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Repositioning aspirin and metformin to improve prostate cancer treatment and outcome

Repositioning aspirin and metformin to improve prostate cancer treatment and outcome

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A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Doctor of Philosophy in the Faculty of Medicine and Dentistry.

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Abstract

Prostate cancer is the second most common cancer in males worldwide and the fifth most common cause of cancer death among men. Identifying drugs which prevent prostate cancer initiation and development is a major goal in the field of cancer research, with a large focus on repositioning established medicines to reduce the time and cost of drug development and decrease adverse side effects in patients. Epidemiological and *in vitro* studies suggest that aspirin (NSAID) and metformin (first-line agent in type II diabetes) are negatively associated with prostate cancer risk and mortality although considering their overlapping function, it is perhaps surprising that little is known about using these drugs in combination for the prevention or treatment of prostate cancer.

The aim of this PhD thesis was to assesses the effect of aspirin and metformin on prostate cell proliferation and migration to determine whether the drugs in combination have the potential to reduce tumour growth and prevent the formation of secondary cancer sites. Four prostate cell lines: prostate epithelial cells, PNT2 and three cancer cell lines; PC3, DU145 and LNCaP were treated with therapeutic concentrations of aspirin (up to 2 mM) and metformin (up to 30 μM). Cell counting, tritiated thymidine incorporation assays and western blotting measured changes in cell growth and markers of proliferation, while annexin V and Q-VD-OPh assays measured apoptosis. In addition, PC3 cells were grown as 3D spheroids to observe the effect of the drugs in a model which better represents the cell to cell interactions and surrounding environment seen *in vivo*. Wound healing assays were used to examine drug induced changes in cell migration and western blotting and qPCR assessed associated changes in key signalling molecules.

The results show that aspirin alone was highly effective at inhibiting 2D culture cell proliferation and inducing cell death in the androgen dependent, p53 positive LNCaP cell line while therapeutic concentrations of metformin had no effect on either of these outputs. In the more advanced androgen independent, p53 negative, PTEN negative PC3 cell line treatment with aspirin alone decreased cell proliferation while cells were unresponsive to metformin. Together the drugs additively inhibited cell proliferation in 2D culture and importantly the combination decreased spheroid growth in 3D cell culture more than when dosed with each drug individually. With the non-tumorigenic PNT2s, neither metformin nor aspirin significantly affected cell proliferation or apoptosis, highlighting the possibility that the drugs more readily target cancer cells as reported in other studies. Both aspirin and metformin reduced the migratory capacity of the highly invasive PC3 cells, promoting a more epithelial phenotype as seen with an increase in E-cadherin and a decrease in N-cadherin, Slug and MMP-9. This effect was enhanced when the drugs were used in combination although it was not additive.

In conclusion, the data presented in this thesis supports the use of aspirin and metformin in combination to reduce prostate cancer proliferation and cell migration in a subset of cancers. As aspirin and metformin appear to target two distinct cancer hallmarks they have a greater chance of efficacy in heterogenous tumours, creating a strong therapeutic argument for this drug combination.

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To my friends, family and Nick, I am so lucky to be surrounded by such a loving, enthusiastic group of people. Thank you for always believing in me.

Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Table of Contents

List of Figures

List of Tables

Abbreviations

4E- BP1	4E-binding protein 1
5- AR	5α-reductase
7- AAD	7- aminoactinomycin
ACC	Acetyl-CoA carboxylase
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
AMP	AMP-activated protein kinase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APAF	Apoptotic protease activating factor
APC	Adenomatous polyposis coli
AR	Androgen receptor
Aspirin	Acetylsalicylic acid
ATP	Adenosine triphosphate
BAK	BCL-2 homologous antagonist killer
BAX	BCL associated X protein
BCL-2	B-cell lymphoma 2
bHLH	Basic helix-loop-helix
BID	BH3-interacting domain death agonist
BIM	BCL-2 interacting mediator of cell death
BRCA1/2	Breast cancer gene 1/2
BSA	Bovine serum albumin
CDKs	Cyclin-dependent kinases
COX	Cyclooxygenase
CTBP	C-terminal binding protein
DAPI	4',6-diamidino-2-phenylindole
DHT	Dihydrotestosterone
DPM	Disintegrations per minute
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
EP3	Prostaglandin EP3 receptor

EZH2	Zeste homologue 2
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin embedded
FGF	Fibroblast growth factor
FOXO	Forkhead box O3
GM	Growth media
GPCR	G-protein coupled receptor
HIF-1α	Hypoxia-inducible factor 1α
HOXB13	Homeobox 13
IGF	Insulin-like growth factor
IGF1R	Insulin growth factor receptor 1
IKK	I_KB kinase
IL-6	Interleukin 6
IRS-1	Insulin receptor substrate 1
JAMS	Junctional adhesion molecules
LKB1	Liver kinase B1
LSD1	Lys-specific demethylase 1
Mcl-1	Myeloid leukemia cell differentiation protein
MCM-2	Minichromosome maintenance complex component 2
Metformin	1,1-dimethylguanidine
MMP-9	Matrix metalloproteinase-9
MOMP	Mitochondrial outer membrane permeabilization
mTOR	Mechanistic target of rapamycin
N-cadherin	Neural cadherin
NF_KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKT	Natural Killer T
NSAID	Nonsteroidal anti-inflammatory drug
OCT	Organic cation transporter
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PCNA	Proliferating cellular nuclear antigen
PDGF	Platelet derived growth factor
PFA/PBS	Paraformaldehyde in PBS

PGE2	Prostaglandin E2
PGH2	Prostaglandin H2
PGI2	Prostacyclin
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIN	Prostatic intraepithelial neoplasia
PIP_3	Phoshatidylinositol (3,4,5) triphosphate
PRC2	Polycomb repressive complex 2
PSA	Prostate specific antigen
Puma	p53 upregulated modulator of apoptosis
RCT	Randomized control trials
S6K1	S6 kinase 1
SCC	Squamous cell carcinoma
SFM	Serum free media
SHBG	Sex hormone-binding globulin
SMAC	Second mitochondria-derived activator of caspases
SNAI1	Snail
SNAI2	Slug
SNAI3	Smuc
SPARC	Secreted protein acidic and rich in cysteine
Src	Steroid receptor coactivator
STAT3	Signal transducer and activator of transcription 3
SUV39H1	Suppressor of variegation 3–9 homologue 1
TIMP1	TIMP metallopeptidase inhibitor 1
TBST	Tris buffered saline tween
TCA	Trichloroacetic acid
TEMED	N,N,N,N- tetramethyethylenediamine
Triton X-100	Iso-octylphenoxypolyethox-ethanol
TSP-1	Thrombospondin-1
TTi	Tritiated thymidine incorporation
Tween 20	Polyoxyethylene-sorbitan monolaurate
TXA2	Thromboxane A2
UGE	Urogenital sinus epithelium
VEGF	Vascular endothelial growth factor
ZO	Zonula occludens

Chapter 1

Introduction

1.1 The prostate gland

1.1.1 The structure of the prostate gland

The prostate is a walnut sized gland found in men, situated under the bladder and surrounding the urethra. Its main function is to produce the fluid portion of semen which contains the proteins and minerals that will nourish sperm. The prostate is formed during late embroyogenesis from the urogenital sinus epithelium (UGE) in a process that is dependent on androgen synthesis (1). While the androgen receptor (AR) is not initially required for development it is necessary for epithelial differentiation and expression of secretory proteins. Several other signalling pathways which mediate interactions during prostate organogenesis include the Wnt, fibroblast growth factor (FGF) and Hedgehog pathways. Inhibition of noncanonical Wnt5a signalling have been shown to lead to defects in ductal formation (2) and mutants for FGF-10 lack prostate budding (3). Similarly, the Shh ligand of the Hedgehog pathway is expressed in the UGE and loss of its signalling leads to defective prostate formation (4).

Histologically, the gland is composed of three differentiated epithelial cell types; luminal, basal and neuroendocrine which are supported by the surrounding stroma (Figure 1.1). The luminal cells form an epithelial layer which expresses characteristic luminal markers such as Nkx3.1, cytokeratins 8 and 18 and the androgen receptor (5). They also produce prostate-specific antigen (PSA), a protease which breaks down the seminal coagulum high molecular weight polypeptides into smaller proteins and causes the semen to become more liquid. PSA is also found in serum and so can be measured in the blood. Basal cells are situated between the luminal epithelium and the basement membrane and express p63, cytokeratins 5 and 14 and low or undetectable levels of AR. Some rare basal cells have unique marker expression comprising of cytokeratins 5, 14, 8, 18, and 19, GSTpi, and p63 (6) and high expression of α2β1-integrin (7) which make them candidates for epithelial stem cells (1), however there is much controversy over this with studies originally suggesting that luminal cells are the cells of prostate cancer origin (8) (9). Neuroendocrine cells are very sparse within the prostate and express endocrine markers such as chromogranin A and are AR negative. Together these cells cause the prostate to have a zonal structure consisting of the central, periureteral transition and peripheral zones surrounded by stroma (10).

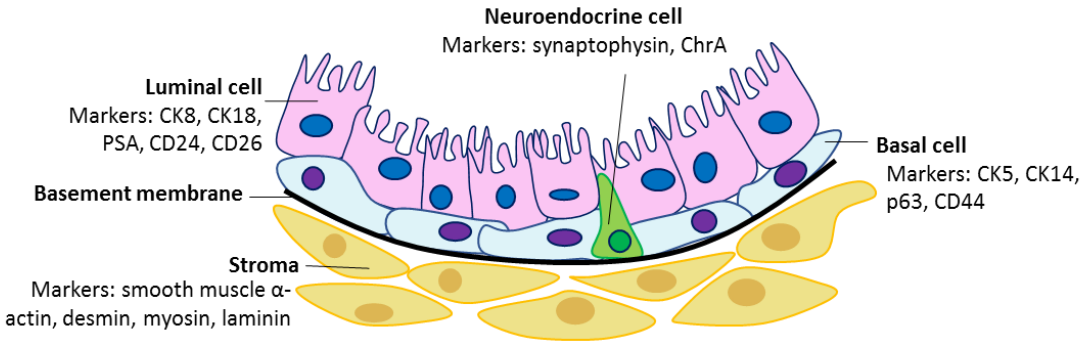


Figure 1.1: Prostate Gland Structure

In a benign prostate gland luminal, basal and neuroendocrine cells are surrounded by the stroma composed of smooth muscle tissue, fibroblasts, nerves and lymphatics. The secretory luminal cells produce prostate specific antigen (PSA) and prostatic acid phosphatase (PAP). The prostate tissue is formed of interconnected glands. Figure adapted from graphic created by *Rybak et al, 2015* (11).

1.1.2 Functional regulation of the prostate gland

The prostate requires androgens, male hormones, which are essential for prostatic development and male sex characteristics to function properly. The Leydig cells in the testes produce approximately 90% of androgens which are secreted as testosterone. The other 10% are produced by the adrenal cortex as dehydroepiandrosterone (DHEA) which can be converted to testosterone in other tissues. Testosterone is the main male hormone and it circulates around the body bound primarily to either albumin or sex hormone-binding globulin (SHBG) (12). Only 1-2% is free (13). When it reaches the prostate, it enters cells either through passive or active diffusion (14). Free testosterone or testosterone which has dissociated from albumin enters passively while testosterone which is bound to SHBG requires a membrane receptor to enter. In the cytoplasm it will either remain as testosterone or will be reduced to a more potent androgen, dihydrotestosterone (DHT), by 5α-reductase (5-AR) (15). Both testosterone and DHT can bind to the androgen receptor (AR), a commonly expressed receptor in the prostate, which will bind to specific DNA segments in promoters of hormone regulated genes called androgen response elements (AREs). The receptor-DNA complex associates with co-activators and transcriptional elements to control prostate cell division and production of PSA (Figure 1.2). Activation of this receptor in androgen dependent cells, such as those of the prostate, enhances the activity of cyclin-dependent kinases and down regulates cell cycle inhibitors such as p16 and p21 which leads to increased cell proliferation. Androgens can also block apoptosis through inhibition of caspase activity, promoting cell survival (16). Therefore, androgen receptor stimulation leads to prostatic growth and maintenance (17).

