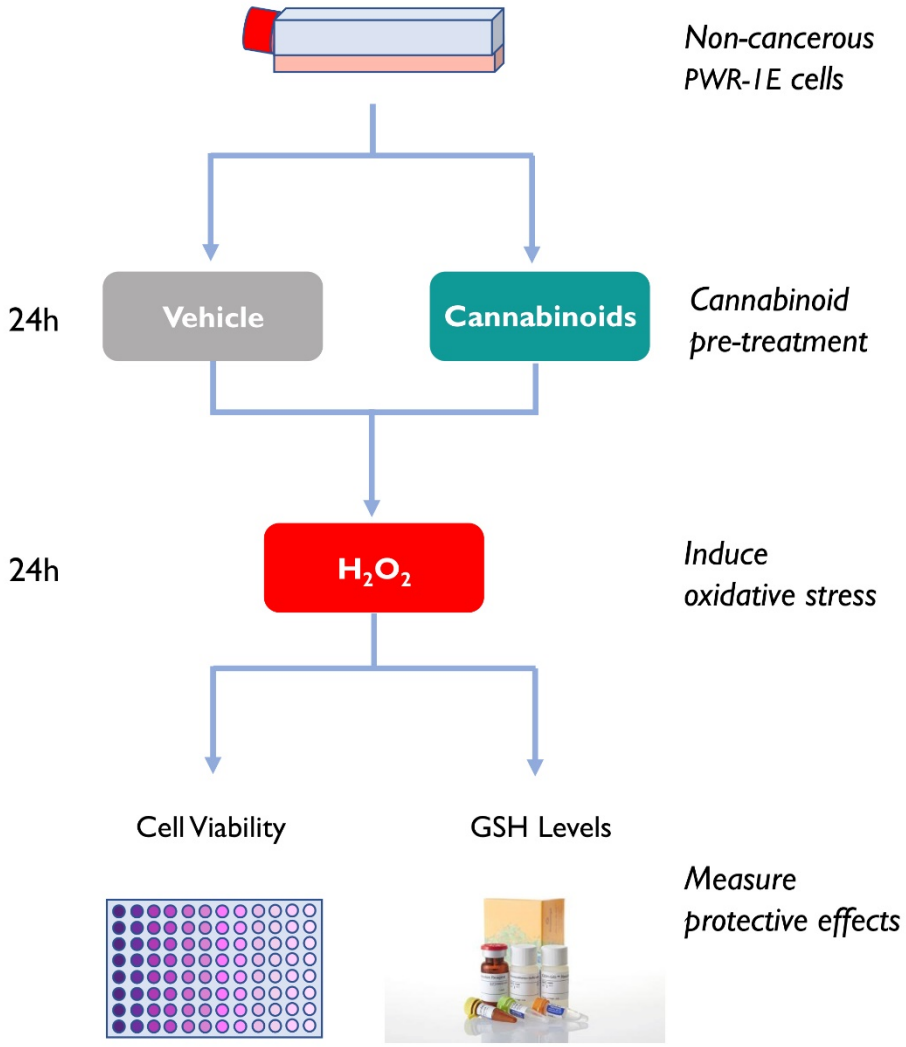


Figure 5.3. Experimental approach used to assess cytoprotective and antioxidant effects of cannabinoids against H_2O_2-induced oxidative stress.

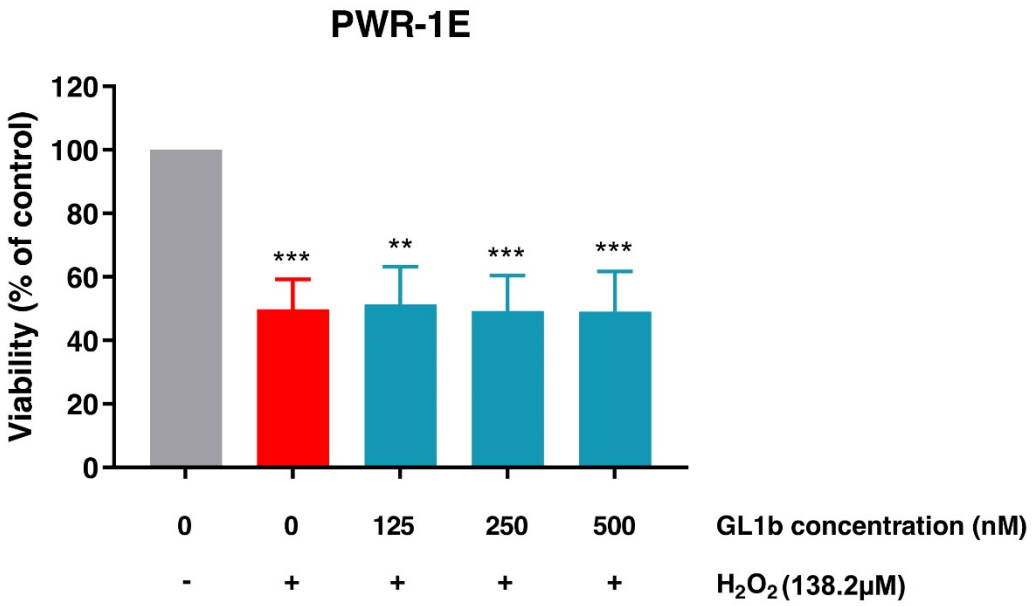


Figure 5.4. Effect of GL1b pre-treatment on H_2O_2-induced inhibition of cell viability. PWR-1E cells were seeded in triplicate. After 24h, cells were treated with low doses of GL1b (0-500nM) for 24h, before the addition of an IC_{50} dose of H_2O_2 (138.2μM) for a further 24h. Cell viability was measured using the MTT assay. GL1b did not significantly protect PWR-1E cells against cytotoxicity induced by an IC_{50} dose of H_2O_2 (1-way ANOVA, $*p<0.05$). Data are reported as the mean ± SD calculated from 3 independent experiments.

5.2.4. PROTECTIVE EFFECTS OF GL1A AGAINST HYDROGEN PEROXIDE-INDUCED CYTOTOXICITY

To assess the protective potential of GL1a against H_2O_2-induced cytotoxicity, PWR-1E cells were pre-treated with a range of low doses of GL1a (0-2000nM) for 24h, prior to 24h co-treatment with an IC_{50} dose (138.2μM) of H_2O_2. Cell viability was measured using the MTT assay. While no significant protective effect was observed when cells were pre-treated with GL1a (1-way ANOVA, $*p<0.05$), there appeared to be a trend towards increasing viability in GL1a pre-treated cells at doses up to 500nM, though this did not reach statistical significance (1-way ANOVA test for linear trend, $p=0.08$). Cells pre-treated with 500nM GL1a showed viability approximately 20% higher than those treated with H_2O_2 alone (**Figure 5.5**). We considered whether the IC_{50} dose of H_2O_2 used was too high and produced an excessive oxidative stress response, too strong to be counteracted by GL1a treatment. To assess this, we repeated the above experiment, but pre-treated the cells with an IC_{25} dose of H_2O_2. At this dose, H_2O_2 reduced viability by

approximately 15%, though this was not statistically significant. However, H_2O_2 produced no reduction in viability in cells pre-treated with GL1a at doses up to 500nM, and cell viability in GL1a pre-treated cells was similar to the viability of vehicle-treated cells (no H_2O_2). In both cases, a GL1a dose of 500nM appeared to display the greatest cytoprotective potential.

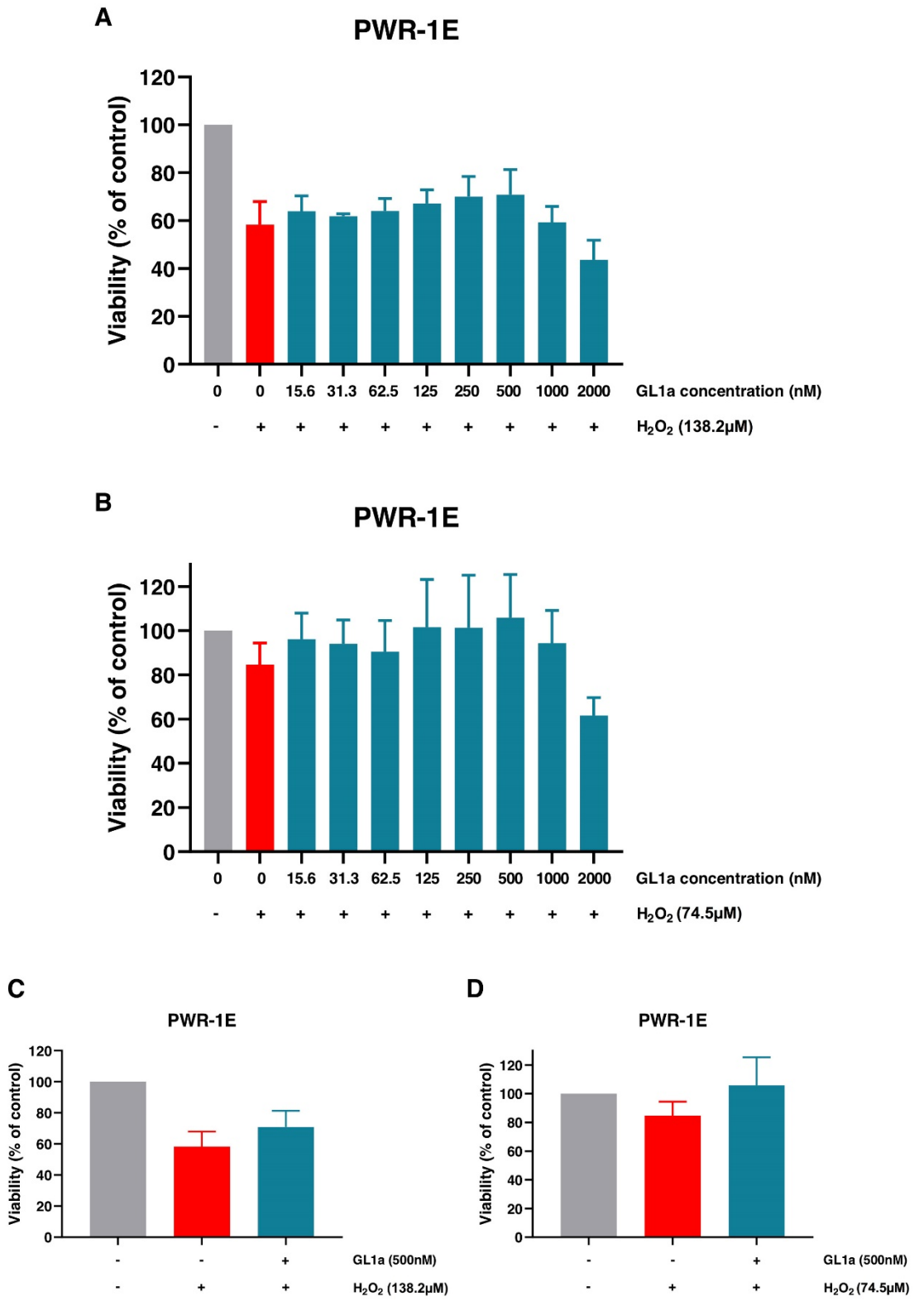


Figure 5.5. Effect of GL1a pre-treatment on H_2O_2-induced inhibition of cell viability. PWR-1E cells were seeded in triplicate. After 24h, cells were treated with low doses of GL1a (0-2000nM) for 24h, before the addition of H_2O_2 for a further 24h. Cell viability was measured using the MTT assay. (A) GL1a did not significantly protect PWR-1E cells against cytotoxicity induced by an IC_{50} dose of H_2O_2 (1-way ANOVA, *p<0.05). (B) GL1a did not significantly protect against

cytotoxicity induced by an IC_{25} dose of H_2O_2 (1-way ANOVA, *p<0.05). (C, D) The GL1a doses producing the greatest cytoprotective effect are highlighted. Data are reported as the mean ± SD calculated from 3 independent experiments.

5.2.5. PROTECTIVE EFFECTS OF SULFORAPHANE AGAINST HYDROGEN PEROXIDE-INDUCED CYTOTOXICITY

To determine the suitability of this model for assessing the antioxidant and chemopreventive potential of a drug compound, the above experiment was repeated using sulforaphane (SFN), in place of GL1a. SFN is a known antioxidant which has been investigated in clinical trials for chemoprevention in prostate cancer[25,26,291,292]. PWR-1E cells were treated for 24h with SFN (0-50μM) before 24h treatment with an IC_{50} or IC_{25} dose of H_2O_2. The SFN dose range was selected based on doses used previously in the literature[293,294]. Cell viability was measured using the MTT assay. The results observed with this known antioxidant were similar to those observed with GL1a, with some evidence of a small increase in viability with SFN pre-treatment up to a dose of 1.56μM, but no significant cytoprotective effect observed at any of the doses tested (**Figure 5.6**). This suggests that to determine the chemopreventive potential of these compounds, drug exposure times and treatment doses may require further optimisation.