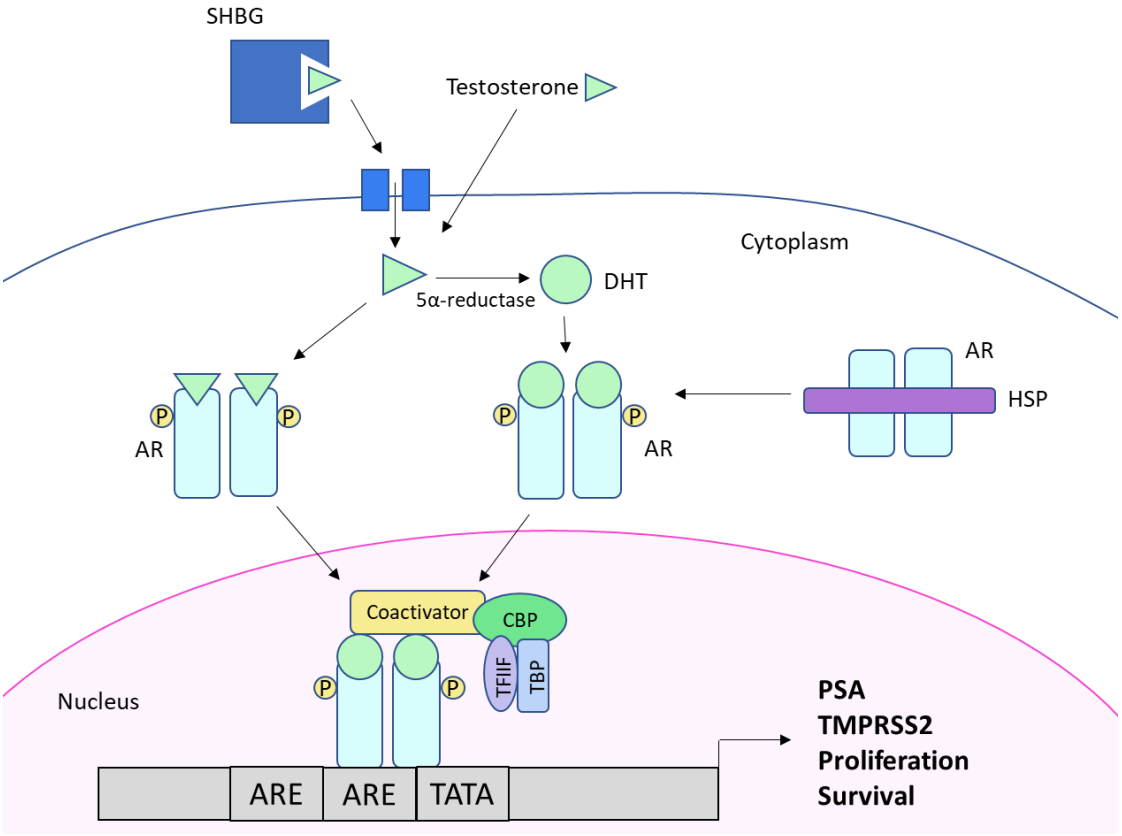


Figure 1.2: Androgen receptor activation in androgen dependent prostate cells.
Testosterone circulates in the blood bound to SHBG. In androgen dependent cells some of the free testosterone is converted to DHT by 5α-reductase. Testosterone or DHT binds to the androgen receptor which causes the androgen receptor to dissociate from the heat shock factor proteins (HSP) to which it is bound and becomes phosphorylated. The androgen receptor dimerizes and is translocated to the nucleus where it binds to AREs and increases expression of genes involved in cell proliferation and PSA production.

DHT also activates several other growth factors, including insulin like growth factor-1 (IGF-1) and epidermal growth factor (EGF) to drive cell division in the prostate (18). Other male androgens which are important for prostate development and maintenance include androstenedione, 5α-androstenedione and dehydroepiandrosterone which are converted to higher potency sex steroids and act directly on the prostate. The normal prostate gland undergoes two growth phases during the a man's lifetime (19). The first is at birth, when the prostate proliferates until it reaches the full adult size, and the second is where the prostate ceases its net growth and only proliferates for prostatic maintenance. At this stage the rate of cell turnover is balanced by the rate of apoptosis. Any imbalances to this cycle, such as androgen receptor activation, will result in abnormal growth and potentially prostate cancer progression. In the later stages of prostate cancer, the androgen receptor can be activated even in androgen deprived conditions, leading to uncontrolled tumour proliferation (20).

1.2 The hallmarks of cancer

Cancer cells are characterised by specific traits which distinguish them from normal cells (21). Acquiring these traits is a multi-step process, with a build-up of genetic and epigenetic alterations conferring growth advantages and resistance to cell death. These traits are commonly known as the hallmarks of cancer. Originally six traits were identified, including; sustained proliferative signalling, evading growth suppressors, resisting cell death, triggering migration and invasion, enabling replicative immortality, and inducing angiogenesis and vascularization (Figure 1.3) (21). However, since the original hallmarks of cancer paper was published in 2000 other traits have been acknowledged, including; deregulating cellular energetics, evading immune responses, tumour promoting inflammation, genome instability and mutation (22) and creating a tumour microenvironment (23). Each of these traits contributes to the transformation of normal cells to malignant ones and promotes cancer development and survival. In this thesis, three of the cancer hallmarks are focused on; sustained proliferative signalling, resisting cell death and triggering migration and invasion.

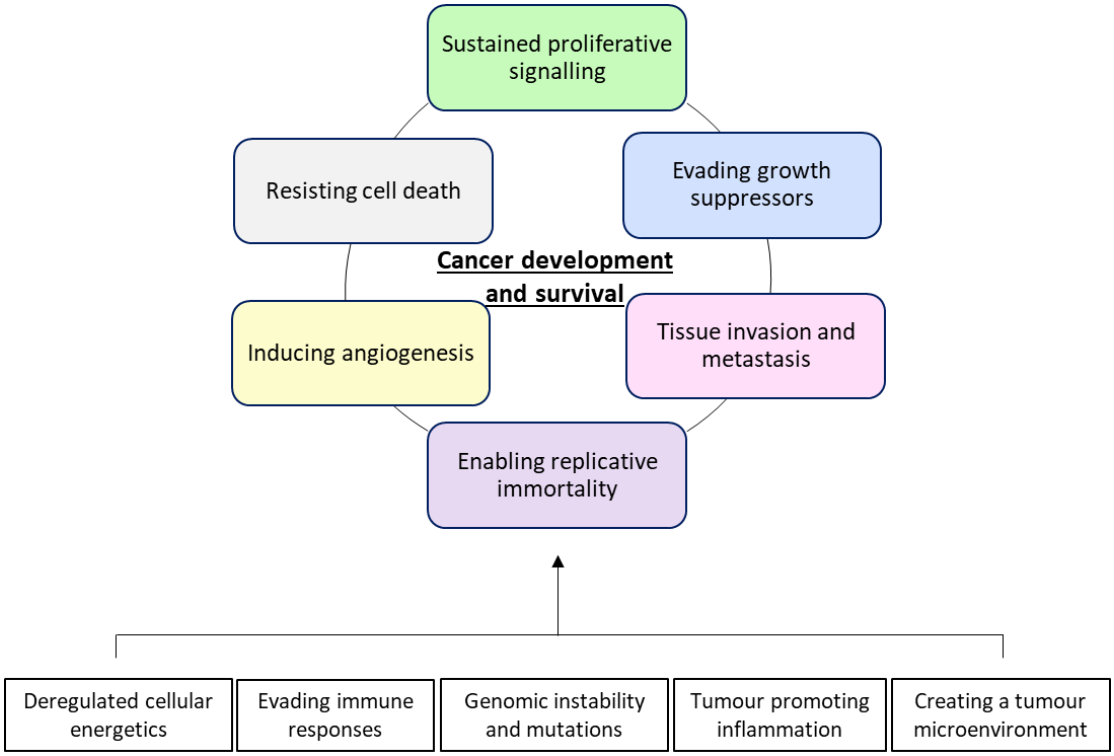


Figure 1.3: The hallmarks of cancer.
The development of cancer is a multi-step process originally defined by the cells acquisition of six biological capabilities. An additional five hallmarks have since been included, adding to the complexity that is cancer.

1.2.1 Sustained proliferative signalling

Cell proliferation is a requirement for normal tissue maintenance and development. It is a carefully regulated process, with signals which both promote and inhibit the cell cycle when necessary, carefully balancing the need for growth and repair whilst preventing uncontrolled expansion. In cancer cells however, this regulation is lost and the cells continue to proliferate even when division is no longer required. Uncontrolled growth can occur in a number of ways in cancer, most commonly because cells stop responding to inhibitory signals, they upregulate growth promoting factors, signalling receptors become overactive or receptor expression is increased (24). Cancers can also become growth factor independent, no longer requiring ligand stimulation by activating pathways downstream of the receptor. Usually these alterations are caused by mutations in genes encoding tumour suppressors or oncogenes or mutations to the receptors themselves. Retinoblastoma protein (Rb), p53 and breast cancer 1 (BRCA1) are commonly mutated tumour suppressor proteins as these proteins are fundamental for cell cycle control and regulation of apoptosis (25). Rb is expressed in every cell type, acting on the cell cycle to prevent a cell advancing to S phase from G1. Normally Rb binds to the E2F transcription factor, inactivating it, but loss of Rb causes E2F to bind to the promoter of the cyclin E gene, resulting in synthesis of Cdk2-cyclin E complexes which drive cell cycle progression (26). Mutations in genes such as cyclin D or CDK4 which control Rb phosphorylation can also mimic the effect of Rb loss. p53 is the most commonly mutated gene in cancer, deregulation of which is found in almost every cancer type. In response to DNA damage and stress p53 activates p21, a cyclin dependent kinase (cdk) inhibitor gene, which binds to various cdks to inhibit cell proliferation (27). p53 also induces apoptosis by regulating transcription of the pro-apoptotic members of the BCL-2 family amongst others (28). BRCA1/2 act to maintain genomic integrity and are nuclear proteins which co-localize with RAD-51 at sites of DNA damage to cause homologous recombination repair of double stranded DNA breaks. BRCA1 mutations cause problems with cell cycle checkpoints, chromosome duplication and DNA damage repair leading to genomic instability thereby exposing the cells to a high risk of malignant transformation (29). Oncogenes such as Ras are often activated in cancers causing enhanced proliferative signalling due to activation of pathways such as the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and the Rac/Rho signalling pathways (30).

1.2.2 Evading growth suppressors

Evading growth suppressors is a fundamental trait of cancer cells and connects to their ability to sustain proliferative signalling. Often, growth suppression is avoided through mutations to tumour suppressor genes as discussed above. However, cancer cells can also avoid growth suppression by preventing contact inhibition, a process by which cells inhibit proliferation when they become confluent, preventing the formation of dense populations and ensuring normal tissue homeostasis within the monolayer. One of the ways in which cancer cells do this is through inactivation of the neurofibromatosis type 2 (NF2) tumour suppressor gene. The NF2 gene encodes the protein product Merlin which is a downstream component of the hippo pathway and localized at the cell to cell contacts (31). Loss of NF2 causes Merlin to couple cell-surface adhesion molecules such as E-cadherin to transmembrane receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR), strengthening the cadherin mediated cell to cell contacts and preventing contact inhibition.

1.2.3 Resisting cell death

When cells become damaged they are destroyed in a genetically regulated process called apoptosis. This programmed cell death prevents the propagation of DNA errors in cells with damaged DNA and limits growth. Apoptosis can be activated by two pathways; the intrinsic (mitochondrial) or extrinsic (death receptor) pathways (Figure 1.4) (32). For both pathways caspases are central to the mechanism, initiating (caspase-2, -8, -9, or -10) but also executing (caspase-3 or -7) the process. In the extrinsic pathway extracellular ligands such as Fas or Tumour necrosis factor (TNF) bind to cell surface receptors which triggers the formation of a death-inducing signal complex (DISC) and activates initiator caspases 8 and 10. In the intrinsic pathway, following an intrinsic lethal stimulus such as genomic stress, BH3-only proteins such as BCL-2 interacting mediator of cell death (BIM), BH3-interacting domain death agonist (BID) and BCL-2 associated agonist of cell death (BAD) are activated which leads to oligomerization of BCL-2 associated X protein (BAX) or BCL-2 homologous antagonist killer (BAK). This triggers mitochondrial outer membrane permeabilization (MOMP) and causes the release of pro-apoptotic proteins cytochrome c and second mitochondria-derived activator of caspases (SMAC) into the cytoplasm. Cytochrome c then binds to apoptotic protease activating factor (APAF-1) to form the apoptosome which is a caspase 9 activating complex (33). SMAC increases caspase activation by binding and neutralizing X-linked inhibitor of apoptosis protein (XIAP) which is an inhibitor of caspase 3, 7 and 9. Anti-apoptotic members e.g. BCL-2, B-cell lymphoma extra-large (BCL-XL) and myeloid leukaemia cell differentiation protein (MCL-1) sequester pro-apoptotic family members, counteracting this process. The balance between pro and anti-apoptotic BCL-2 members is essential for the initiation of MOMP (34).

While these pathways work well in normal cells, cancer cells are constantly under stress due to genomic instability, hypoxia and oncogene activation and so must avoid the intrinsic apoptotic pathway to survive. Cancer cells can bypass the apoptotic response either transcriptionally, translationally or post-translationally. An upregulation of anti-apoptotic and downregulation of pro-apoptotic gene expression confers apoptotic resistance by reducing the signalling for cell death. In normal cells, p53 controls the activation of many pathways involved in apoptosis following cellular stresses including APAF-1, BAX, p53 upregulated modulator of apoptosis (PUMA) and NOXA. Similarly, cancer cells can induce stabilization or destabilization of pro and anti-apoptotic proteins. While BCL-2 and BCL-XL are relatively stable, MCL-1 is rapidly turned over, with its protein levels quickly dropping once the apoptotic response has begun (35). Its transcription is activated by pro-survival factors such as cytokines and its rapid turnover has

been shown to be reduced in cancer cells, preventing the intrinsic apoptotic process. Post translational modifications such as ubiquitination or phosphorylation can also change the functions of pro or anti-apoptotic proteins. Ribosomal s6 kinase (RSK), a family of proteins which are increased in ~50% of prostate cancers compared to normal tissue (36) have been shown to phosphorylate APAF-1, preventing its activation and activation of downstream executer caspase 9.

The ability of cancer cells to dampen the apoptotic response promotes cell survival and makes the cells more resistant to therapies such as chemo or radiotherapy, allowing the cancer to survive even when the treatment induces cellular stress and injury.

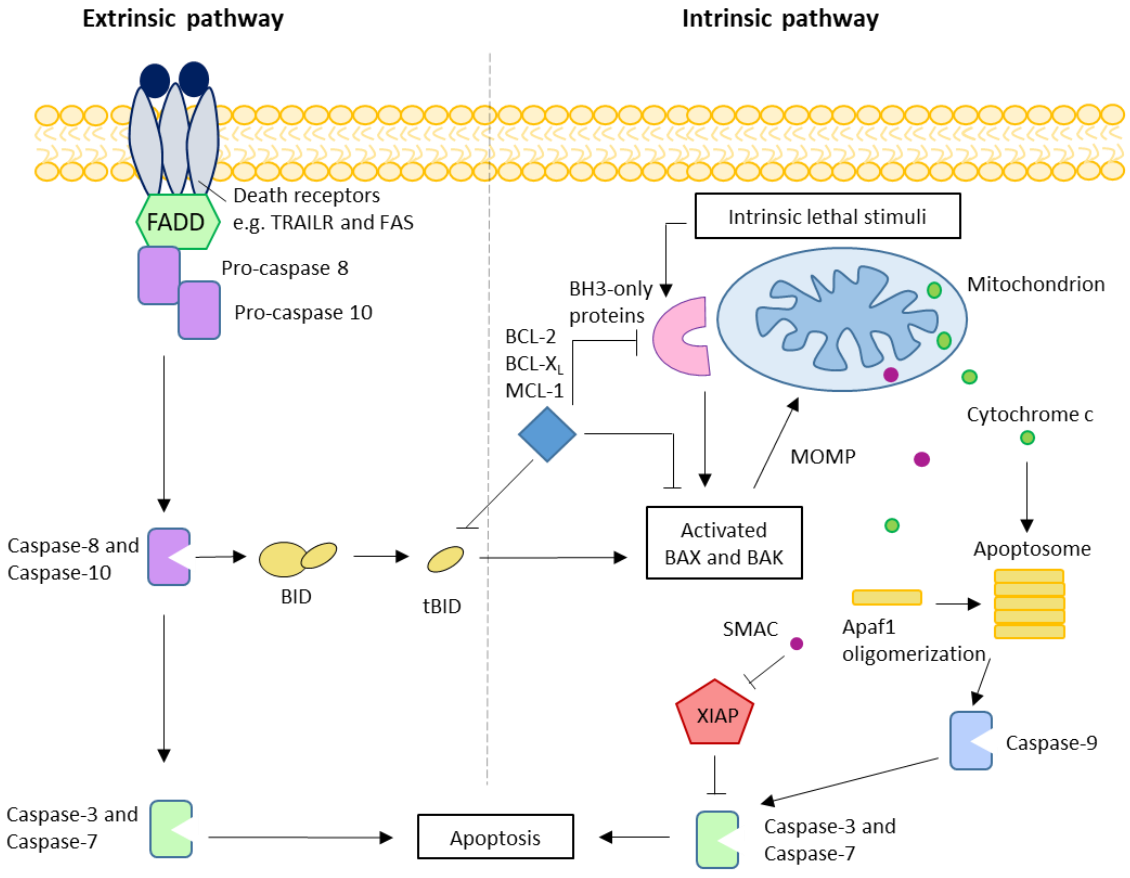


Figure 1.4: The extrinsic and intrinsic apoptosis pathways.
The extrinsic pathway is a receptor mediated programmed cell death pathway while the intrinsic pathway is initiated by intrinsic lethal stimuli such as DNA damage, ER stress, hypoxia and metabolic stress. Both pathways lead to the activation of initiator and executer caspases resulting in cell death. Image adapted from graphic created by *Marquez et al, 2013* (37).

1.2.4 Invasion and migration

Cell invasion and migration is a multi-step process by which tumour cells move out from the primary tumour and enter the circulatory and lymphatic systems to form a new tumour at a distant site. This occurs via a decrease in expression of cell-cell adhesion and attachment proteins and an increase in expression of migratory and invasive molecules in a process known as the epithelial to mesenchymal transition (EMT) (38).

The change from an epithelial cellular profile to a more mesenchymal one is seen normally in the development of structures such as neural tube formation and in wound healing but is also upregulated in cancer (39). The transition is mediated by key genes, most importantly E-cadherin, N-cadherin, vimentin, claudins, occludin, fibronectin, matrix metalloproteinases (MMPs) and a variety of transcription factors (Figure 1.5). Epithelial (E)-cadherin forms tight junctions with neighbouring cells, holding the cells firmly together. Its expression is frequently lost in cancer progression, loosening contact with adjacent cells and allowing the cells to migrate away from the primary tumour. The repression of genes encoding claudins, occludin and desmoplakin accompanies the downregulation of E-cadherin, loosening contact with the apical tight junctions (40). Conversely, Neural (N)-cadherin is frequently upregulated in cancer, enhancing cell migration and invasion and promoting cell survival. It does this by inducing cellular polarity and organising the actin cytoskeleton to form actin bundles for migration. The alteration in gene expression to promote EMT requires multiple transcription factors, including SNAIL, homodimeric and heterodimeric basic helix–loop–helix (bHLH) and zinc-finger E-box-binding (ZEB) transcription factors. Each of these transcription factor families contains several transcription factors including SNAI1 (Snail) and SNAI2 (Slug) for the SNAIL group, E12, E47, and TWIST1/2 for the bHLH group and ZEB1/2 for the ZEB group (41). Their expression is activated early in EMT and they play a central role in development, fibrosis and cancer (40). Together they regulate the expression of each other and the expression of genes involved in EMT, repressing epithelial associated genes and activating mesenchymal genes.

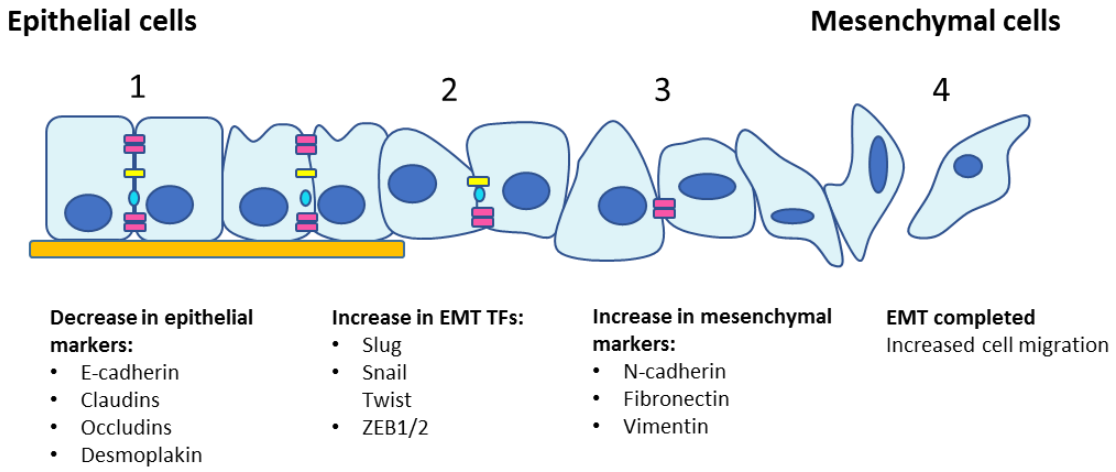


Figure 1.5: Cellular changes in the epithelial to mesenchymal transition.
Epithelial cells have an intact basement membrane (BM) and high expression of epithelial, cell adhesion and junction proteins. As the EMT process begins, these proteins decrease and matrix metalloproteinases (MMP2,3 and 9) increase to degrade the BM. EMT transcription factors also increase as do mesenchymal markers. In the final stages of EMT there is loss of cellular polarity resulting in increased cell migration.

1.2.5 Enabling replicative immortality

In normal tissue, cells undergo cell growth and division to repair and expand damaged tissues. Cells only pass through a limited number of division cycles before they either become senescent, remaining viable but entering a non-proliferative state, or die. In cancer however, cells require unlimited replicative potential to generate tumours and manage to overcome this state of crisis, becoming immortal. Evidence supports the notion that telomeres which protect the ends of chromosome are involved in this transition to immortality (42). Telomeres are composed of many hexanucleotide repeats which shorten with each cell division. Dysfunctional telomeres resulting from telomere shortening, activate DNA damage responses (DDRs) leading to cellular senescence (43). Therefore, the length of telomeric DNA determines how many cell divisions can take place. In cancer the oncogenic alterations allow the cells to bypass senescence, continuing to divide and shortening the telomeres. Critically shortened telomeres can undergo breakage-fusion-bridge (BFB) cycles where two sister chromatids which lack telomeres fuse together (44). During anaphase the chromatids are pulled apart leading to uneven chromatids and genomic instability. The absence of a functional p53 gene in cancer has been shown to allow cancer cells to survive despite telomere erosion leading to chromosomal BFB cycles (45), increasing the mutations within the tumour and accelerating the acquisition of mutant oncogenes and tumour suppressors. In addition, cancer cells activate the human TERT gene (hTERT) which encodes telomerase, a DNA polymerase which is absent in normal cells but is expressed in almost 90% of human cancers (46). Telomerase adds TTAGGG repeats to the end of the telomere by using its RNA as a template for reverse transcription (47) thereby preventing senescence and cell death. Two cancer specific hTERT promoter mutations have been identified (48) which leads to increased telomerase activity, making hTERT a potential therapeutic target for cancer. Additionally, the role of the epidermal growth factor receptor (EGFR) pathway as an important regulator of telomerase activity is becoming more apparent. The binding of a ligand to the EGFR activates three pathways which are often associated with increased proliferation; the MAP kinase pathway, the PI3K pathway and the JAK/STAT pathway. In recent studies, EGFR activation also induces overexpression of hTERT promoting telomerase expression and driving cancer progression (49). Therefore, drugs which target the EGF pathway may reduce cancer immortality as well as proliferation.