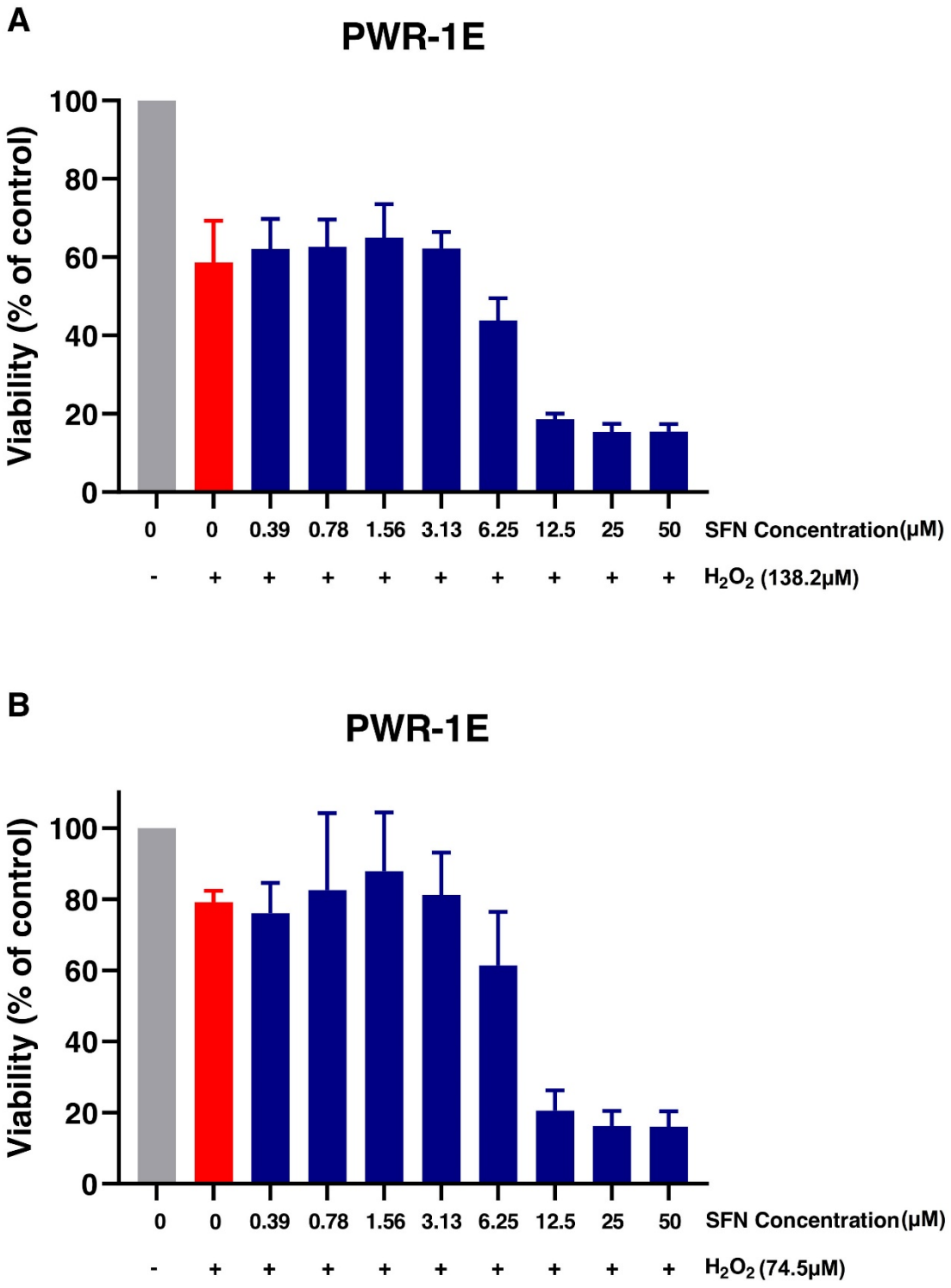


Figure 5.6. Effect of sulforaphane pre-treatment on H_2O_2-induced inhibition of cell viability. PWR-1E cells were seeded in triplicate. After 24h, cells were treated with various doses of sulforaphane (SFN) (0-50μM) for 24h, before the addition of H_2O_2 for a further 24h. Cell viability was measured using the MTT assay. (A) SFN did not significantly protect PWR-1E cells against cytotoxicity induced by an IC_{50} dose of H_2O_2 (1-way ANOVA, *p<0.05). (B) SFN did not significantly protect against cytotoxicity induced by an IC_{25} dose of H_2O_2 (1-way ANOVA, *p<0.05). Data are reported as the mean ± SD calculated from 3 independent experiments.

5.2.6. INVESTIGATION OF PROTECTIVE EFFECTS OF GL1B AGAINST HYDROGEN PEROXIDE-INDUCED OXIDATIVE STRESS

To further assess the chemopreventive potential of cannabinoids, protective effects against H_2O_2-induced oxidative stress were measured using the GSH-Glo assay. The GSH-Glo assay is used to measure glutathione (GSH) concentration, which is reduced under oxidative stress conditions. PWR-1E cells were treated with GL1b (500nM) for 24h, before 24h treatment with an IC_{50} dose of H_2O_2 (138.2μM). The H_2O_2 alone, at this dose and timepoint, produced no change in GSH concentration, indicating no increase in oxidative stress (**Figure 5.7**). We considered that the H_2O_2 treatment time may have been too long, and that the cells had recovered from the oxidative stress by the experiment endpoint. Optimisation of H_2O_2 antioxidant doses and treatment times by other members of our group revealed that a shorter 20 min H_2O_2 treatment produced the greatest reduction in GSH concentration (**Appendix Figure A7.6,Figure A7.7**).

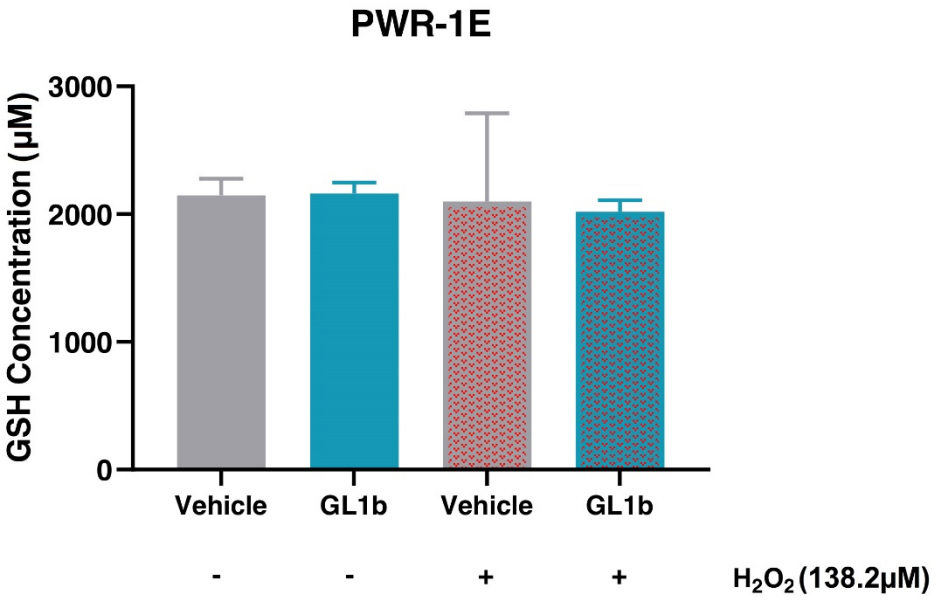


Figure 5.7. Effect of GL1b pre-treatment on H_2O_2-induced oxidative stress. PWR-1E cells were seeded in triplicate. After 24h, cells were treated with vehicle (DMSO) or 500nM GL1b for 24h, before the addition of an IC_{50} dose of H_2O_2 (138.2μM) for a further 24h. Oxidative stress was measured using the GSH-Glo assay. At the dose and timepoint tested, H_2O_2 did not significantly reduce the GSH concentration. GL1b produced no significant antioxidant effect, in the presence or absence of H_2O_2. (1-way ANOVA, *p<0.05). Data are reported as the mean ± SD calculated from 3 independent experiments.

5.2.7. INVESTIGATION OF PROTECTIVE EFFECTS OF GL1A AGAINST HYDROGEN PEROXIDE-INDUCED OXIDATIVE STRESS

The GSH-Glo assay experiments were repeated with GL1a using a shorter 20 min H_2O_2 treatment time (**Figure 5.8**). The antioxidant N-acetyl cysteine (NAC) was included as a positive control, using treatment conditions optimised previously by other members of our group (**Appendix Figure A7.8**). Cells were pre-treated with GL1a or NAC for 40 min, prior to 20 min treatment with H_2O_2 (138.2µM). However, no significant reduction in GSH concentration was observed following H_2O_2 treatment (**Figure 5.9**). Furthermore, while NAC slightly increased the GSH concentration, this effect was not statistically significant, due to large variability between the replicates (1-Way ANOVA, $*p<0.05$). Further optimisation of GL1a and H_2O_2 doses and treatment times may provide a greater insight into whether GL1a has any antioxidant or chemopreventive potential in prostate cancer.

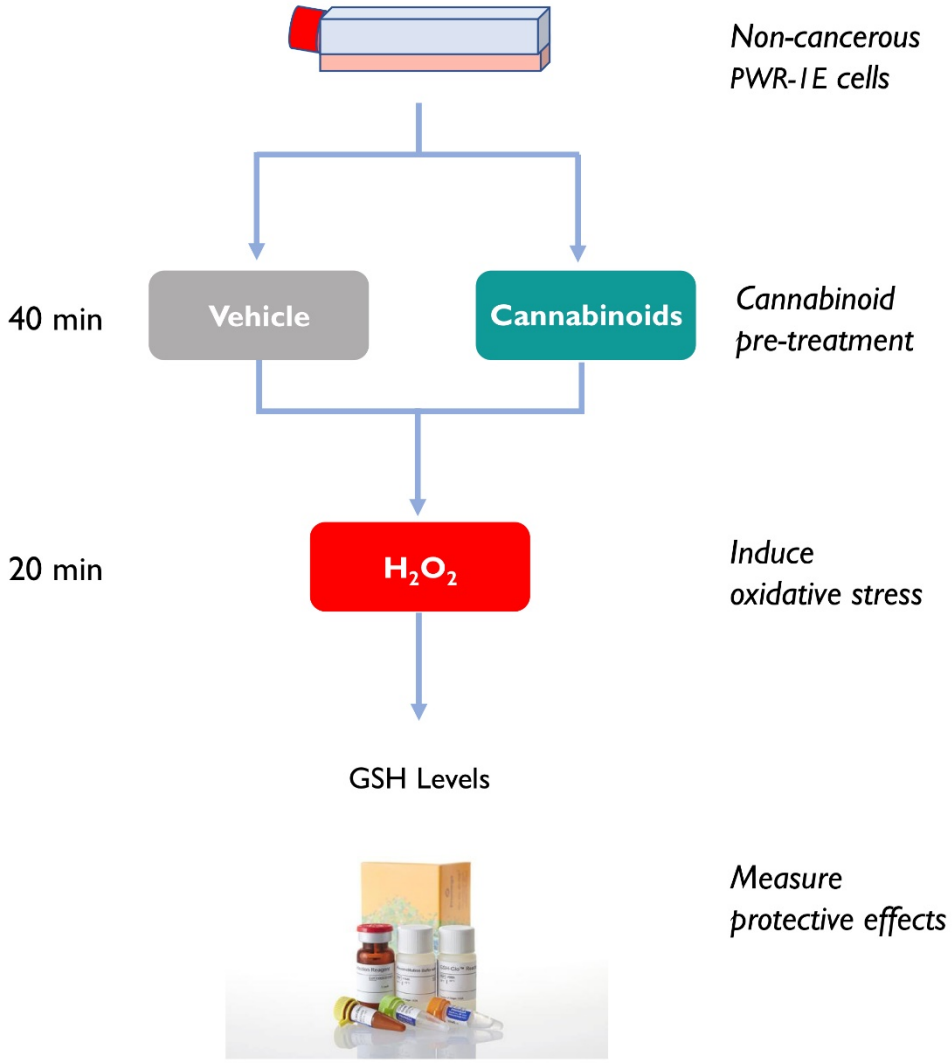


Figure 5.8. Revised experimental approach used to assess antioxidant effects of GL1a against H_2O_2-induced oxidative stress.

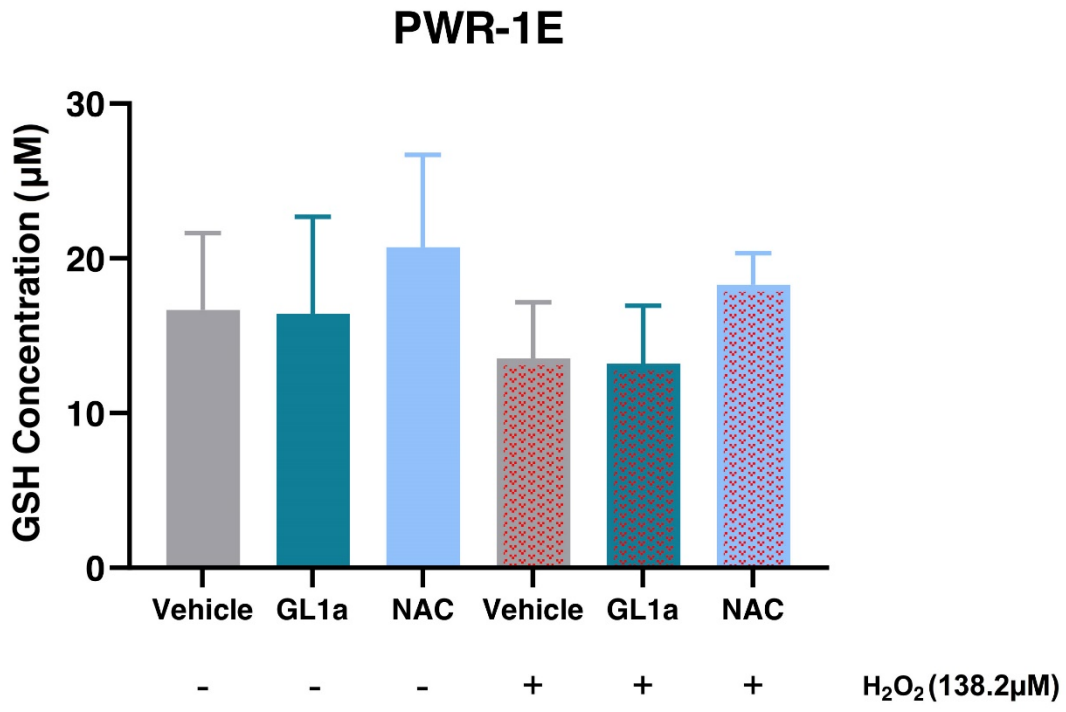


Figure 5.9. Effect of GL1a pre-treatment on H_2O_2-induced oxidative stress. PWR-1E cells were treated with vehicle (DMSO), GL1a (500nM), or NAC (10mM) for 40 min before treatment with an IC_{50} dose of H_2O_2 (138.2µM) for 20 min. GSH concentration was measured as a marker of oxidative stress using the GSH-Glo assay. NAC was included as a positive control. H_2O_2 did not significantly reduce the GSH concentration at the dose tested. GL1a did not produce a significant antioxidant effect, in the presence or absence of H_2O_2. NAC slightly increased the GSH concentration, though this was not statistically significant (1-way ANOVA, $*p<0.05$). Data are reported as the mean ± SD calculated from 3 independent experiments.

5.3. DISCUSSION

The aim of this chapter was to investigate the chemopreventive potential of plant-derived cannabinoids in prostate cancer. As oxidative stress is one of the major drivers of prostate cancer development and progression[21], we determined whether cannabinoids could protect against oxidative damage in non-cancerous prostate cells. Specifically, we assessed whether low doses of cannabinoids could protect PWR-1E cells from H_2O_2-induced cytotoxicity and oxidative stress.

Several plant-derived compounds show potential as chemopreventive agents, including sulforaphane, found in cruciferous vegetables, curcumin, found in turmeric, and resveratrol, found in grapes and berries[103,105,109,295]. These compounds have

strong antioxidant properties and may inhibit carcinogenesis by reducing oxidative stress[104,105].

Oxidative stress is one of the major causes of cancer development and progression. Oxidative stress occurs when an imbalance exists between the production of ROS and the body's ability to neutralise the effects of those reactive species or repair the resulting oxidative damage. Production of ROS at high levels causes damage to DNA, proteins, and lipids, and plays a role in the initiation, promotion, and progression stages of carcinogenesis[22]. In prostate cancer, increased oxidative stress is associated with cancer development, progression, and response to therapy[21]. Importantly, substantial preclinical evidence suggests that antioxidants can reduce oxidative stress in prostate cancer and may inhibit prostate carcinogenesis, though clinical trials have thus far yielded mixed outcomes[286,296,297].

While cannabinoids can increase oxidative stress in cancer cells by stimulating ROS production leading to increased apoptosis, substantial evidence indicates that, at lower doses (typically up to 10μM) and in certain cell types, CBD can protect cells from oxidative stress[298-302]. The antioxidant or pro-oxidant activities of CBD may be dependent on the dose used, as effects of CBD on other cellular parameters such as cell proliferation and cell death appear be dose-dependent in certain models[299,303,304]. Research in neurons shows that CBD increases oxidative stress and neurotoxicity at high doses, while producing antioxidant and neuroprotective effects at low doses[288]. To identify potential chemopreventive doses of cannabinoids, non-cancerous PWR-1E cells were treated with various low doses of cannabinoids and cell viability was measured. At doses in the nanomolar range, GL1a and GL1b caused no reduction in cell viability after 72h. As GL4a produced some cytotoxicity, even at low doses, GL1a and GL1b were selected for further investigation.

To determine the cytoprotective potential of phytocannabinoids, PWR-1E cells were pre-treated with various low doses of cannabinoids before inducing oxidative stress using H_2O_2. The MTT assay was used to measure the cytoprotective effects of cannabinoid pre-treatment against H_2O_2-induced oxidative damage, as described previously by Liu *et al.* [289,290]. At the doses tested, pre-treatment with GL1b did not alter the effects of H_2O_2 on PWR-1E cell viability. As the effects of cannabinoids are often highly dose-dependent, we considered that testing a wider range of cannabinoid doses could identify a more effective protective dose. When investigating the cytoprotective potential of GL1a, a wider range of doses were tested. While GL1a appeared to display some

cytoprotective potential at doses up to 500nM, the effects were not statistically significant. To assess the effectiveness of this experimental approach for determining chemopreventive potential, the above experiments were repeated using the antioxidant sulforaphane (SFN), which has been assessed in clinical trials as a chemopreventive agent for prostate cancer[25,26]. In the current study, while a small increase in cell viability was observed with SFN pre-treatment up to a certain dose, the effect was not statistically significant. Notably, the results obtained with GL1a pre-treatment were similar to those obtained with SFN pre-treatment, albeit at much lower doses for GL1a than SFN. Thus, the above findings do not necessarily rule out the potential use of these cannabinoids as chemopreventive agents, though assessing the cytoprotective potential of GL1a will require further optimisation of drug exposure times and treatment doses.

Studies of the chemopreventive potential of other plant-derived compounds have measured the ability of the compounds to protect cells from H_2O_2-induced oxidative stress[305-307]. Here, the antioxidant activity of GL1b was assessed by measuring levels of reduced glutathione (GSH) in cells exposed to H_2O_2, with or without cannabinoid pre-treatment. However, no reduction in GSH levels was detected in prostate cells after 24h H_2O_2 exposure. We considered that the long duration of the H_2O_2 treatment had allowed time for normal antioxidant defence mechanisms to counteract the increased oxidative stress before the experimental endpoint. Therefore, H_2O_2 treatment times were optimised by other members of our group, to identify the timepoint where the greatest effect on GSH levels was observed. The above antioxidant experiments were repeated with GL1a pre-treatment, using shorter treatment times for both GL1a and H_2O_2. The antioxidant N-acetyl cysteine (NAC) was included as a positive control. Although a small reduction in GSH concentration (~20%) was seen after H_2O_2 treatment, this was not statistically significant. Similarly, while GSH levels were higher in NAC-treated cells, the difference was not significant. Furthermore, no difference in GSH levels was detected when cells were treated with GL1a, with or without H_2O_2 insult. It is important to note that the above experiments are exploratory in nature, due to the lack of previous similar *in vitro* studies. Therefore, further optimisation of the experimental design is needed, particularly in relation to drug exposure time and treatment dose for both cannabinoids and H_2O_2.

Additionally, it would be useful to assess other markers of oxidative stress, such as ROS levels, catalase activity, or expression of inducible nitric oxide synthase (iNOS), which have previously been altered by cannabinoids in non-cancer disease models[22,300,301,308]. Furthermore, it would be interesting to measure the effect of

cannabinoids on the expression of Nrf2, a key regulator of antioxidant defences[22]. Previous studies have shown that CBD produces antioxidant effects by altering the expression of Nrf2 and its target genes[302,309].

We have shown in Chapter 1 and Chapter 2 that cannabinoids can produce some phenotypic and intracellular effects that are characteristic of chemopreventive agents, including inhibition of cell proliferation and invasiveness and downregulation of cyclin D expression and AKT phosphorylation[91]. Assessing whether cannabinoids can protect cells from oxidative stress-induced DNA damage could also provide an insight into their chemopreventive potential. Notably, Aviello *et al.* reported that CBD pre-treatment protected DNA from oxidative damage in colon cancer cells and, importantly, that this translated to inhibition of AOM-induced formation of aberrant crypt foci, polyps, and tumours by CBD *in vivo*[153], indicating that cannabinoids may have chemopreventive potential in cancer.

Another useful *in vitro* method for assessing chemopreventive potential is measuring the ability of a drug to protect against malignant transformation of healthy cells. For example, the cadmium-transformed prostate epithelial (CTPE) cell line has been developed from non-cancerous RWPE-1 cells and used to study the process of malignant transformation in prostate cancer[310-313]. Once cannabinoid treatment conditions are optimised, future studies should investigate whether chronic treatment with low-dose cannabinoids can protect non-cancerous prostate cells from malignant transformation. This will provide an insight into whether cytoprotective or antioxidant effects of cannabinoids translate to inhibition of carcinogenesis.

Together, our findings suggest that GL1a may have some cytoprotective activity against H_2O_2 toxicity in non-cancerous prostate cells. No significant antioxidant effects of cannabinoids were observed in PWR-1E cells. Further optimisation of cannabinoid doses and treatment times is needed to determine whether cannabinoids can produce antioxidant effects that may protect healthy prostate cells from malignant transformation.

CHAPTER 6
Discussion

In 2020, prostate cancer was responsible for an estimated 375,000 deaths worldwide[1]. Novel treatments, particularly for advanced prostate cancer, are urgently needed. While substantial preclinical evidence demonstrates anti-cancer properties of cannabinoids in many cancer types, few studies have investigated the chemotherapeutic or chemopreventive potential of cannabinoids in prostate cancer. Here, we investigated the phenotypic effects and mechanisms of action of the phytocannabinoids GL1a, GL1b, and GL4a in cell line models of prostate cancer. Data from our fluorescence microscopy and transwell assay experiments demonstrate that GL1a alters key hallmarks of cancer, specifically cell proliferation and cell invasion (**Figure 6.1**). GL1a inhibited prostate cancer cell proliferation by altering the expression of cell cycle proteins and reducing the phosphorylation and activation of AKT. Furthermore, GL1a reduced the invasiveness of highly metastatic PC-3 cells. Combining GL1a with GL4a in specific dose combinations produced enhanced effects on cell viability. However, GL1a also reduced viability and induced apoptosis in non-cancerous prostate cells, and these off-targets effects require further investigation. We found no significant antioxidant or chemopreventive effects of GL1a or GL1b in non-cancerous prostate cells, though the dosing and timing of the cannabinoid treatments require further optimisation. Most existing anti-cancer drugs are capable of altering multiple hallmarks of cancer. Therefore, the ability of GL1a to inhibit both the proliferation and invasion of prostate cancer cells supports its potential as a chemotherapeutic agent in prostate cancer. Overall, our results suggest that phytocannabinoids may have potential for the treatment of prostate cancer.

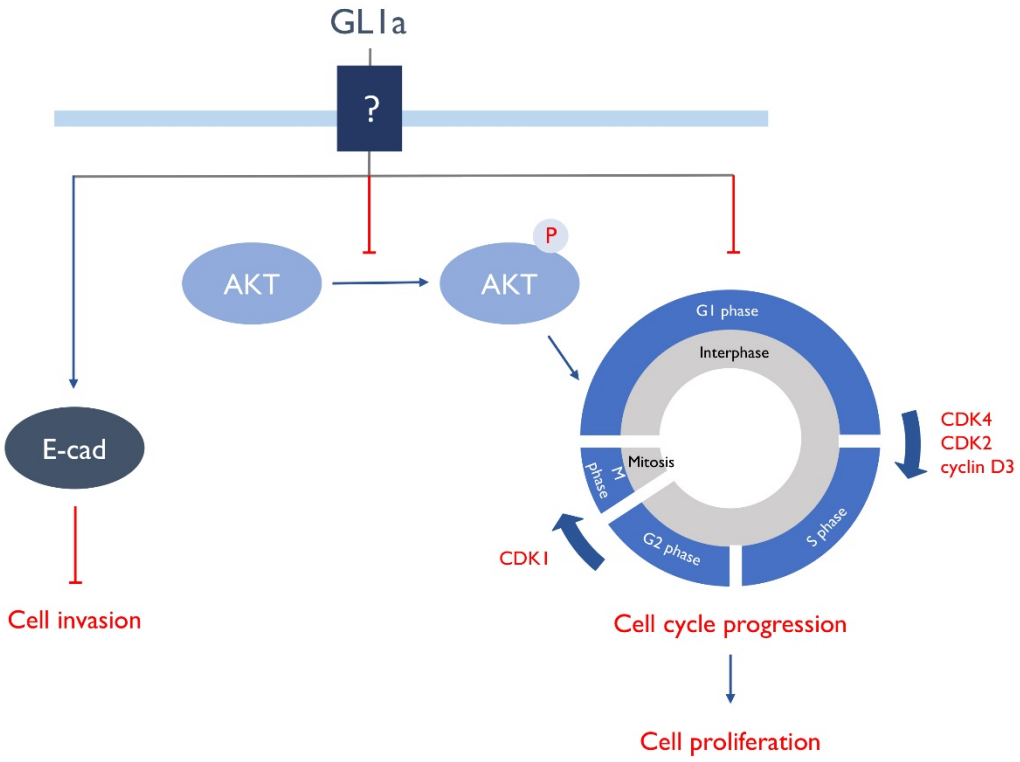


Figure 6.1. Proposed mechanisms underlying the phenotypic effects of GL1a in prostate cancer cells. GL1a inhibits AKT phosphorylation and reduces the expression of CDK4, CDK2, cyclin D3, and CDK1, leading to inhibition of cell cycle progression and cell proliferation. GL1a increases the expression of E-cadherin, leading to reduced cell invasion. The receptor target mediating the effects of GL1a in prostate cancer cells is unknown. Blue arrows indicate increased expression/activation. Red arrows and text indicate reduced expression/inhibition. E-cad – E-cadherin, CDK – cyclin dependent kinase.