1.2.6 Angiogenesis and vascularization

Vascularization and angiogenesis are important to all tissues, generating new blood vessels to provide nutrients and oxygen and to remove metabolic wastes and carbon dioxide. These processes are activated during embryogenesis, causing vascular endothelial cells to assemble into tubes and sprout new vessel branches. After this, vascularization and angiogenesis are only activated transiently in adulthood, during wound healing or female reproductive cycling. In tumours however, normally quiescent vasculature begins to grow, in a process known as the angiogenic switch, helping to sustain and expand cancerous growths. In animal models and humans, angiogenesis is induced early on during cancer development. The angiogenic switch is controlled by factors such as signalling proteins which bind to stimulatory or inhibitory receptors to either induce or prevent angiogenesis. Vascular endothelial growth factor-A (VEGF-A) is a well-known inducer of angiogenesis, encoding ligands to generate new blood vessels (Figure 1.6). VEGF signalling can be upregulated by hypoxia and oncogenic signalling. Conversely, thrombospondin-1 (TSP-1) is a well-known inhibitor of angiogenesis which binds to receptors displayed by vascular endothelial cells to promote suppressive signals which reduce endothelial cell migration and proliferation necessary for vessel formation (50). In some tumours, oncogenes within tumour cells such as Myc and Ras can also increase proangiogenic factors through downregulation of TSP-1 (51) and indirectly promoting hypoxia to induce VEGF activation (52). Cells within the bone marrow such as neutrophils, macrophages and myeloid progenitors are also important for angiogenesis, helping to induce the angiogenic switch and sustain the formation of new blood vessels. Often the blood vessels produced by tumours display leakiness, erratic blood flow, convoluted and excessive vessel branching, distorted and enlarged vessels and are prone to microhemorrhaging due to the aberrant pro and inhibitory angiogenic signalling. Despite this, vascularization within a tumour supports cancer growth and survival.

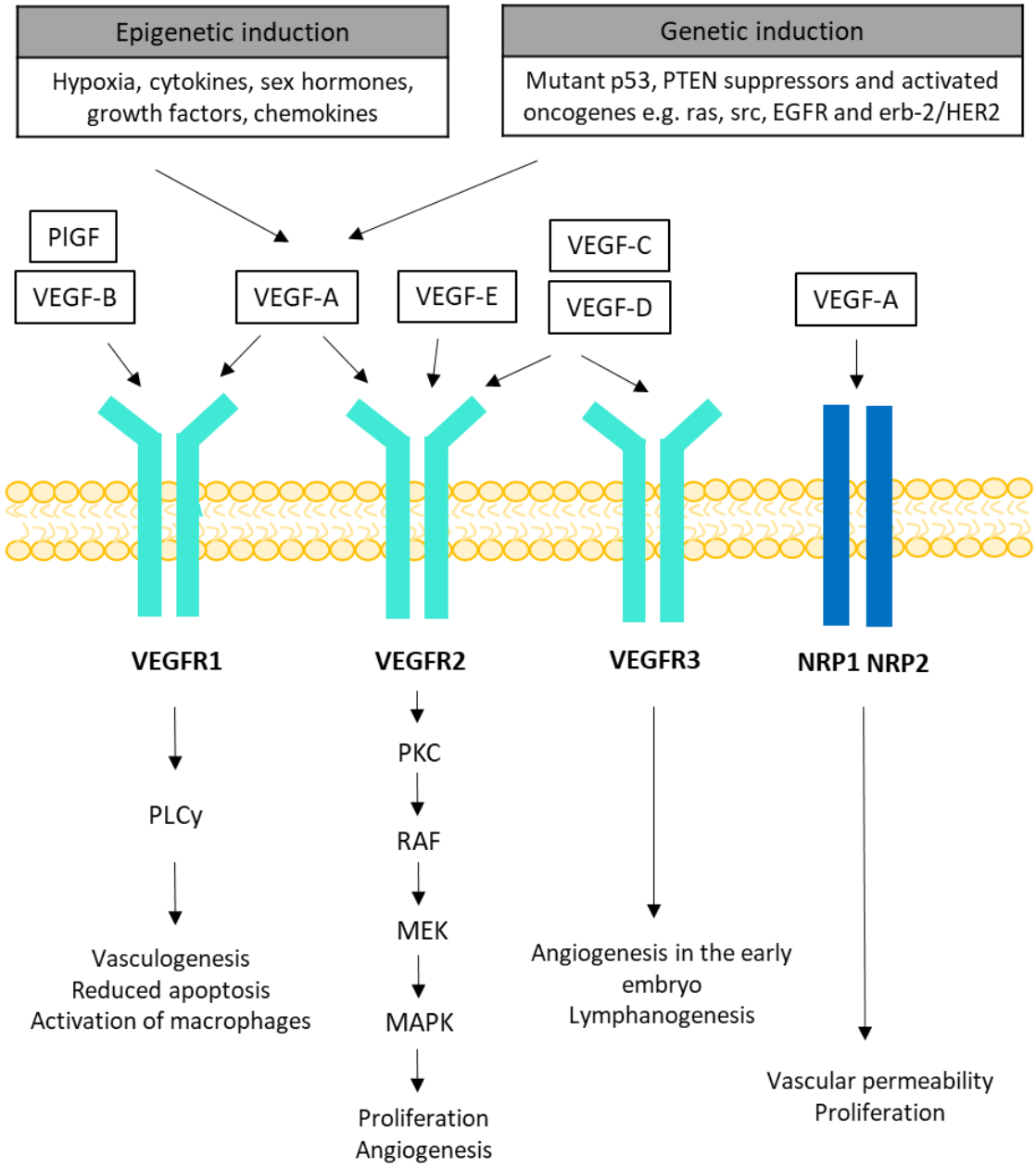


Figure 1.6: Activation of VEGFR1-3 and NIPR1/2 promotes angiogenesis and vascularization.
Epigenetic and genetic induction causes production of VEGF-A leading to activation of VEGFR1/2 and inducing vasculogenesis and angiogenesis.

1.3 Prostate Cancer - Initiation and development

Prostate cancer is the second most common cancer in males worldwide and the first among men in 84 countries (53). Its incidence is rising, with over 1.1 million new cases diagnosed in 2012 alone (54) and although this is in part due to new and improved screening methods and more frequent testing, the numbers are still staggering.

As the prostate develops, genes such as NKX3.1, FOXA1, and the AR are required for cellular differentiation during gland formation and maturation (55). However, deregulated activation of these genes within a differentiated prostate can promote excessive proliferation and the development of prostate cancer. Prostate cancer development is a multifactorial process, driven by the interplay of genetic and epigenetic aberrations which cause abnormal gene regulation. High grade prostatic intraepithelial neoplasia (PIN) is one of the most established precursors for prostate cancer with many studies identifying a strong association between PIN and adenocarcinoma (56) (57). PIN is characterized by increased cellular proliferation within the ducts of the prostate gland which results in an increase in cellular proliferation markers, luminal epithelial hyperplasia, enlargement of nuclei, cytoplasmic hyperchromasia and a reduction in basal cells (58). It shares many similar genetic alterations with prostate cancer such as the frequent 8p12-21 allelic loss, loss of heterozygosity at chromosomes 6 and 8, gain of chromosomes 7, 8, 10, and 12 and decrease in telomere length. However, when PIN progresses to cancer there is an elimination of basal cells which can be confirmed by the absence of basal cell markers such as p63 and cytokeratins 5 and 14 in biopsy specimens (59). Once the cancer has formed, other genetic alterations including myc upregulation, TMPRSS2-ERG translocations, PTEN inactivation and EZH2 overexpression promote further cancer progression driving the disease to the metastatic form (5) (Figure 1.7).

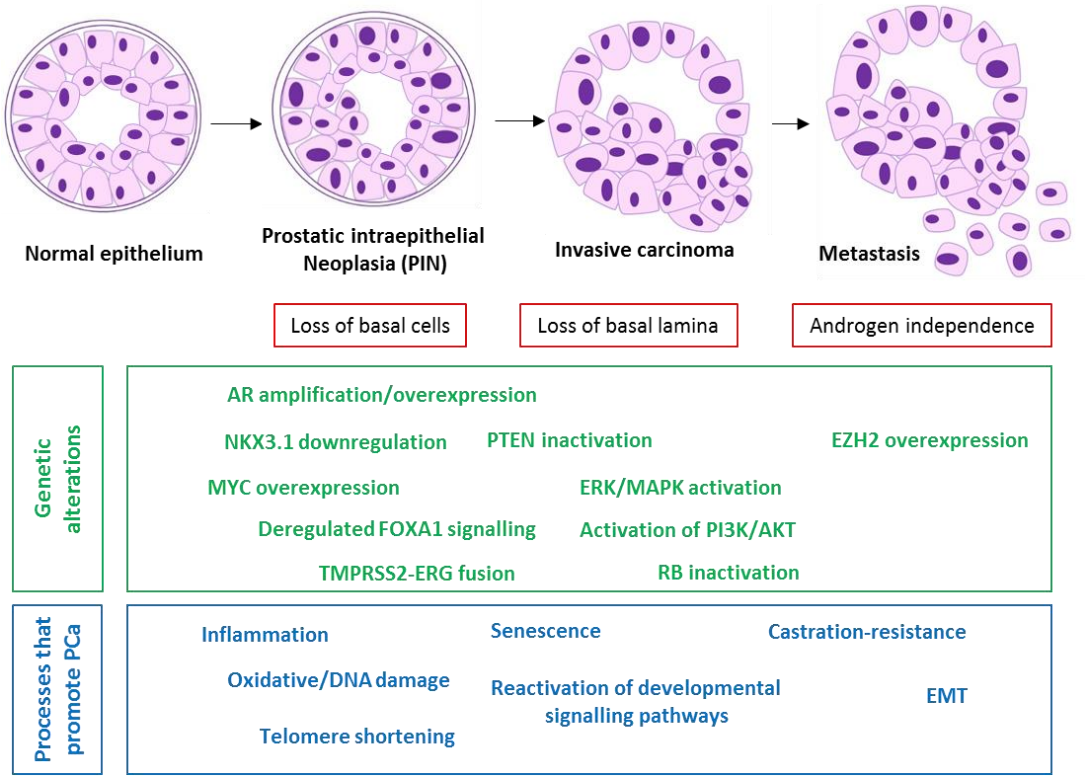


Figure 1.7: Genetic alterations and processes which promote prostate cancer development.
The development of prostate cancer is a multi-step process by which cells become malignant through a series of genetic alterations and processes.

The genetic alterations that occur during prostate cancer development also cause deregulation of key signalling pathways involved in tissue homeostasis. These pathways include the PI3K/AKT, Wnt/β-catenin, NF_KB, JAK/STAT, mitogen activated protein kinase (MAPK) pathways and growth factor and androgen receptor signalling (60).

The PI3K/AKT pathway has been shown to be increased in about 30-50% of prostate cancer patients primarily due to loss of the tumour suppressor phosphate and tensin homolog (PTEN) which inhibits PI3K/ATK signalling by dephosphorylation of PIP_3, a lipid second messenger (61). It has also been shown that PI3K/AKT signalling also favours androgen independent growth which occurs in the more advance stages of the disease by disturbing the action of ERKs. This results in increased cell proliferation despite androgen withdrawal. PI3K/AKT is also known to enhance prostate cancer invasion and metastasis by increasing the expression of metalloproteinase receptors MT1-MMP, promoting the development of metastatic cancer (62).

Aberrant AKT signalling as well as mutant forms of β-catenin which have been identified in prostate cancer (63) are known to cause phosphorylation and inactivation of glycogen synthase

kinase 3 (GSK3) resulting in β-catenin stabilization and nuclear localization (64). Upregulation of Wnt pathway members such as frizzled-4 (FZD4) in prostate cancer (65) are correlated with higher Gleason scores, increased PSA and metastasis.

The NF_KB pathways also plays a large role in prostate cancer progression and is often found to be activated due to increased expression of receptors such as tumour necrosis factor (TNF) receptor 1/2 which increases IkB degradation. The NF-$_K$B signaling pathway has an essential role in inflammation and innate immunity and has increasingly been recognized as a central player in cancer initiation and development (66). Crosstalk between this pathway and transcription factors such as p53 and STAT3 modulate NF_KB activity, causing an increase in the immune defense but can also lead to constitutive activation of NF_KB and a pro-tumorigenic effect (67). The p65 subunit of NF_KB is also reported to increase AR expression (68), and NF_KB expression is increased at both the mRNA and protein level in androgen independent prostate tumours (69).

The JAK/STAT3 pathway has long been implicated in prostate cancer development, with the DNA repair gene BRCA1 shown to increase cell proliferation and survival through interaction with JAK1/2 and STAT3 phosphorylation. In addition, amplification of the STAT5A/B locus and activation of STAT3 in prostate cancer results in increased expression and nuclear localization of STAT5 and are associated with cell cycle progression, cell survival, angiogenesis and tumour invasion.

Overexpression of IGF, FGF, EGF and KGFs in prostate cancer often results in the activation of endogenous Ras and MAPK pathways. Both pathways have been shown to induce prostate cancer cell growth independently of the AR by activating transcription factors such as c-myc and AP-1, leading to the development of castration resistant prostate cancer (70) (71). Abnormal signalling within these pathways promote cancer progression, making these pathways attractive therapeutic targets.

1.4 Prostate cancer grading and staging

Prostate cancer develops when cells in the prostate lose the ability to regulate growth. The cells grow and divide at varying rates, but prostate cancer is often a slow developing cancer contained in small foci within the prostate. This slow progression can allow men to live for decades without displaying any symptoms or requiring treatment but the risk of developing the disease increases with age, with 60% of cases occurring in men over the age of 65 (72). While some cases progress slowly, others develop rapidly and are only identified once they have become metastatic and localized treatment is no longer an option. Using drugs which could be given before diagnosis without affecting a person's quality of life to slow down cancer progression could benefit these cases, giving more time to identify the cancer in its earlier stages.

Prostate cancer is divided into stages depending upon its location and how advanced it is, most commonly using the American Joint Committee on Cancer (AJCC) TNM system. This system separates cancers depending on five key points; the main tumour (T) (
Table **1-1**), whether the cancer has spread to nearby nodes (N) (Table 1-2), whether the cancer has metastasised to other parts of the body (M) (Table 1-3), the PSA level at time of diagnosis and the Gleason score taken during a biopsy or surgery. Once the stage for each category has been determined they can be combined to give the overall cancer stage (73).

Table 1-1: Stages of prostate cancer according to the size and localization of the main tumour.

Tumour (T) stage	Explanation
TX	The main cancer site cannot be assessed.
T0	There is no sign of cancer.
T1a	Cancer is usually found during surgery for benign prostatic hyperplasia but is in no more than 5% of the tissue removed during surgery.
T1b	Cancer is found during surgery for benign prostatic hyperplasia but is in more than 5% of the tissue removed.
T1c	Cancer is found by needle biopsy that was done because of an increased PSA.
T2a	Cancer is in half of one side of the prostate gland.
T2b	Cancer is in more than half of one side of the prostate gland.
T2c	Cancer is in both sides of the prostate gland.
T3a	Cancer has broken through the capsule of the prostate gland.
T3b	Cancer has spread to the seminal vesicles.
T4	Cancer has spread to other organs nearby.

Table 1-2: Stages of prostate cancer depending on whether the cancer has spread to the lymph nodes.

Node (N) stage	Description
NX	The lymph node cannot be assessed.
N0	There are no cancer cells in nearby lymph nodes.
N1	There are cancer cells in lymph nodes near the prostate.

Table 1-3: Stages of prostate cancer depending on whether the cancer has metastasised and its location.

Metastatic (M) stage	Explanation
M0	Cancer has not spread to other organs.
M1a	There are cancer cells in the lymph nodes outside the pelvis.
M1b	There are cancer cells in the bone.
M1c	There are cancer cells in other places.

PSA is a protein produced by both normal and cancerous prostate cells so in males it is normal to have small amounts of PSA in the blood. A raised PSA level can be indicative of prostate cancer and as a rough guide men with a PSA level of above 3 ng/ml in the age group 55-60 are often sent to a specialist for further testing (74). However due to a high number of false positive or false negatives results, the use of PSA as a biomarker for prostate cancer is controversial. Indeed, in a recent randomized control trial examining the use of PSA screening intervention vs standard practice the study did not support the use of PSA testing for population-based screening (75). Similarly, the prostate, lung, colorectal and ovarian (PLCO) Cancer Screening Trial in 2009 indicated no difference in mortality between the PSA screening and control group after a 7 to 10 years follow-up (76). Contradictory to this, the European Randomized study of Screening for

Prostate Cancer trial showed a 27% decrease in mortality for those who had undergone PSA testing after a follow-up of 13 years (77). While the benefit of PSA testing is debated, it has been the most prominent screening tool for prostate cancer since the 1980s (78) and is still recommended to be used before further testing.

A Gleason score is a prostate cancer grade between 1 and 5 given by pathologists when they examine cancerous cells taken during a biopsy or surgery (Figure 1.8). Since not all areas of the prostate will be the same, two scores are given, the first describes the most common grade that the tumour is made up of and the second score is the second most common grade (79). Grades 1 and 2 are not commonly used for biopsies as the biopsy is only taken when there is an indication of cancer and so most samples tend to score a 3 or higher. The two scores are added together to give the Gleason score.

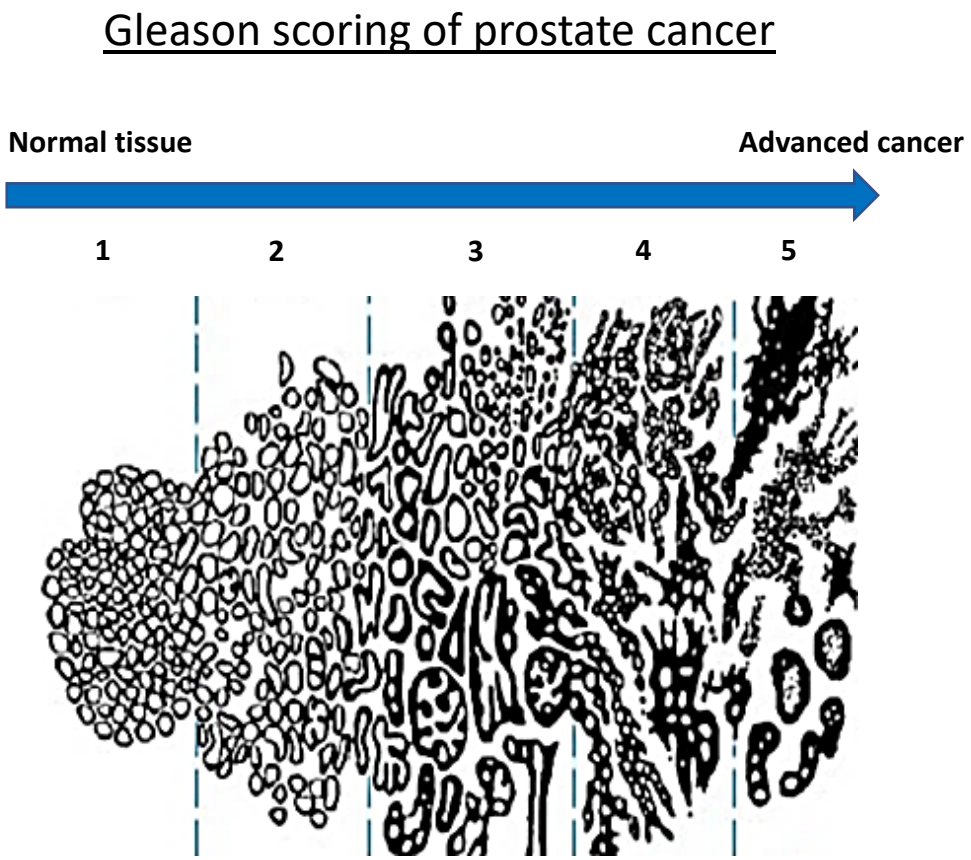


Figure 1.8: Gleason scoring in prostate cancer.
A score of 1 usually represents normal prostate tissue whereas the closer the score is to 5 the more advanced the cancer is. **1-** The cells are well differentiated with small, uniform glands. **2-** There is more space between the glands. **3-** There is infiltration of cells from glands at the margins. **4-** There are irregular masses of neoplastic cells with few glands. The cells are poorly differentiated. **5-** There are sheets of cells with no or only the occasional gland. Figure adapted from figure on the prostate conditions education council website (80)

While the TNM grade system is commonly used to characterise prostate cancer, prostate cancer can also be more simply divided into three stages; localized to the prostate, locally advanced and metastatic prostate cancer (81) (Table 1-4).

Table 1-4: Stage of prostate cancer compared to TNM staging.

Stage of cancer	TNM staging
Localized	T1, N0, M0 T2, N0, M0
Locally advanced	T3, N0, M0 T4, N0, M0 Any T, N1, M0
Metastatic	Any T, any N, M1

1.5 Prostate cancer risk factors

The risk of prostate cancer and whether it develops to a clinically relevant disease is influenced by many genetic, lifestyle and environmental factors with the most prominent risk factor being age (Figure 1.9). The risk of developing prostate cancer increases with age, with over 60% of cases being diagnosed in men over 65 (72).

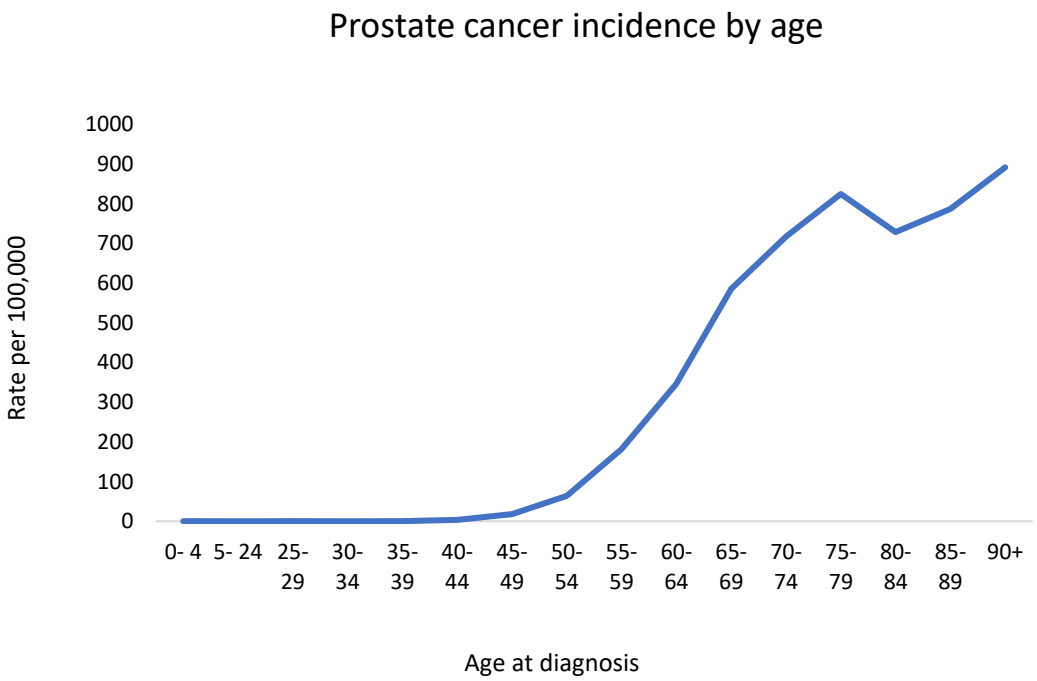


Figure 1.9: Prostate cancer incidence by age.
Graph created from data obtained from Cancer Research UK. Original data was provided by the Office for National Statistics, July 2017, ISD Scotland, August 2017, the Welsh Cancer Intelligence and Surveillance Unit, Health Intelligence Division, Public Health Wales, October 2017 and the Northern Ireland Cancer Registry, July 2017 (82).