6.1. ANTI-PROLIFERATIVE EFFECTS OF CANNABINOIDS IN PROSTATE CANCER

Uncontrolled cell proliferation is the most fundamental hallmark of cancer. One of the key findings of this study was that GL1a inhibits the proliferation of prostate cancer cells. First, we determined that GL1a inhibited the viability of both androgen-dependent and androgen-independent prostate cancer cell lines. Microscopy analysis revealed that the reduced viability was associated with inhibition of cell proliferation. These findings are consistent with previous studies demonstrating anti-proliferative effects of CBD in several cancer types, including glioma, breast cancer, lung cancer, leukaemia, and gastric cancer[51,52,54,114,174,179]. Investigation into the mechanisms underlying the anti-proliferative effects of GL1a revealed that GL1a reduced the expression of the key cell cycle proteins cyclin D3, CDK4, and CDK2, which drive the G1/S phase transition of

the cell cycle. Overexpression and hyperactivation of cyclin D, CDK4, and CDK2 are common features of prostate cancer, particularly of high-grade disease[41,42]. Accordingly, previous preclinical studies have indicated that the CDK4/6 inhibitor palbociclib inhibits prostate cancer cell proliferation, and several clinical trials are currently investigating the efficacy of various CDK4/6 inhibitors for the treatment of patients with prostate cancer[42,46]. GL1a also altered the expression of proteins involved in the G2/M phase transition, inducing a strong inhibition of CDK1, particularly in DU145 cells. CDK1 expression is also increased in prostate cancer and is associated with high-grade disease[42,314]. Additionally, we showed that GL1a inhibits AKT phosphorylation and activation in DU145 cells. The AKT signalling pathway is one of the most important pathways in cancer, regulating cell proliferation, cell survival, and cell death. Inactivation of the AKT inhibitor PTEN is one of the most common genetic alterations in prostate cancer and is observed more frequently in castration-resistant disease[48]. Increased AKT activity is observed in 50% of prostate cancers and is associated with increased tumour grade and reduced survival[48,49]. Therefore, the inhibition of AKT phosphorylation and cyclin and CDK expression by GL1a, leading to reduced cell proliferation, indicates its potential as a chemotherapeutic agent in prostate cancer.

6.2. ANTI-INVASIVE EFFECTS OF CANNABINOIDS IN PROSTATE CANCER

Activating invasion and metastasis is one of the hallmarks of cancer cells. Targeting cell invasion is particularly important, as colonisation of tissues by metastases leads to organ dysfunction and organ failure[315]. Consequently, metastasis is the cause of approximately 90% of cancer deaths[315]. Therefore, novel drugs that can inhibit metastasis are urgently needed. Previous studies have demonstrated anti-metastatic effects of phytocannabinoids *in vitro* in various cancer types, including lung cancer, breast cancer, cervical cancer, and glioblastoma[52,197-200,202,203]. Moreover, studies have shown that cannabinoids can also inhibit metastasis *in vivo*[52,178,187]. However, to our knowledge, no existing studies have investigated the anti-metastatic potential of cannabinoids in prostate cancer. Here, we showed that GL1a reduced the invasiveness of the highly metastatic PC-3 cell line, a cell line originating from a bone metastasis. Investigation into the mechanisms underlying the anti-invasive effect of GL1a revealed that GL1a induced a two-fold increase in the expression of the adhesion

protein E-cadherin, indicative of a non-invasive epithelial phenotype, though this was not statistically significant. Garcia-Morales *et al.* showed previously that CBD can restore an epithelial phenotype and reduce migration in breast cancer cells that have undergone epithelial mesenchymal transition[197]. In that study, fluorescence microscopy analysis revealed that GL1a relocalised β-catenin and E-cadherin at the adherens junctions and inhibited nuclear translocation of β-catenin, leading to reduced expression of pro-metastatic genes. Similar analysis in prostate cancer cells may provide an insight into the anti-invasive mechanisms of GL1a. Another potential target of GL1a in prostate cancer cells is the metastatic regulator Id-1. Id-1 is overexpressed in prostate cancer and promotes cell invasion[283-285]. Notably, cannabinoids have been shown to inhibit Id-1 expression in breast cancer and glioblastoma[178,200,282]. Future studies should assess the involvement of Id-1 in the anti-metastatic effects of GL1a. Despite the importance of metastasis in cancer, the ability of compounds to inhibit metastasis has been largely ignored by standard drug development pathways, in favour of identifying compounds with anti-proliferative or anti-tumourigenic properties[315]. This may be because several agents with promising preclinical anti-metastatic activity failed to show efficacy in clinical trials[315]. However, this lack of clinical research has meant that, over time, the survival of patients with metastatic disease has not improved to the same extent as that of patients with localised cancer[315]. Identifying agents that can effectively inhibit cancer metastasis could substantially reduce cancer-related mortality. Our research indicates that GL1a may have potential as an anti-metastatic agent in prostate cancer, though further research is needed to elucidate its underlying mechanisms of action.

6.3. CANNABINOID MECHANISMS OF ACTION IN PROSTATE CANCER

Due to their diverse pharmacological activities, natural products have been the source of many existing medicines, including cancer chemotherapeutics[316]. However, identifying molecular targets and deciphering mechanisms of action of natural products can be difficult due to their complex pharmacology[316]. Indeed, one of the major challenges in cannabinoid research is deciphering the underlying mechanisms of action. Plant-derived cannabinoids such as CBD can activate and inhibit many receptors and targets, and alter diverse signalling pathways, leading to modulation of a wide range of physiological processes throughout the body[260]. While we have demonstrated that GL1a can modulate the phosphorylation of AKT and the expression of cyclins and CDKs,

questions remain regarding the activity of GL1a upstream and downstream of these targets. Sequencing analysis of GL1a-treated and non-treated cells may provide a greater insight into these mechanisms. For example, transcriptome sequencing can be used to simultaneously measure the expression levels of a large number of genes in response to a drug treatment[317]. Accordingly, transcriptome analysis can identify drug targets, mechanisms of action, and unintended off-target effects[318,319]. Transcriptome analysis has provided insights into the pharmacological activity of numerous other plant-derived compounds, such as the effects of shikonin, a component of the Chinese herbal plant *Lithospermum erythrorhizon*, in breast cancer and the effects of withaferin A, a component of the medicinal plant *Withania somnifera*, in prostate cancer[317,320]. In recent years, transcriptome sequencing has improved our understanding of the mechanisms of action of phytocannabinoids in other disease models, including multiple sclerosis and Alzheimer's disease[321-323]. Notably, one recent study used RNA sequencing to investigate the mechanisms of action underlying the cytotoxic effect of CBD in cancer cells[175]. They showed that CBD treatment reduced the expression of genes involved in DNA repair, cell proliferation, and cell division in a cell line model of head and neck squamous cell carcinoma. The use of transcriptome sequencing and/or other sequencing techniques to analyse gene expression patterns in prostate cells following GL1a treatment could provide valuable insights into the mechanisms of action of GL1a in prostate cancer. Deciphering underlying mechanisms of action is an important step in the clinical development of a drug. Understanding how a drug produces its therapeutic effects can help in determining which patients are most likely to benefit from a particular treatment, identifying combination treatments that may provide enhanced benefits, understanding off-target effects and potential for toxicity, and possibly even providing new insights into the mechanisms involved in cancer development and progression.

6.4. RECEPTOR TARGETS OF CANNABINOIDS IN PROSTATE CANCER

One of the key questions remaining following this study is the identification of the receptor target(s) of GL1a in prostate cancer cells. Identifying the receptor target of a drug compound is an important step in understanding its mechanism of action. Existing studies have reported the involvement of various receptors and targets in mediating the anti-cancer effects of cannabinoids. For example, CBD inhibited proliferation in

colorectal cancer through activation of CB_1 and TRPV1[153,224]. Similarly, the anti-invasive effects of CBD in lung cancer were blocked by antagonists of CB_1, TRPV1, and CB_2[199,324]. Meanwhile, the effects of CBD on cell proliferation and cell death in glioma, multiple myeloma, and leukaemia, and on apoptosis and metastasis in lung cancer were mediated through increased activation of TRPV2[50,114,179,187]. Additionally, some evidence indicates the involvement of GPR55 in the anti-cancer activity of CBD in pancreatic cancer and glioblastoma[53,184]. Here, we assessed the involvement of several common cannabinoid targets in the phenotypic effects of GL1a in prostate cancer cells. We showed that the inhibition of viability by GL1a was not blocked by the addition of CB_1 or CB_2 receptor antagonists, a TRPV ion channel antagonist, or a GPR55 agonist, suggesting that the effects of GL1a in prostate cancer cells are not mediated by activity at these targets. The cells were treated with antagonists at various doses, including doses close to the known *K*i values of the drugs, and doses used previously in the literature. However, we cannot rule out the possibility that the receptors were not effectively bound by the antagonists/agonists at the doses tested. Therefore, receptor knock-down using siRNA may be a more effective method of assessing the involvement of the above targets in GL1a activity and confirming that GL1a inhibits cell viability independently of these receptors. Many additional cannabinoid targets have been identified in cancer cells, including TRPA1, TRPM8, PPARγ, and serotonin receptors[139,142,154,190]. Further research is needed to determine whether any of these targets are modulated by GL1a treatment in prostate cells. Future studies should assess the expression levels of various cannabinoid targets across different cancerous and non-cancerous prostate cell lines. This may provide an insight into the cell-specific effects of cannabinoids, the mechanisms underlying the observed phenotypic effects, and the potential for cancer-specific activity. Additionally, identifying receptors that are overexpressed in cancer and/or high-grade cancer may yield new diagnostic or prognostic biomarkers. In fact, existing studies show that increased expression of the CB_2 receptor in HER2-positive breast cancer is correlated with reduced overall survival and increased likelihood of recurrence and metastasis[167]. Overexpression of HER2-CB_2 heteromers is also correlated with poor patient prognosis[170]. These findings suggest that cannabinoid receptors may have potential as prognostic biomarkers in cancer. Moreover, some existing research indicates that cannabinoid targets including CB_1, TRPV1, and TRPV2 are overexpressed in prostate cancer, particularly in high-grade disease[134]. Evaluating the expression levels of common cannabinoid targets across different cancerous and non-cancerous cell and tissue types could identify new diagnostic and/or prognostic biomarkers for prostate cancer. Furthermore, identifying the

target receptors of GL1a and other cannabinoids may provide new predictive biomarkers to help identify the patients most likely to benefit from specific cannabinoid treatments.

6.5. EFFECTS OF CANNABINOIDS IN NON-CANCEROUS PROSTATE CELLS

One important finding of the current study is the cytotoxic effect of GL1a treatment in non-cancerous prostate cells. We have shown that cannabinoid effects in cancerous cells are enhanced under serum deprivation conditions, in agreement with previous studies. Because the non-cancerous prostate cell lines are routinely cultured in serum-free media, enhanced effects of cannabinoids are expected. However, even when the cancerous cells were treated in the absence of serum, the IC_{50} values for inhibition of viability by GL1a were lower in the non-cancerous cell lines. This is in contrast with several existing studies showing that cannabinoids reduce the viability of colon cancer, breast cancer, gastric cancer, and HNSCC cells without affecting the viability of corresponding non-cancerous cells[175,203,224,254]. Moreover, Sharma *et al.* reported that cannabinoid effects in prostate cancer were cancer-specific, as no cytotoxic effect was observed in the non-cancerous cell lines BPH-1 and PNT1B[226]. However, both of these cell lines are grown with serum, and the effects of CBD in serum-deprived non-cancerous cells were not assessed in that study. Notably, all the above studies used a shorter 24h cannabinoid treatment, and longer treatments may induce cytotoxic effects in those models. In fact, Deng *et al.* reported that the effect of a 72h cannabinoid treatment in glioblastoma was not cancer cell specific, as similar CBD-induced reductions in viability were observed in non-cancerous neural progenitor cells[255].

Determining the precise mechanisms of action of GL1a in various cancerous and non-cancerous cell lines may provide an insight into the observed lack of cancer-specificity. Due to their complex pharmacology, cannabinoids can modulate the activity of a wide range of target receptors, which may be expressed at varying levels in different cancerous and non-cancerous cell lines. For instance, Sharma *et al.* reported that the cancer-specific effects observed in prostate cancer cells may be due to increased expression of CB_1 and CB_2 receptors in those cell lines[226]. Measuring the expression levels of various cannabinoid targets in these cells may improve our understanding of the potential off-target effects of GL1a.

Additionally, it should be noted that the current study used artificially immortalised non-cancerous cells. Immortalisation is known to alter many cellular characteristics and processes, including cellular differentiation, DNA damage response, and chromosome structure, all of which may be targeted by treatment with anti-cancer drugs[256,257]. Indeed, ongoing research in our laboratory has shown that the non-cancerous prostate cells used in the current study are particularly sensitive to treatment with various plant-derived and synthetic agents. Therefore, it would be useful to determine whether GL1a also produces cytotoxic effects in primary prostate cell lines. Although culture of primary prostate cells is technically challenging, they are likely more representative of normal, healthy prostate cells. Furthermore, it will be important to test the safety of GL1a in animal models of prostate cancer to ensure that GL1a treatment does not induce unintended toxic effects in healthy prostate cells.

More targeted drug delivery approaches could also be explored to minimise any off-target toxicity of cannabinoids. For example, drug compounds can be conjugated with nanoparticles to improve drug delivery and specificity. Nanoparticles can specifically target tumour cells, minimising off-target effects and toxicity[325-327]. The direct delivery of drugs to target sites improves efficacy and maximises damage to tumour cells, which can reduce the therapeutic dose required, further reducing potential toxicity[325]. Moreover, conjugating drugs with nanoparticles can enhance their stability and allow for the controlled release of the drug over time[325,327,328]. Cannabinoids have already been identified as attractive candidates for nanoparticle-conjugated therapy[325-327]. Indeed, a nanomicellar formulation of the psychoactive synthetic cannabinoid WIN55,212-2 (WIN) enhanced the anti-tumour effects of WIN in a mouse model of triple-negative breast cancer[329]. Additionally, side effects were milder when WIN was conjugated to the nanomicelles. Furthermore, in a cell line model of glioblastoma, delivery of CBD using lipid nanocapsules improved targeting of glioma cells and allowed extended release of the CBD treatment[328]. Notably, like GL1a, existing standard of care treatments for prostate cancer, such as docetaxel, also produce similar cytotoxic effects in cancerous and non-cancerous cells[330]. However, conjugation of docetaxel to nanoparticles can protect primary non-cancerous cells from its cytotoxic effects[330].

Importantly, many previous studies have shown that CBD has a favourable safety profile in humans and animals. Doses up to 1,500mg/day are safe and well-tolerated in humans[258]. Moreover, CBD-based medicines such as Epidiolex (CBD) and Sativex (THC:CBD) have already been approved for the treatment and management of various medical conditions, including epilepsy and multiple sclerosis. Therefore, the observed

effects of GL1a in immortalised non-cancerous cells do not rule out its potential clinical use. However, further research is needed to understand the mechanisms of action of GL1a in cancerous and non-cancerous cells and to assess its safety *in vivo*.

6.6. CHEMOPREVENTIVE POTENTIAL OF CANNABINOIDS

Because up to 50% of cancers may be preventable, identifying novel chemopreventive agents has the potential to substantially reduce cancer incidence and mortality. However, no chemopreventive agents for prostate cancer have yet been granted FDA approval. Because oxidative stress is a major driving force in prostate cancer development, several antioxidant compounds, including selenium, vitamin E, sulforaphane, lycopene, and curcumin have been investigated in clinical trials for prostate cancer chemoprevention, with some promising results[23-26,110]. Notably, substantial evidence indicates that phytocannabinoids display antioxidant activity in various non-cancer disease models[298-302]. However, to date, research of phytocannabinoids in cancer has primarily focussed on their chemotherapeutic properties, and few studies have assessed the antioxidant and chemopreventive potential of cannabinoids in cancer. In the current study, we found no evidence of significant antioxidant or cytoprotective effects of GL1a or GL1b in non-cancerous prostate cells. It should be noted that these experiments are exploratory in nature, due to the dearth of previous similar *in vitro* studies. Therefore, the experimental design should be further optimised, specifically in terms of exposure time and treatment doses for both cannabinoids and hydrogen peroxide. Here, we assessed antioxidant effects in a single non-cancerous cell line, and future studies should incorporate additional non-cancerous cell lines. Effects could also be assessed in cancerous cell lines, to determine whether cannabinoids may be capable of inhibiting markers of cancer progression. Future studies should also assess the ability of cannabinoids to protect against additional types of carcinogenic insult, as various carcinogens can induce cancer development and progression through different mechanisms[91]. While we did not observe any evidence of cannabinoid chemopreventive effects in the current study, this does not rule out the possibility that, under optimised treatment conditions, cannabinoids may produce antioxidant effects that could protect prostate cells from malignant transformation. Interestingly, some evidence in other cancer types indicates that phytocannabinoids may have chemopreventive potential. For example, Aviello *et al.* investigated the chemopreventive effects of cannabinoids against colorectal cancer development and found that CBD protected cells

from oxidative stress-induced DNA damage and inhibited cancer cell proliferation[153]. Moreover, pre-treatment of mice with CBD prior to treatment with the carcinogenic agent AOM inhibited AOM-induced formation of polyps, aberrant crypt foci, and tumours. Additionally, a CBD-rich whole plant extract produced similar effects[224]. These findings suggest that plant-derived cannabinoids may have potential as future chemopreventive agents.

6.7. EXPERIMENTAL MODELS FOR CANNABINOID RESEARCH

One of the limitations of the current study is that the compounds were investigated using 2D cell culture models. 2D cell culture has been a well-accepted and widely used technique in biological research, which has greatly improved our understanding of cell biology and provided important insights into cell behaviour and drug response[331]. 2D cell culture is particularly useful for preliminary screening of drug compounds, due to its simplicity, efficiency, reproducibility, low cost (particularly for large-scale studies), and suitability for long-term culture[331,332]. Thus, 2D cell culture has played a crucial role in biological research. However, there are several major limitations of 2D cell culture models. Cells in 2D culture grow by adhering to a flat surface in a monolayer, which alters cell morphology, polarity, and bio-activity[331,333]. Furthermore, 2D models are not capable of fully representing the complexity of the tumour microenvironment and cellular responses to signalling factors and other environmental cues[331]. Consequently, cells grown using 2D platforms display altered cell behaviour and drug response[332].

Our growing understanding of the importance of the tumour microenvironment in cancer development and progression has led to the development of new 3D cell culture approaches such as spheroid culture, organoid culture, and patient explant models. Spheroids are spherical aggregates composed of tumour cells, alone or in combination with other cell types[333,334]. Organoids represent a more complex 3D model, grown to resemble the organ in question, structurally and functionally[334]. Meanwhile, patient explants models are cultured from pieces of excised human tumours from biopsy[333]. In explant models, the original tissue structure is preserved and both cellular and non-cellular components of the tumour microenvironment are present. 3D models are capable of more accurately mimicking the structural complexity and heterogeneity of tumours *in vivo*[333]. Furthermore, 3D cell culture models more accurately represent the

in vivo tumour microenvironment[331,333]. These models can therefore provide information about the interactions of cells with cellular and non-cellular components of the microenvironment, including cancer-associated fibroblasts, tumour infiltrating immune cells, growth factors, cytokines, chemokines, and the extracellular matrix, all of which influence cell behaviour[331,333]. Therefore, 3D models can be useful in bridging the gap between 2D cell culture and *in vivo* models[332].

Substantial evidence indicates that cells behave differently under 2D vs 3D cell culture conditions. For instance, spheroid cell cultures show distinct sub-populations of cells, with highly proliferative cells localised on the surface of the aggregates and a higher proportion of quiescent and necrotic cells in the interior[331,333]. Generally, slower cell proliferation rates are observed in cells grown using 3D platforms, as well as differences in cell survival, differentiation, and motility[331,332]. Additionally, cells in 3D cultures can display different characteristics to 2D cells in terms of deposition of extracellular matrix, growth factor secretion, and gene and protein expression patterns[331-333]. Cells grown under 2D vs 3D conditions also show differences in drug response. For example, a study investigating carboplatin response in various models of ovarian cancer found that 3D models, and particularly *ex vivo* models, showed a stronger correlation with *in vivo* response than 2D models[335]. Interestingly, some evidence indicates that phytocannabinoid pharmacological activity also differs between 2D and 3D models. A study in triple-negative breast cancer showed that IC_{50} values for CBD were six- to ten-fold higher in 3D hydrogel-based cultures compared to in 2D cell line models[202]. Future studies of cannabinoids in prostate cancer should test their efficacy in more relevant 3D models.

Clinical development of cannabinoids will require validation of *in vitro* findings in relevant animal models. Several existing studies have demonstrated anti-cancer activity of phytocannabinoids *in vivo*. For example, Vara *et al.* showed that THC treatment reduced tumour growth by more than 50% in a mouse model of hepatocellular carcinoma, an effect that was dependent on increased activation of PPARγ[182]. Similarly, CBD inhibited tumour growth in animal models of colorectal cancer and glioma, accompanied by increased apoptosis in tumour cells[119,188]. Furthermore, CBD treatment reduced metastasis in mouse models of breast cancer and lung cancer[52,178,187]. Some evidence also suggests that CBD can inhibit angiogenesis *in vivo*[206]. Additionally, several studies show that cannabinoids can enhance the effects of existing anti-cancer therapies on tumour growth in mice[175,218]. While the effects of cannabinoids in animal models of prostate cancer have not been thoroughly investigated, De Petrocellis *et al.*

reported that a plant extract rich in CBD reduced tumour growth in mouse models of hormone-sensitive prostate cancer[155]. Further studies should determine whether GL1a and other purified cannabinoid compounds can produce similar effects, and whether they can produce anti-tumour effects in models of castration-resistant prostate cancer, where new effective treatments are urgently needed. Additionally, future studies in 3D and animal models should aim to identify safe and effective cannabinoid doses and ensure that cannabinoids do not produce toxicity in healthy prostate cells. If preclinical studies can identify safe and effective cannabinoid doses in prostate cancer models, clinical trials can then be developed to assess the safety of phytocannabinoids for the treatment of patients with prostate cancer.

6.8. COMBINING CANNABINOIDS WITH EXISTING ANTI-CANCER AGENTS

Clinically, cannabinoids may provide the greatest therapeutic benefit through use in combination with existing anti-cancer therapies. Several existing preclinical studies have shown that the addition of cannabinoids to standard chemotherapeutic agents enhances anti-cancer efficacy. In 2008, Liu *et al.* showed that the addition of THC sensitised leukaemia cells to treatment with the cytotoxic chemotherapy drugs cytarabine, doxorubicin, and vincristine[215]. Several studies indicate that CBD can also enhance the effects of anti-cancer therapies. CBD increased the uptake of temozolomide, carmustine, and doxorubicin by glioma cells, enhancing their cytotoxic activity[152]. CBD also sensitised triple-negative breast cancer cells to doxorubicin[336]. In multiple myeloma cells, CBD acted synergistically with bortezomib to inhibit cell proliferation and induce cell death[50]. Additionally, CBD enhanced the effects of cisplatin, 5-fluoracil, and paclitaxel in HNSCC cells[175]. Furthermore, in a HNSCC mouse model, the combination treatment of cisplatin and CBD significantly reduced tumour growth compared to either compound alone. Similarly, a combination of CBD and paclitaxel synergistically reduced the viability of breast cancer cells[337]. Moreover, in a breast cancer mouse model, the addition of CBD reduced paclitaxel-induced neuropathic pain, suggesting that combining existing treatments with cannabinoids could alleviate some unwanted side effects of chemotherapy[337]. Notably, evidence from preclinical studies showing that cannabinoids synergistically enhance the effects of temozolomide on glioblastoma cell viability *in vitro*, and on tumour growth and survival *in vivo*[173,211,218,220] has led to the development of several clinical trials to assess the

safety and efficacy of combining cannabinoids with temozolomide treatment in patients with newly-diagnosed or recurrent glioblastoma (NCT01812603, NCT01812616, NCT03529448)[223].

Some evidence suggests that cannabinoids may enhance the effectiveness of standard prostate cancer treatments. For example, in an animal model of hormone-sensitive prostate cancer, combining bicalutamide with a CBD-rich cannabis extract inhibited tumour growth and prolonged survival more effectively than treatment with bicalutamide alone[155]. The study also provided some evidence that the plant extract enhanced the effect of docetaxel in a model of castration-resistant prostate cancer. Future studies should assess whether the combination of GL1a and other purified cannabinoid compounds with these and other existing prostate cancer treatments produces enhanced anti-cancer effects, particularly in castration-resistant models which may be more likely to benefit patients with advanced prostate cancer.