Most cases of prostate cancer are not caused by inherited genes and occur due to somatic mutations which build up naturally over time due to DNA damage. However, inherited mutations found in the breast cancer 1 (BRCA1), breast cancer 2 (BRCA2) and homeobox 13 (HOXB13) genes do account for about 3% of the hereditary cases (83) (84) (85). Lifestyle and environmental factors also play a large role in the development of the disease, influencing overall health and exposure to risk factors. Studies have shown that the cancer risk for second generation migrants shifts after adopting the lifestyle of the country they have moved to (86). In 2004, the The Multiethnic Cohort (MEC) Study examined cancer rates in Japanese migrants who had moved to Hawaii, comparing colorectal, breast and prostate cancer rates in the Japanese, Hawaiian Caucasians and Japanese's migrants (87). The study found that Japanese

cancer rates were lower than in Hawaiian Caucasians but that cancer rates in first and second-generation Japanese migrants who had moved to Hawaii were similar to those of Hawaiian Caucasians. Other migration studies have also highlighted the effect of lifestyle and environmental exposure on cancer rate (88). In addition, ethnicity has also been shown to play a role in prostate cancer risk with men of African origin having a higher risk of prostate cancer than White and Asian males (89). Comparing age standardised rates for each group, White, Black and Asian males have a rate of prostate cancer of 96-66, 120.8- 247.9, and 28.7-60.6 per 100,000 men respectively (82). It is thought that genetic and epigenetic differences contribute to the differences in risk however no clear molecular basis has been identified (90).

1.6 Prostate cancer treatment

Prostate cancer treatment is selected depending on the grade and stage of the cancer with the main treatment options including surgery, radiotherapy, chemotherapy and hormone therapy (91) (Table 1-5). As these options can have long-term side effects, doctors tend to avoid treatment if it is low risk, localized prostate cancer and suggest active surveillance unless it is unsafe to do so. Regular tests such as PSA testing, digital rectal examinations, prostate biopsies and MRI scans are conducted to monitor the cancer (92). Treatment occurs if tests show that the cancer is growing or if the person requests treatment. However, once the cancer has progressed to advanced localized or metastatic, treatment is necessary.

Table 1-5: The most commonly used treatments for prostate cancer.

Surgery- Radical prostatectomy	Radiotherapy	Hormone Therapy	Chemotherapy	Other treatments
Laparoscopy (keyhole surgery)	External Beam radiation	Adrenal inhibitors e.g. Abiraterone Acetate	Docetaxel	Radium 223
Robotic prostatectomy	Brachytherapy (internal radiation)	Androgen receptor inhibitors e.g. enzalutamide	Cabazitaxel	High-intensity focused ultrasound (HIFU)

A radical prostatectomy is an option for men who have localized or advanced localized prostate cancer and in this procedure the prostate is completely removed. This aims to remove all cancerous cells to reduce recurrence, however, the side effects can include problems urinating and impotence as the nerves surrounding the prostate may be damaged.

For treatment with radiotherapy the structure of the prostate is mapped out and radiotherapy and in the UK is given in 20 fractions over 4 weeks (93). This treatment is much less invasive than the prostatectomy allowing the person to carry on with normal activities while having treatment, but the procedure has side effects and if the cancer comes back or spreads surgery may no longer be possible.

Hormone replacement therapy is often used in combination with other treatments and causes the patient's body to stop producing androgens or prevents them from reaching the cancer cells (94). Luteinizing hormone-releasing hormone (LHRH) agonists, gonadotrophin releasing

hormone (GnRH) agonists and anti-androgens are the most common forms of hormone treatment. LHRH agonists bind to the LHRH receptor to stimulate the pituitary gland to produce luteinizing hormone (LH) which in turn stimulates the production of testosterone by the testes (95). Negative feedback due to high levels of LH prevents LH production and causes a reduction in testosterone. Similarly, GnRH blockers bind to GnRH receptors in the pituitary gland which block the production of LH. Anti-androgens such as bicalutamide antagonize the AR by binding to a binding pocket next to the hormone binding site which distorts the coactivator binding site and prevents gene transcription (96). While hormone therapy will not remove the cancer, it will delay symptoms and cancer growth. However, over time prostate cancer cells can become androgen independent, continuing to grow even during androgen deprivation (20). At this stage hormone therapy will no longer be beneficial. The side effects can also negatively impact on a person's standard of life causing changes such as, extreme tiredness, weight gain, bone thinning and alterations in mood.

Chemotherapy is a treatment often used alongside hormone therapy or after hormone therapy has been given, most often to treat advanced prostate cancer. It uses anti-cancer drugs to target all the rapidly dividing cells in the body with the aim of shrinking the cancer and slowing its growth. The most commonly used chemotherapeutic drug for prostate cancer is docetaxel which binds to microtubule binding sites to increase microtubule assembly and stabilize polymers to prevent cell division (97). This causes cell cycle arrest and apoptosis. Other taxels such as Cabazitaxel and paclitaxel are also commonly used in prostate cancer treatment (98), disrupting microtubule dynamics and inhibiting mitosis. Unlike taxels, mitoxantrone intercalates with DNA causing single and double stranded breaks and suppresses DNA repair by inhibition of topoisomerase II, sending the cells into programmed cell death (99).While chemotherapy is beneficial in controlling symptoms, it is not a localized treatment and has side effects such as hair loss, tiredness and sickness.

Although these are the main treatment options others are sometimes selected depending on the person's requirements. Each option is different, but all have their own advantages and limitations. While men can live for decades with localized prostate cancer identifying drugs which can delay the need for treatment or help to control the cancer without such a large effect on the person's health is an extremely important goal.

1.7 Drug repurposing for cancer treatment

Drug repurposing is the application of an existing therapeutic to the treatment of a new disease or indication (100). It is an attractive prospect due to the expense and time it takes to bring a drug to market which is usually 10-15 years (101). Furthermore, most non-repurposed drugs fail during clinical trials with less than 15% of drugs that undergo clinical development receiving approval (102). For drug repurposing computational screening identifies the most promising candidate for a disease while high-throughput testing examines the effect of many drugs at once. Identifying new uses for drugs which are known to be well tolerated brings benefits to patients much more quickly and without the risks of a new, unfamiliar drug. In cancer treatment, repurposing drugs may delay the need for aggressive treatments such as chemo- and radiotherapy and improve patient survival without adding unknown risks (103). Two such drugs which have been suggested to possess anti-cancer effects are aspirin and metformin. While

studies have shown that both drugs may reduce cancer progression, their mechanism of action is still not fully understood as discussed in Sections 1. 4 and 1.5.

1.8 Aspirin

Aspirin (acetylsalicylic acid) is a non-steroidal anti-inflammatory drug (NSAID), exerting its anti-inflammatory effects by interfering with the production of prostaglandins and thromboxanes through the inhibition of cyclooxygenase 1 and 2 (COX 1 and 2) enzymes (Figure 1.10) (104). What differentiates aspirin from other NSAIDs, such as ibuprofen, is that it is an irreversible COX inhibitor, acetylating the active site serine residues of the COX enzymes, serine 530 (COX-1) and serine 516 (COX-2). This prevents arachidonic acid from binding to the catalytic site, inhibiting its metabolism and the formation of prostaglandins. There are four principal prostaglandins generated *in vivo:* prostaglandin D_2 (PGD_2), prostaglandin (PG) E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostacyclin (PGI_2) (105). Aspirin is most commonly prescribed for its anti-inflammatory and analgesic properties as well as its ability to prevent blood clotting and reduce fever. Numerous clinical trials have identified aspirin as a novel candidate for the treatment of a variety of different diseases including cardiovascular disease (106) and arthritis (107). However, in the past few decades it has also been suggested to possess anti-cancer effects, with the potential to be used as a cancer treatment.

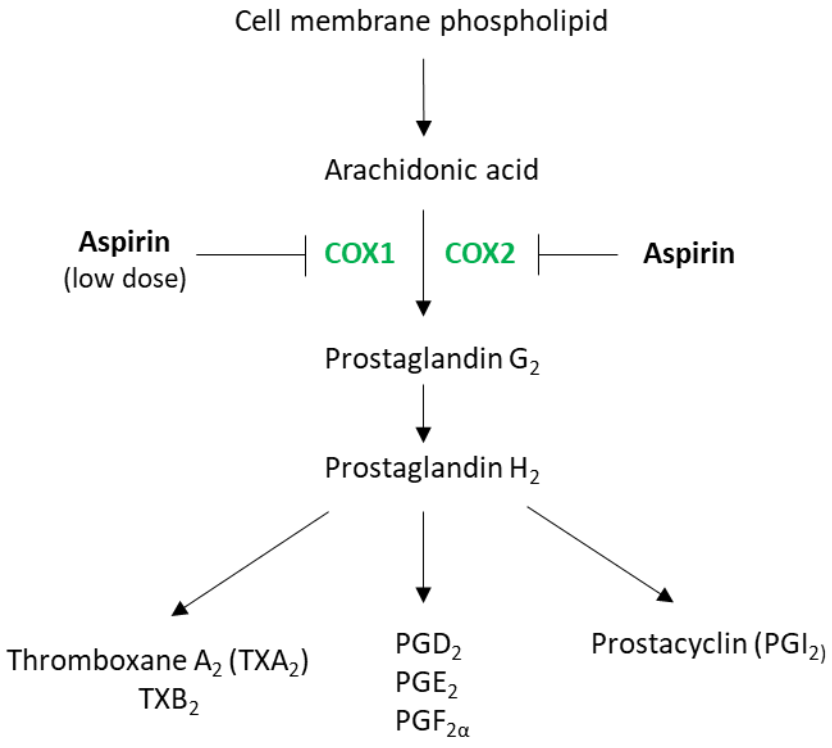


Figure 1.10: Aspirin inhibits COX1/2 to prevent the production of thromboxane's and prostaglandins. Membrane phospholipids are acted upon by phospholipase A_2 to form arachidonic acid. Aspirin acetylates COX1 and 2 enzymes, inhibiting arachidonic acid from binding to the COX catalytic site and preventing the synthesis of thromboxanes and prostaglandins

1.8.1 Epidemiological studies examining aspirin and cancer treatment

Aspirin is one of the most commonly used drugs in the world which makes epidemiological studies extremely powerful in providing evidence to determine whether aspirin is beneficial for cancer prevention and treatment or not. In a study that pooled data from seven randomised trials to collect information on 23,535 patients it was seen that after 5 years of aspirin use the risk of cancer death was 20% lower in the aspirin groups than in the control groups (108). This risk remained lower after 20 years and the benefit of aspirin increased with the duration of the trial treatment. For stomach, colorectal and prostate cancers the effect of aspirin on cancer death was more delayed than for oesophageal, pancreatic, brain and lung cancers. This study also determined that the cancer benefit did not increase when doses beyond 75 mg of aspirin were taken daily. Currently aspirin is used at a range of doses depending on what the treatment is for. Generally, the doses are around 75 mg for antiplatelet activity, 325-600 mg for analgesic properties, and 1200 mg for anti-inflammatory action (109). The fact that a benefit with aspirin treatment at doses as low as 75 mg has been seen is encouraging, indicating that anti-cancer effects occur at doses which are known to be well tolerated. A number of other population studies have also supported the idea that aspirin possesses anti-cancer effects and have stimulated huge interest in aspirin's association with cancer risk (110) (111) (112) (113) (114).

Epidemiological studies examining the effect of aspirin specifically on prostate cancer have also found an inverse association with aspirin consumption and prostate cancer risk. In a meta-analysis involving 24 studies, 14 case-control and 10 cohort studies that assessed the effect of NSAIDs on prostate cancer risk they concluded that taking aspirin regularly was associated with a reduction in overall and advanced prostate cancer risk (pooled RR= 0.86, 95% CI =0.81–0.92; pooled RR= 0.83, 95% CI= 0.75–0.91, respectively) (115). This reduction in risk became stronger when patients took aspirin for 4 or more years indicating that the benefit increases with a longer duration of use. Other meta-analyses have also found similar benefits (116). Interestingly, it has also been seen that the associations been aspirin use and prostate cancer mortality were stronger before PSA testing was common (117). PSA testing has led to earlier prostate cancer diagnosis, on average by 11 years. This change in strength of association may indicate that the benefit of aspirin occurs later in the disease progression and so with more people being diagnosed in the earlier stages of prostate cancer by PSA, less of an effect with aspirin is seen.