As with other anti-cancer properties of cannabinoids, synergistic effects with existing drugs can be highly dose-dependent. In glioma, combining high doses of CBD with the DNA-damaging agents temozolomide, carmustine, and cisplatin produced limited synergistic anti-proliferative and pro-cell death effects[255]. However, when low CBD doses were used, antagonistic effects occurred between CBD and other drugs in certain glioma cell lines, highlighting the importance of identifying the most effective cannabinoid doses, and the need for caution when combining cannabinoids with existing anti-cancer agents. Future preclinical studies should aim to determine safe and effective dose combinations, identify drugs with complementary mechanisms of action that can produce synergistic activity, and test the efficacy of combination treatments in relevant 3D and animal models.

6.9. THE ENTOURAGE EFFECT

Existing research into cannabis-based medicines has focussed primarily on single purified cannabinoid compounds. However, several studies have shown that mixtures of phytocannabinoids can produced enhanced biological activity compared to individual compounds[137]. The synergistic activity between different components of the cannabis plant has been termed the ‘entourage effect’[137]. The mechanisms underlying the entourage effect are not fully understood, though possible explanations include the modulation of multiple molecular targets, enhanced bio-availability of compounds, or

inhibition of adverse events[338]. In the current study, we found some evidence that combining GL1a and GL4a at specific doses may produce enhanced effects on cell viability. This is consistent with existing studies which have shown that combination treatments of CBD and THC produce enhanced anti-cancer effects in multiple myeloma and glioma cells compared to treatment with either compound alone[205,210]. Furthermore, combining CBD and THC at submaximal doses produced strong anti-tumour effects in a mouse model of glioma[211]. Some evidence suggests that whole plant extracts may provide even greater therapeutic benefits. For example, in breast cancer, a botanical drug preparation produced more potent effects than purified THC on cell viability *in vitro* and on tumour growth in a mouse model[213]. Further research showed that whole cannabis extracts inhibited cell proliferation and survival and induced apoptosis more effectively than pure THC in cancer cell line models of various origins, including prostate cancer[135]. Additionally, De Petrocellis *et al.* reported that several whole plant extracts were more effective than isolated compounds at reducing cell viability in prostate cancer[155]. Moreover, phytocannabinoids can act synergistically with other non-cannabinoid cannabis components such as terpenoids, further enhancing their therapeutic effects. For instance, a combination treatment of THC or CBD with co-related terpenoids in breast and colon cancer cells produced cytotoxic activity not observed following treatment with any of the compounds in isolation[214]. Interestingly, the most potent cytotoxic effects were observed when cells were treated with cannabinoids and terpenoids at ratios most similar to those occurring naturally in the cannabis plant. The development of cannabis strains containing specific ratios of various cannabinoid compounds is now possible through selective Mendelian breeding[156-158,339]. Future studies should assess the anti-cancer efficacy of various phytocannabinoid compounds, alone and in combination, in prostate cancer cells, identify the most effective synergistic cannabinoid doses and combinations, and determine whether whole plant extracts rich in specific cannabinoid compounds can provide enhanced therapeutic effects. Additionally, further research is needed to understand the mechanisms underlying the anti-cancer and synergistic effects of various cannabinoid and non-cannabinoid components of the cannabis plant. Exploiting the entourage effect to develop new therapies based on cannabinoid compounds and whole plant extracts may be beneficial for the treatment of prostate cancer.

6.10. CANNABINOID CLINICAL RESEARCH

The cannabis plant has been cultivated for thousands of years by cultures across the world and used as a source of medicine since as early as 400 A.D.[111]. Cannabinoid research is an example of 'reverse drug discovery', where scientific research is conducted based on anecdotal evidence from patients reporting therapeutic benefits of smoked cannabis for various conditions[340]. While a large body of preclinical evidence indicates therapeutic potential of cannabinoids in range of conditions, many barriers to cannabinoid research have delayed the validation of these reported therapeutic benefits in clinical studies. The cannabis plant has generated much controversy due to the psychoactive effects produced by certain components of the plant, leading to the classification of cannabis as an illegal drug in most countries. The illegal status of cannabis has created challenges for researchers in gaining access to cannabinoid compounds for research[341]. Additionally, the prohibition of cannabis has led to many misconceptions and much stigma surrounding cannabis and cannabinoid research[340,341]. Historically, the above barriers, in addition to a lack of knowledge of cannabinoid therapeutic benefits among many medical professionals, may have contributed to a lack of interest from pharmaceutical companies in developing cannabis-based medicines[342].

In recent years, many governments have begun legalising cannabis and cannabis products, particularly for medical use[113]. Consequently, patients and members of the public have increased access to cannabis-based medicines[341]. Public knowledge of and access to cannabis-based medicines could be risky, as many claimed benefits of cannabinoids have not yet been proven in robust scientific studies[342]. Therefore, high-quality scientific research into the therapeutic effects of cannabinoids is essential if we are to understand and accurately communicate the benefits, and potential risks, of cannabis-based medicines. In 2017, the National Academies of Sciences, Engineering, and Medicine (NASEM) reported that "conclusive or substantial" evidence supported the use of cannabinoids for the management of pain, muscle spasticity, and nausea and vomiting[160]. Meanwhile, the review reported "low-quality evidence" for the use of cannabis for most conditions and "insufficient" evidence of cannabinoid anti-cancer effects. Consequently, the NASEM recommended further research, and particularly clinical research, into cannabinoid efficacy in cancer.

Randomised clinical trials are essential to determine whether pharmacological activities observed in preclinical models translate to therapeutic benefits in patients. To date, the

emphasis of cannabinoid clinical trials has been around dosing, safety, and chronic pain/symptom relief in multiple advanced cancer types. For example, several large-scale trials have demonstrated that Sativex, a THC:CBD combination treatment, effectively reduces pain in patients with advanced malignancies, with a relatively low incidence of adverse events (NCT00674609, NCT00530764, NCT01262651, NCT01361607). However, the efficacy of plant-derived cannabinoids for the treatment of cancer remains largely unexplored. Of note, a recent phase 1b trial provided some evidence that Sativex may improve survival in patients with glioblastoma[223].

In the current study, we provide evidence that GL1a inhibits prostate cancer cell proliferation and invasion *in vitro*. If these findings translate to more relevant 3D cell culture and animal models, Phase I clinical trials should be designed to determine the safety of GL1a in patients with prostate cancer and to assess any adverse events. Larger-scale trials can then be conducted to determine whether the anti-cancer activity of GL1a *in vitro* can translate to therapeutic benefits and improved survival in patients with prostate cancer.

Further clinical studies will be essential to determine whether the anti-cancer effects observed in preclinical studies can translate to therapeutic benefits for cancer patients. With changing legislation, increased accessibility of cannabis and cannabis products, and increasing public interest in cannabis-based medicines, researchers must generate robust scientific data to inform patients, healthcare providers, and legislators of the potential benefits and risks of cannabis-based medicines. Phytocannabinoids show considerable potential as anti-cancer agents in the preclinical setting, but further research will be needed to determine whether cannabis-based medicines can improve outcomes for cancer patients.

6.11. CONCLUSION

Here, we provide novel insights into the phenotypic effects and mechanisms of action of cannabinoids in prostate cancer. The phytocannabinoid GL1a inhibited the viability, proliferation, survival, and invasiveness of prostate cancer cells. GL1a-induced inhibition of proliferation was accompanied by reduced expression of the cell cycle regulators cyclin D3, CDK4, CDK2, and CDK1, and reduced phosphorylation and activation of the protein kinase AKT. These findings are consistent with previous studies showing that cannabinoids reduce cancer cell proliferation by modulating cell cycle regulation and

protein phosphorylation. Further research is needed to identify the receptor target(s) of GL1a in prostate cancer cells, to gain a deeper understanding of the underlying mechanisms of action, and to investigate the effects of GL1a on non-cancerous cells. Additionally, testing of GL1a in more biologically relevant models will determine whether the promising effects observed *in vitro* are likely to translate to therapeutic benefits. Ultimately, clinical trials should be developed to assess the safety and efficacy of phytocannabinoids, alone or in combination with existing treatments, particularly in patients with castration-resistant prostate cancer for whom effective treatment options are limited. GL1a shows promising potential as a chemotherapeutic agent in the *in vitro* setting, though further research is needed to determine whether cannabinoid treatments can improve outcomes for patients with prostate cancer.

APPENDIX

7.1. GL1A CERTIFICATE OF ANALYSIS

PROVERDE LABORATORIES

Test Certificate

Certificate ID: **20794**
Client Sample ID: **Cherry ISO**
Matrix: **Concentrates/Extracts - Isolate**
Date Received: **8/30/2017**

Commonwealth Extracts
11907 Lilac Way
Louisville, KY 40243
Attn: John Taylor

This test method was performed in accordance with the requirements of ISO/IEC 17025. These results relate only to the test article listed in this report. Reports may not be reproduced except in their entirety.

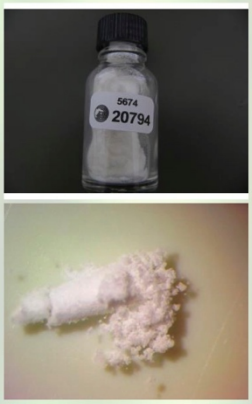
Authorization:	Signature:	Date:
Chris Hudalla, Chief Science Officer	Christopher Hudalla	9/11/2017

CN: Cannabinoid Profile & Potency [WI-10-04] *Analyst: JFD* *Test Date: 9/11/2017*

The client sample was analyzed for plant-based cannabinoids by Convergence Chromatography (CC). The collected data was compared to data collected for certified reference standards at known concentrations.

20794-CN

ID	Weight %	Conc.
Δ9-THC	-	-
THCV	-	-
CBD	99.74 wt %	997.40 mg/g
CBDV	0.07 wt %	0.67 mg/g
CBG	-	-
CBC	-	-
CBN	-	-
THCA	-	-
CBDA	-	-
CBGA	-	-
Total	99.81 wt%	998.07 mg/g
Max THC	-	-
Max CBD	99.74 wt%	997.40 mg/g



Max THC (and Max CBD) are calculated values for total cannabinoids after heating, assuming complete decarboxylation of the acid to the neutral form. It is calculated based on the weight loss of the acid group during decarboxylation: Max THC = (0.877 x THCA) + THC.

FM-10-10, Rev. 1, DCN:15-0003

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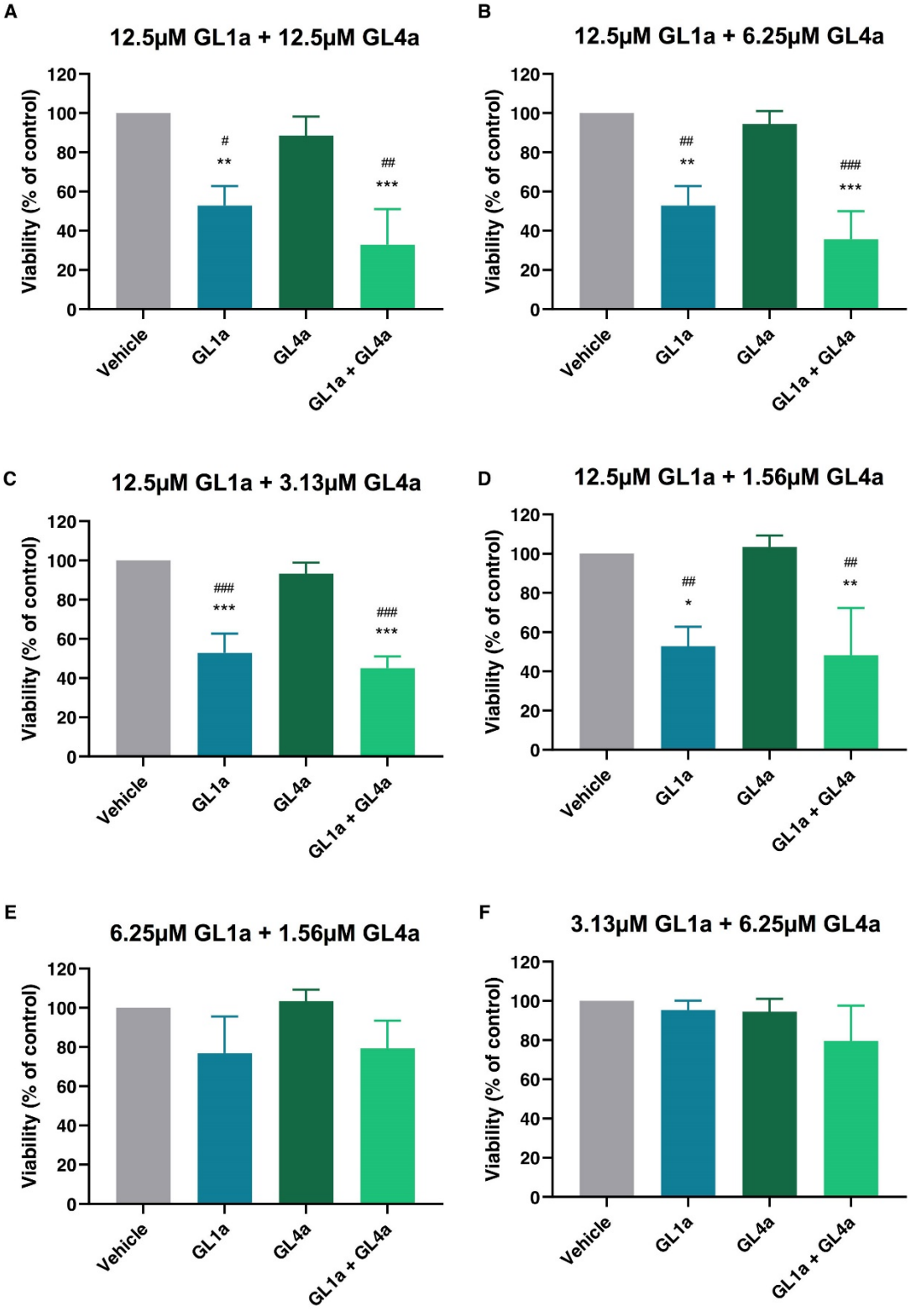
Figure A7.1. Certificate of analysis for GL1a. GL1a is composed of 99.74% CBD.

7.2. EFFECT OF CANNABINOID COMBINATION TREATMENTS ON PROSTATE CANCER CELL VIABILITY



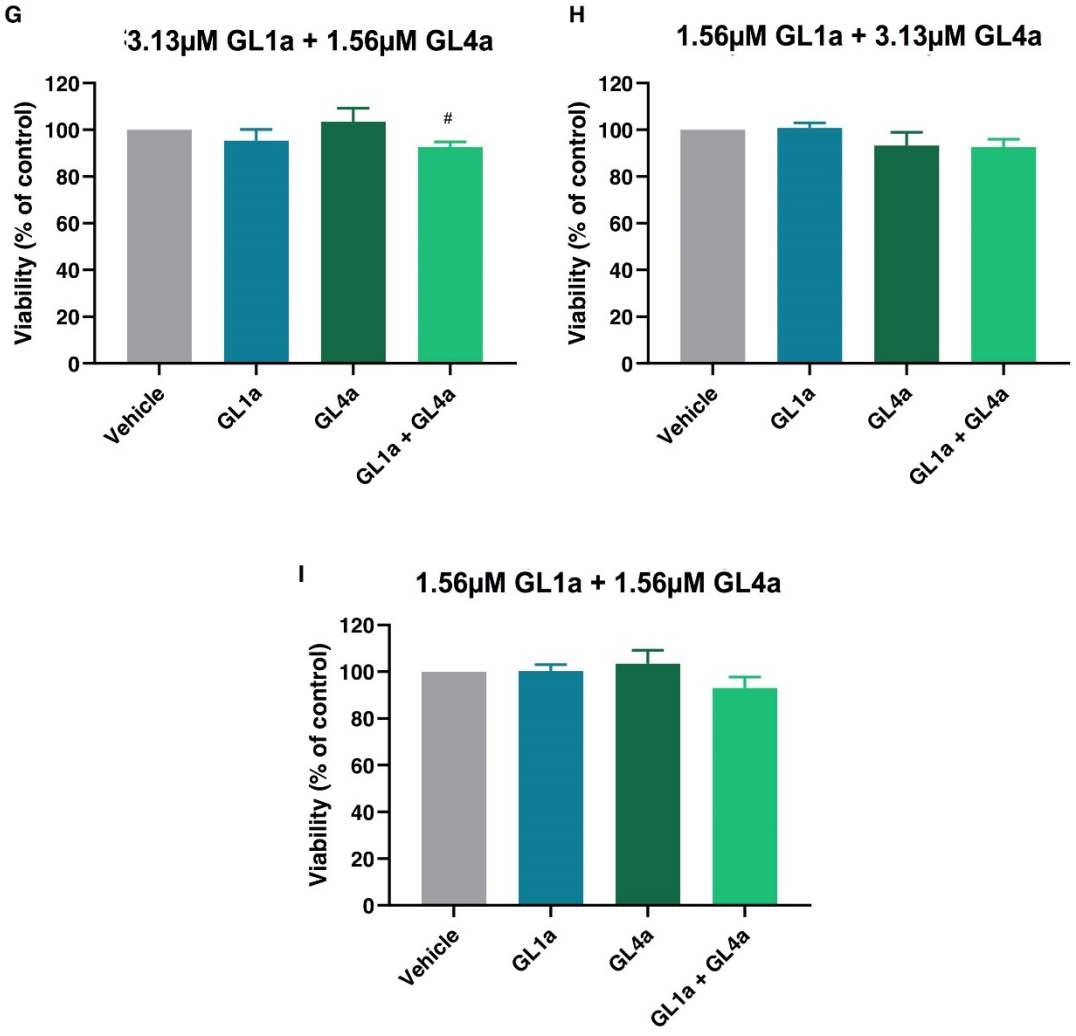


Figure A7.2. Effects of cannabinoid combination treatments on prostate cancer cell viability. (A-I) PC-3 cells were treated for 72h with various doses of GL1a and GL4a, alone or in combination. Cell viability was measured using the MTT assay. 1-way ANOVA *p<0.05 **p<0.01 ***p<0.001 compared to vehicle alone; #p<0.05 ##p<0.01 ###p<0.001 compared to GL4a alone. Results are representative of 3 biological replicates and 3 technical replicates.

7.3. INVOLVEMENT OF THE CB_1 RECEPTOR IN GL1A EFFECTS

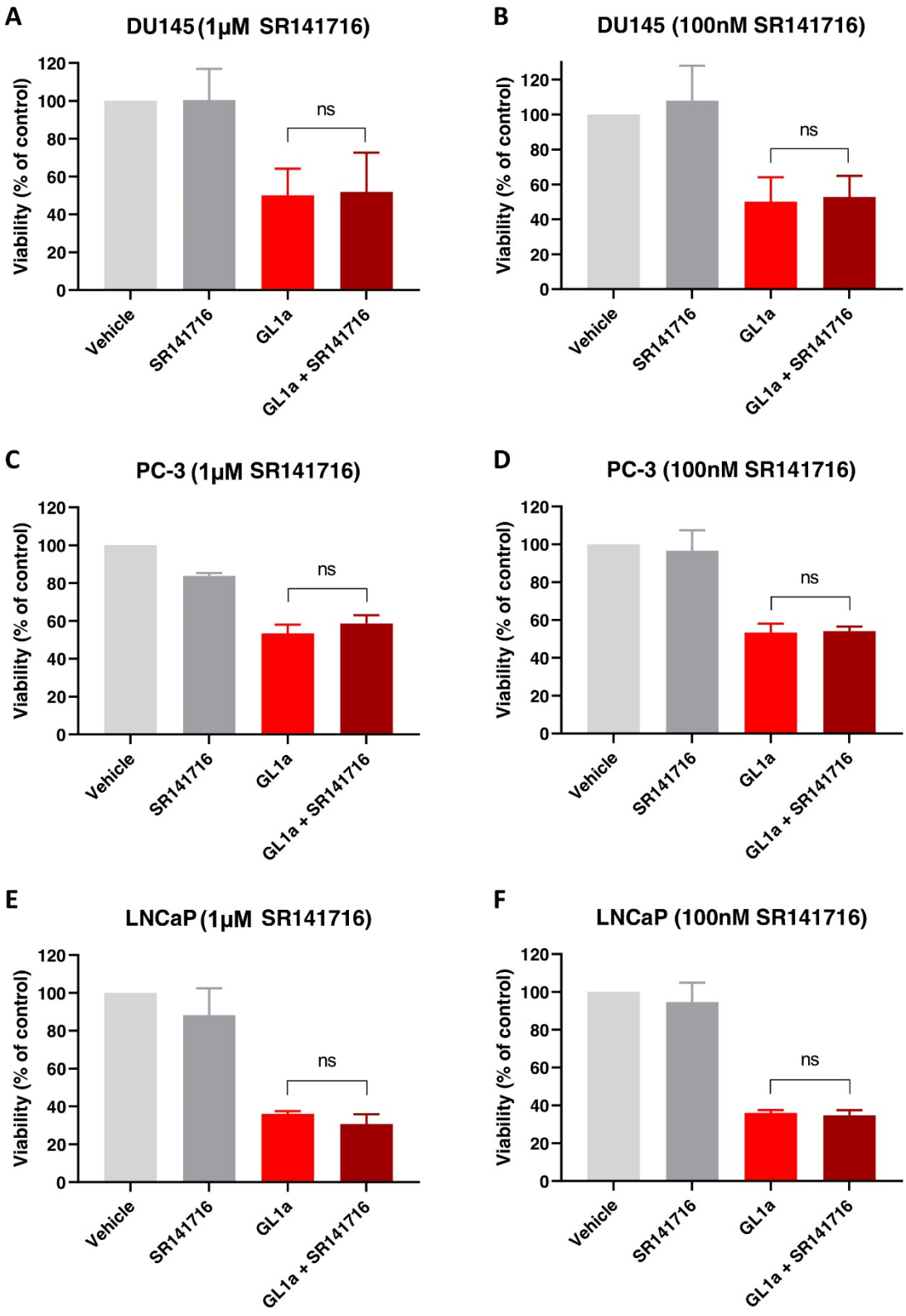


Figure A7.3. The effect of GL1a on cell viability is not blocked by a CB_1 receptor antagonist. Prostate cancer cells were pre-treated with the CB_1 antagonist SR141716 (1μM, 100nM) for 1h

before treatment with an IC_{50} dose of GL1a for 72h. Cell viability was measured using the MTT assay. (A-F) SR141716 did not alter the effect of GL1a on cell viability (1-way ANOVA, *p<0.05). Results are representative of 3 biological replicates and 3 technical replicates.

7.4. INVOLVEMENT OF THE CB_2 RECEPTOR IN GL1A EFFECTS

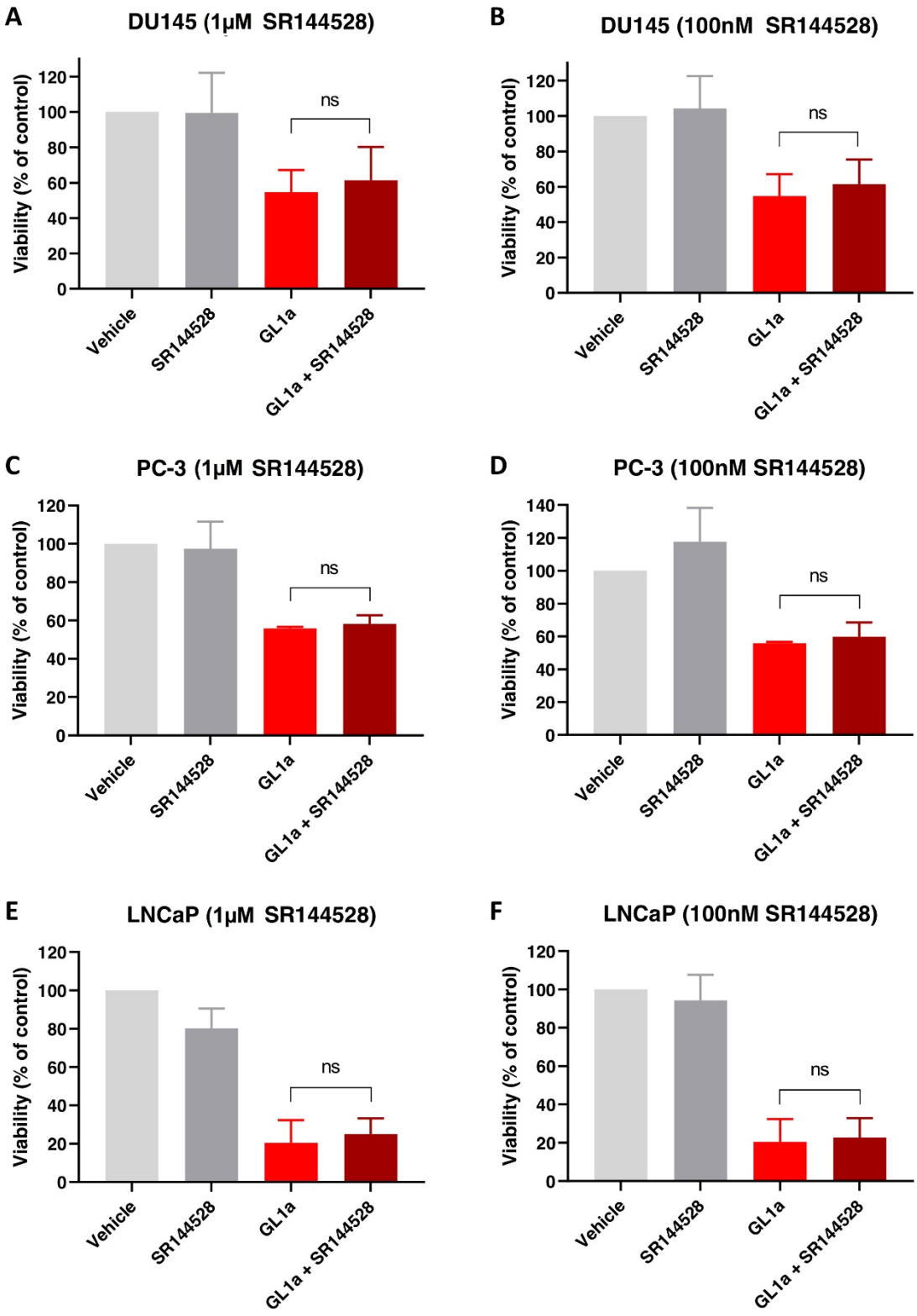


Figure A7.4. The effect of GL1a on cell viability is not blocked by a CB_2 receptor antagonist. Prostate cancer cells were pre-treated with the CB_2 antagonist SR144528 (1μM, 100nM) for 1h before treatment with an IC_{50} dose of GL1a for 72h. Cell viability was measured using the MTT

assay. (A-F) SR144528 did not alter the effect of GL1a on cell viability (1-way ANOVA, *p<0.05). Results are representative of 3 biological replicates and 3 technical replicates.