Cancers which have shown an inverse association with aspirin consumption include pancreatic (118), prostate (111), breast (119), squamous cell carcinoma of the oesophagus (120) and ovarian (121) cancers.

While the bulk of studies indicate a benefit of aspirin in the reduction of prostate cancer risk other studies have found no association. In a nested case-control study which examined the effect of five classes of NSAIDs on prostate cancer risk, propionates were seen to cause a modest reduction in prostate cancer risk but no effect of aspirin was observed (OR= 1.01; 95% CI =0.95–1.07) (122). It has been suggested that as aspirin is metabolised extensively in the liver a lower concentration reaches the prostate gland, limiting the effect of aspirin.

Current recruitment is underway for a large clinical trial, Add-Aspirin, which aims to assess whether regular aspirin use in patients after treatment for early stage solid tumours prevents recurrence and prolongs survival (123). It is a phase 3, double-blind, randomized trial and examines patients with breast, colorectal, prostate and gastroesophageal cancers who have undergone standard therapy. These patients will take placebo, 100 mg or 300 mg of aspirin daily for 5 years. The data obtained will provide large scale information on the regular use of aspirin from centres across the UK, republic of Ireland and China and will aid in determining whether aspirin is beneficial in post prostate cancer treatment.

1.8.2 Molecular mechanism of aspirin in cancer treatment

While epidemiological evidence strongly supports an association between aspirin use and a reduction in cancer risk, *in vitro* and *in vivo* work aims to test this claim in a more standardized condition. This allows variables in the experiment to be controlled and so reduces the complexity of the system to give a clearer picture of what effect altering one of the variables will have. *In vitro* and *in vivo* work also attempts to identify the signalling pathways involved to understand the mechanisms behind how the drug is exerting its anti-cancer effects. Aspirin and metformin are pleiotropic drugs in that they impact many different signalling pathways. However, the focus in research has mainly been on the effect of aspirin or metformin on cell proliferation, cell survival and cell migration and invasion as well as testing whether using the drugs in combination with other treatments such as radio and chemotherapy improves patient outcome.

1.8.2.1 The effect of aspirin on cell proliferation

Cox dependent mechanisms

The mechanisms behind aspirins anti-cancer effects are often divided into cyclooxygenase (COX)-dependent and COX-independent pathways. The COX pathway is one of the most well-known targets of aspirin, with the drug irreversibly inhibiting COX-1 and COX-2 (Chapter 1, Section 1.8), disrupting the generation of molecules which play a key role in the generation of the inflammatory response. Chronic inflammation has been suggested to be an important factor in promoting prostate cancer progression and is a common problem in men with prostate cancer, causing unpleasant symptoms due to enlargement of the prostate (124). Indeed, COX-2 has been shown to be up-regulated in several cancers including, breast, colorectal, pancreatic, head and neck and prostate cancer (125). In a study that examined the expression of COX-1 and 2 in tumour specimens it was seen that human prostate carcinoma tissue had higher levels of COX-2 expression than benign prostatic hyperplasia, prostatic intraepithelial neoplasia and normal prostate tissue (126). It is thought that the prostaglandins produced by this enzyme, particularly prostaglandin E2 (PGE2) can promote cell proliferation and migration. PGE2 can bind to four G-protein-coupled receptors: EP1, EP2, EP3 and EP4. Receptors EP2 and EP4 couple to stimulative G proteins and increase intracellular cAMP while EP3 binds to an inhibitory G protein and decreases cAMP. Previously, aspirin has been shown to upregulate EP3 expression which suppresses LNCaP prostate cell proliferation and downregulates the androgen receptor,

preventing progression to castration resistant prostate cancer (127). Furthermore, in mice injected with PC3 prostate cancer cells treatment with a COX-2 inhibitor, NS938, reduced tumour cell growth and a regression of existing tumours (128). This was due to an increase in apoptosis and a reduction in VEGF expression and MVD, indicating that through COX inhibition aspirin reduces both cell proliferation and angiogenesis.

Cox independent mechanisms

In addition to its COX dependent effects aspirin also has been proposed to exert its anticancer action through multiple other signalling pathways. Aspirin is known to interfere with the AKT/PI3K pathway, a major pathway involved in regulating cell metabolism, growth, proliferation, survival and migration. The AKT/PI3K pathway is activated by stimulation of receptor tyrosine kinases, G-protein coupled receptors, integrins, cytokine receptors and other stimuli which induce the production of PIP3 by PI3K. These activate AKT, a protein which controls the expression of several proteins including AMPK, mTOR, cell cycle proteins and FOXO, and is often aberrantly activated in cancer. Aspirin has been shown to reduce mTOR signalling in colorectal cancer cells by inhibiting its effectors, s6K1 and 4E-BP1, causing mTOR induced autophagy (129). In addition, aspirin alters nucleotide ratios in the cells, activating the energy sensor AMPK which has an inhibitory effect on mTOR. Knockdown of AMPK confirms that aspirin inhibits mTOR both in an AMPK dependent and independent manner. This strong inhibition of mTOR is extremely valuable for cancer treatment as mTOR is a key gene involved in protein synthesis and cell proliferation. In another study which examined the effect of aspirin in murine H22 hepatocarcinoma and S180 models it was seen that aspirin decreased angiogenesis and promoted autophagy in tumours (130). p-mTOR, HIF-1α, and VEGF-A expression were decreased while ULK1 and LC3A expression were increased with treatment. This change in protein expression was thought to contribute to the phenotypic effects observed. Aspirin has been shown to activate AMPK/PI3K signalling or inhibit its downstream effector mTOR in multiple other studies (131) (132).

Deregulation of the Wnt/β-catenin signalling pathway is a common occurrence in many types of cancer and has been suggested to be an early event in tumorigenesis. Target genes of the Wnt/β-catenin pathway include those which regulate cell proliferation and apoptosis. Studies have shown an up-regulation of Wnt1 expression in several prostate cancer cell lines and tissues as

well as aberrant β-catenin localization which provides evidence of an important role of Wnt/β-catenin signalling in prostate carcinogenesis (133). Aspirin has long been known to target the Wnt/ β-catenin pathway and in a study which looked at levels of Wnt/β-catenin signalling in different prostate cell lines, it was seen that the highly invasive cancerous cell lines, DU145s and PC3s, exhibited higher levels of Wnt signalling than the less invasive LNCaPs and the non-cancerous cell lines (133). When cells were dosed with a nitric oxide donating aspirin derivate cell proliferation was inhibited and this was in part through the Wnt/β-catenin pathway. Wnt/β-catenin signalling has also been demonstrated to activate the androgen receptor to promote prostate cell proliferation (134). A specific interaction between β-catenin and the androgen receptor has also been demonstrated in both prostate and neuronal cells(135). In this, β-catenin translocates from the cytoplasm to the nucleus bound to the androgen receptor. This movement is stimulated by exogenous androgens and is independent of Wnt molecules.

The NFκB and STAT3 pathways are two major pathways implicated in cancer development, and are both activated due to inflammation, stress and cytokines. Epidemiological evidence has identified a link between inflammation and a predisposition to cancer and has indicated that inflammation can create a microenvironment more suitable for tumour initiation (136). The NFκB pathway has an essential role in inflammation and innate immunity and has increasingly been recognized as a central player in cancer initiation and development (66). Crosstalk between this pathway and transcription factors such as p53 and STAT3 modulate NFκB activity, causing an increase in the immune defense but also can lead to constitutive activation of NFκB and a pro-tumorigenic effect (67). Aspirin inhibits the canonical NFκB pathway, preventing phosphorylation of IκB kinase (IKK) α/β and IκB-α degradation as well as p65 nuclear translocation (137) (138). Studies have shown that aspirins inhibition of IκB phosphorylation induces apoptosis in cancer cells (139).This is also seen in *in vivo* animal models of colorectal cancer after dosing with 40 mg/kg, a concentration which is pharmacologically relevant to humans (140).

1.8.2.2 The effect of aspirin on cell migration

Cox dependent mechanisms

As well as suppressing the production of prostaglandins, aspirin is also known to affect platelet function through inhibition of the COX enzymes, specifically COX-1 (141). Inhibition of COX-1 suppresses thromboxane A_2 (TXA_2) synthesis which inhibits platelet aggregation. Platelet activity has been shown to be increased in cancer patients with extensive evidence demonstrating that platelets play a crucial role in the survival of tumour cells within the vasculature while also supporting the formation of tumour metastases (142). Platelets also facilitate vascular adhesion, growth factor production such as platelet derived growth factor (PDGF), immune evasion by natural killer T (NKT) cells, and pro-angiogenic proteins such as VEGF and thrombospondin-1 (TSP-1). Inhibition of COX enzymes by aspirin prevents angiogenesis and platelet assisted metastasis of tumour cells (Figure 1.11) (143). Co-culturing HT29 colorectal cancer cells with platelets leads to an increase in markers for EMT characterised by a downregulation of E-cadherin and an upregulation of the transcription factor Twist (144). In mice injected with platelet primed HT29 cells, treatment with aspirin prevented the platelet increased rate of metastasis and production of TXA_2 and PGE_2. Aspirin is very effective at inhibiting platelets as they do not contain a nucleus and are unable to resynthesize the COX-1 enzyme as rapidly as nucleated cells (145). Consequently, this allows lower doses of aspirin as well as fewer dosing periods to remain effective.

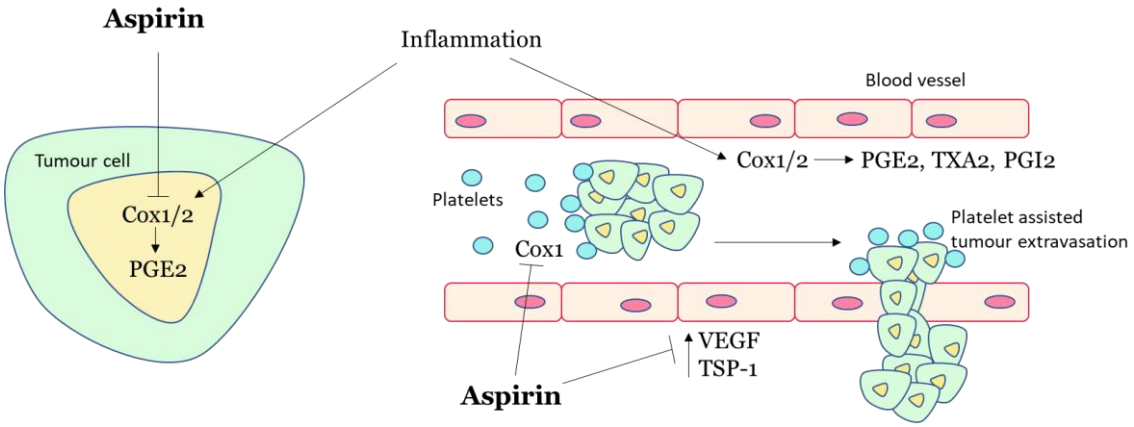


Figure 1.11: COX dependent effects of aspirin on tumour cells
COX1/2 metabolize arachidonic acid to PGH2 which promotes the synthesis of thromboxane A2 (TXA2), prostacyclin (PGI2), and PGE2. Aspirin inhibits the COX enzymes and prevents PGE2 induced tumour cell proliferation, migration, apoptosis and angiogenesis. Aspirin also prevents PGI2 and TXA2 induced platelet aggregation and vasoconstriction in blood vessels and platelet facilitated tumour migration.