7.5. INVOLVEMENT OF TRPV CHANNELS IN GL1A EFFECTS

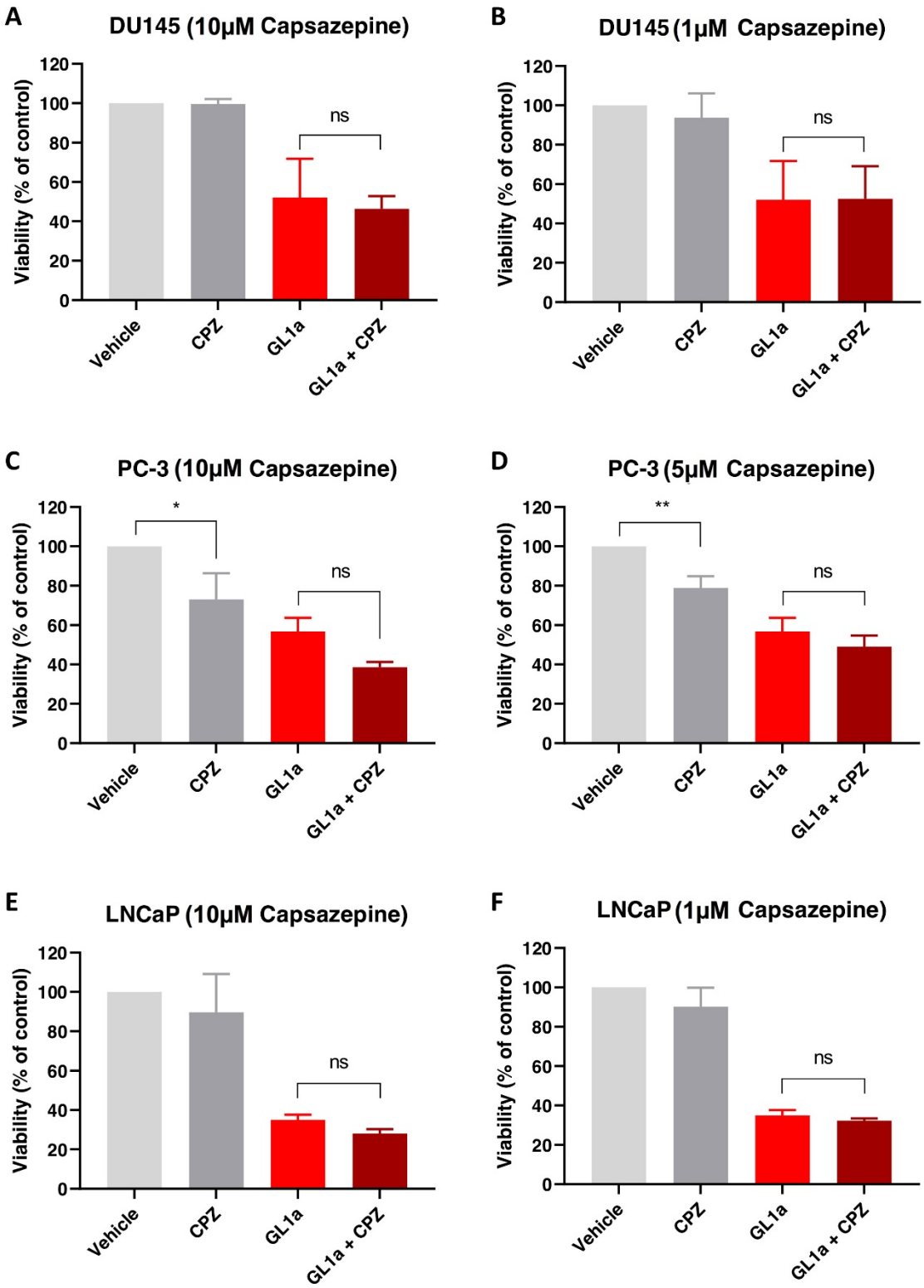


Figure A7.5. The effect of GL1a on cell viability is not blocked by a TRPV channel antagonist. Prostate cancer cells were pre-treated with the TRPV channel antagonist capsazepine (1μM, 5μM, 10μM) for 1h before treatment with an IC_{50} dose of GL1a for 72h. Cell

viability was measured using the MTT assay. (A-F) Capsazepine did not alter the effect of GL1a on cell viability (1-way ANOVA, *p<0.05). Capsazepine alone (5μM or 10μM) significantly reduced the viability of PC-3 cells (*p<0.05 **p<0.01) Results are representative of 3 biological replicates and 3 technical replicates.

7.6. OPTIMISATION OF H_2O_2 ANTIOXIDANT DOSE

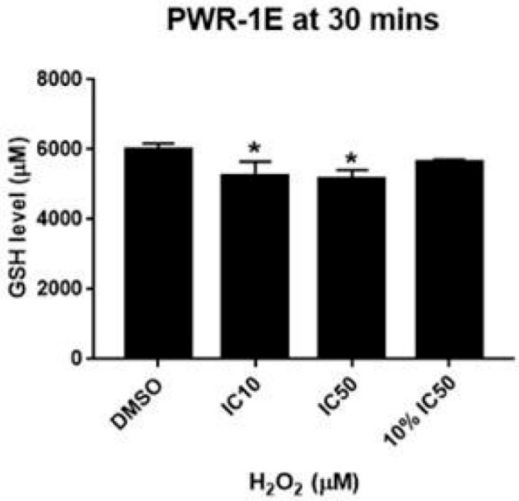


Figure A7.6. Optimisation of H_2O_2 antioxidant dose in PWR-1E cells. After 24h adhesion, cells were treated with various doses of H_2O_2 for 30 min. Reduced glutathione levels were measured using the GSH-Glo assay. IC_{10} and IC_{50} H_2O_2 doses significantly reduced GSH levels (1-way ANOVA, *p<0.05). Results are representative of three biological replicates and three technical replicates.

7.7. OPTIMISATION OF H_2O_2 ANTIOXIDANT TREATMENT TIME

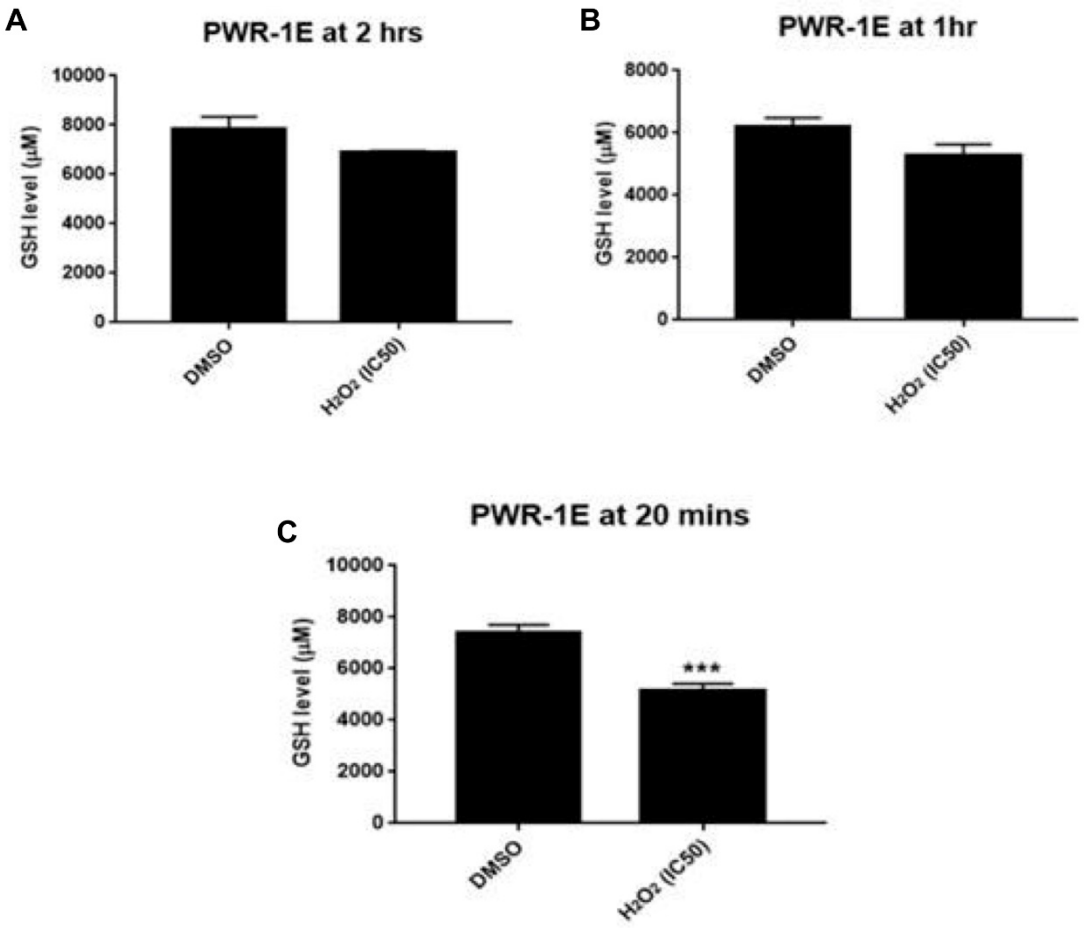


Figure A7.7. Optimisation of H_2O_2 antioxidant treatment time in PWR-1E cells. After 24h adhesion, cells were treated with an IC_{50} dose of H_2O_2 for (A) 2h, (B) 1h, or (C) 20 min. Reduced glutathione levels were measured using the GSH-Glo assay. H_2O_2 significantly reduced GSH levels after 20 min treatment (Student's *t*-test, ***$p<0.001$). No significant effect was observed after 1h or 2h treatments (*$p<0.05$). Results are representative of three biological replicates and three technical replicates.

7.8. OPTIMISATION OF NAC CYTOPROTECTIVE TREATMENT TIME

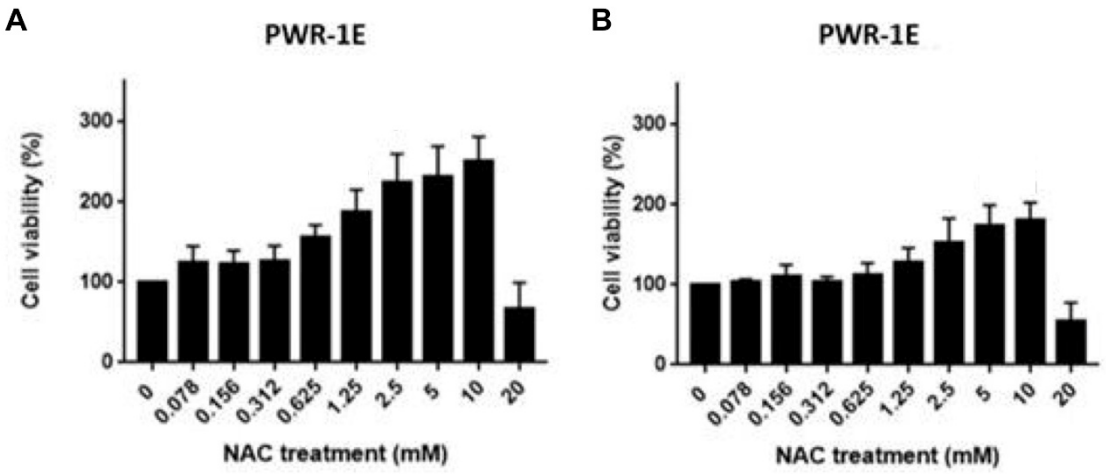


Figure A7.8. Optimisation of NAC cytoprotective treatment time in PWR-1E cells. After 24h adhesion, cells were treated with a range of doses of NAC (0-20mM). Cell viability was measured using the MTT assay, after (A) 1h or (B) 2h treatment. 10mM NAC was the highest dose to produce no cytotoxic effect. Results are representative of three biological replicates and three technical replicates.

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