Cox independent mechanisms

Aspirin has also been shown to target other pathways involved in cell migration such as the NF_KB pathway. In a study by *Shi et al*, through inhibition of NF_KB aspirin suppressed invasion and attachment of prostate cancer cells by reducing matrix metallopeptidase 9 (MMP-9) and upregulating TIMP metallopeptidase inhibitor 1 (TIMP-1), two proteolytic enzymes involved in degrading the extracellular matrix which allows cells to migrate and invade (146). Inhibition of NF_KB by aspirin in highly invasive PC3 prostate cancer cells has also been demonstrated to suppress the secretion of urokinase-type plasminogen activator (uPA), a molecule which degrades the extracellular matrix and reorganises the actin cytoskeleton, resulting in decreased PC3 cell migration and adhesion (147). In non-small cell lung cancer aspirin has also been shown to prevent activation of NF_KB and as a result downregulates Slug transcription, preventing Slug induced EMT and diminishing the metastatic potential of mutant K-Ras cells (148). In addition, aspirin causes ERK pathway inhibition by preventing the binding of c-Raf, a proto-oncogene, to Ras, another oncogene which is often overactive in cancer. Mutations that permanently activate Ras are found in around 20-25% of all human tumours (149) and evidence is accumulating showing that Ras contributes to the invasive responses of cancer cells. Cancers which support a mutation in the Ras allele are often highly aggressive and have a poor prognosis.

1.9 Metformin

Metformin (1,1-dimethylguanidine) is an oral antidiabetic drug and the most commonly prescribed medicine for the treatment of type II diabetes mellitus (150). It is of the biguanide class, found naturally in the French Lilac, but first synthesized in the 1920s (151). However, while metformin has been prescribed since 1957 in Europe for its glucose lowering effect, it is only recently that its mechanism of action is beginning to become understood (152). Because of this, while it is considered a well-established drug for the treatment of type II diabetes, metformin has quite recently sparked interest in the field of cancer research with the realization that its effects could suppress cell growth and development, key to cancer progression (153). Metformin is not metabolized and is excreted into the urine unchanged, with a half-life of ~5 hours. It is widely distributed around the body's tissues through uptake via organic cation transporters, most commonly Organic Cation Transporter 1 (OCT1). Because of this it is thought to be beneficial for the treatment of a number of different cancers with breast, prostate and colon cancers suggested to receive the most benefit (152).

1.9.1 Epidemiological studies examining metformin and cancer treatment

It was first proposed that metformin may possess anti-cancer properties when epidemiological data showed that patients who were prescribed metformin for the treatment of type II diabetes seemed to have decreased overall cancer related mortality and cancer occurrence. In a meta-analysis of 24 studies; 11 observational cohort studies, 3 randomized control trials (RCTs) and 10 case-control studies the use of metformin was associated with a significantly lower risk of cancer mortality and incidence than the non-metformin users (154). The large number of studies assessed in this review as well as the large sample sizes of up to 998,947 patients fully support the use of metformin in cancer treatment. Numerous other population studies have produced similar results, providing the rationale to look further into the mechanism behind metformin's anti-cancer effects. In a study which focused on the use of metformin and prostate cancer and included data from 12 epidemiological studies and 14 datasets, it was found that metformin use was significantly associated with a decreased prostate cancer risk (14 datasets, 963991 male subjects, odds ratio: 0.91, 95% CI: 0.85–0.97) and biochemical recurrence (6 datasets, 2953 patients, hazard ratio: 0.81, 95% CI: 0.68–0.98). However, no significant effect was found to be associated with metformin use and the all-cause mortality of patients (5 datasets, 9241 patients, hazard ratio: 0.86, 95% CI: 0.64–1.14) (155). This implies that metformin delays the development of prostate cancer and aids in preventing recurrence after treatment but that once the cancer has developed to a certain point its effects are reduced.

While studies such as these and others provide strong evidence for the statement that metformin reduces prostate cancer risk there are others that disagree. In a study which examined the effect of metformin on overall risk of colorectal, lung, breast and prostate cancer in diabetic patients no protective effect was identified between metformin use and cancer incidence. It followed patients for a mean follow-up of 4.8 years and compared metformin users to users of sulfonylurea or insulin (156). Another study published in Nature examined 8 cohort studies and 1 nested case-control study and found that metformin was marginally associated with a decrease in risk of biochemical recurrence for prostate cancer but was not associated with metastases, all-cause mortality and prostate-specific mortality (157). It concluded that metformin may reduce the risk of biochemical recurrence in prostate cancer but with such a minor effect that many more studies are needed before it is possible to claim metformin provides an anti-cancer benefit.

In addition, there is also some dispute over which confounders are considered when examining the use of metformin for cancer treatment which may affect the results of epidemiological studies. Epidemiological evidence suggests that people with diabetes are at higher risk of developing certain types of cancer than nondiabetic individuals (158) and that a longer duration of diabetes may be associated with higher cancer incidence (159) . Therefore, when comparing metformin users, of whom the majority are diabetic, with a control group factors such as the duration of diabetes, BMI, modification of cancer treatment due to diabetes and comorbidities must be considered (160). Observational studies are often subject to selection bias and so randomised control trials are needed for a more robust answer. Despite this, current evidence leans towards the benefit of metformin use in cancer prevention with more studies identifying a reduction in cancer risk for patients taking metformin than not.

1.9.2 Molecular mechanism of metformin in cancer treatment

1.9.2.1 The effect of metformin on cell proliferation

Studies have shown that metformin has both direct and indirect effects on cancer cells with its indirect effect mainly acting on insulin, a mitogen which regulates the metabolism of fats and carbohydrates (Figure 1.12). Insulin promotes pathways involved in cell proliferation and survival such as the PI3K pathway and metformin acts by inhibiting hepatic gluconeogenesis and increasing insulin sensitivity. This lowers circulating insulin levels, reducing activation of its downstream pathways and reducing cell growth (161). Its direct effects are the result of an interaction between multiple mechanisms, but the drug largely works through inhibition of complex 1 in the respiratory chain of the mitochondria. This leads to a transient change in the cells energy status, increasing the AMP: ATP ratio and sending cells into energetic stress. Increased levels of AMP lead to the activation of a key energy sensor, AMPK, which regulates many signalling cascades to maintain a normal energy balance. It's seen that this activation of AMPK switches off anabolic ATP-consuming pathways such as those involved in glucose, protein, and lipid synthesis and switches on ATP generating pathways such as fatty acid oxidation (162). AMPK is also a key regulator of several proteins implicated in cancer development including those involved in protein synthesis and growth such as mTOR, cell cycle proteins such as p53 and cyclinD1 and inflammation pathways such as NF_KB and STAT3.

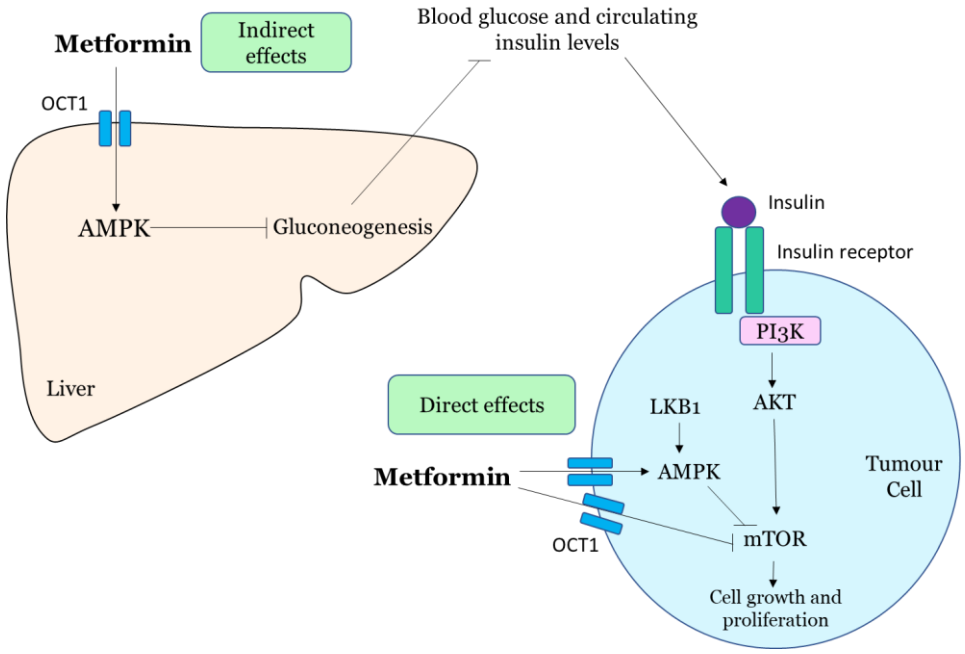


Figure 1.12: Direct and indirect effects of metformin on tumour cells
Metformin has both indirect and direct effects on tumour cells, leading to a reduction in activation of the PI3K pathway and its downstream proliferation promoting pathways as well as activation of AMPK, the energy sensor of the cell.

AMPK is the most well-known target of metformin and many studies have been conducted elucidating how exactly metformin activates AMPK and what this activation does to tumour cells. In a study which examined what effect inactivating AMPK would have on cancer cell either by introducing a stable dominant negative mutant of AMPK into cell lines or its shRNA, it was seen that AMPK inactivation promoted tumorigenic properties (163). Cell proliferation was accelerated and cells became more aggressive, with an increase in migration and anchorage-independent growth. This was associated with a decrease in p53, p21 and an increase in S6K, IGF1R and IGF-1. Use of an AMPK activator, AICAR, caused opposite changes, strengthening the suggestion of AMPK activators, such as metformin, as antitumor agents. Metformin has also been shown to decrease cell number and caused G0/G1 cell cycle arrest but not apoptosis in DU145, PC3 and LNCaP cancerous prostate cell lines (164). There was less of an effect on a normal prostate cell line, P69, with only a 20% decrease in cell viability compared to 52%, 54% and 38% for LNCaP, DU145 and PC3s respectively, indicating that the drug is more effective on abnormal or cancerous cells. In mice bearing LNCaP xenografts metformin caused a 50% and 35% reduction in tumour growth with oral and intraperitoneal treatment respectively. However, while metformin was shown to activate AMPK this growth inhibition was not only dependent on AMPK as the anti-proliferative effect remained even with AMPK knockdown. In both the *in vitro* and *in vivo* work a strong reduction of the cell cycle protein cyclin D1 was seen, indicating that metformins inhibition of the cell cycle progression may contribute to its anti-tumour effects.

The IGF axis is a critical pathway involved in cell growth, development and survival and is commonly deregulated in cancer. Upon binding the IGF ligand the IGF-1R is activated and activates the insulin receptor substrate 1 (IRS-1). This in turn activates PI3K, resulting in the activation of the PI3K/AKT pathway, a key pathway in cancer progression. In parallel, the IGF-1R also activates the Ras/MAPK pathway, another major oncogenic pathway, leading to additional cell growth (165). In a study which examined the effects of metformin on prostate cells it was seen that metformin inhibited proliferation, migration, and invasion as well as decreased IGF-IR mRNA and protein expression in PC3 prostate cells (166). Metformin inhibited IGF-1 stimulation of the ERK and AKT pathways, and it was proposed that this inhibition contributed to the anti-proliferative effects of the drug. IGF-IR is frequently overexpressed in tumours and is often associated with higher Gleason scores. It is also often upregulated in androgen receptor positive cell lines. This study was carried out in PC3s, an androgen independent cell line, and so metformins inhibition of proliferation by decreasing the expression of this receptor could be even stronger in androgen positive cell lines. Insulin, a hormone similar in structure to IGF-1,

also acts to promote cell metabolism and growth and is often implicated in cancer progression. Signaling pathways do not act alone but interact with other pathways to form intricate signaling networks. It has been shown that crosstalk between the IGF-1/insulin receptors and G protein coupled receptors (GPCRs) promotes mitogenic signaling and could lead to cancer development. This point of convergence has been suggested to be at mTOR (168), a protein which promotes protein synthesis and cell growth and is a downstream target of metformin (Figure 1.13). In a review which examined the effect of metformin on the interaction between IGF-1R/insulin and GPCRs in pancreatic cancer, it was stated that recent studies had shown that metformins activation of AMPK also disrupted the crosstalk of these proteins in pancreatic cancer cells and this interaction could be a novel target for metformin (169). These studies also showed that metformin inhibited the growth of pancreatic cancer cells in xenograft models (167). GPCRs are implicated in the formation of solid tumours of several cell types including colon, prostate, breast and pancreas and so a similar crosstalk may be disrupted in these cell types.

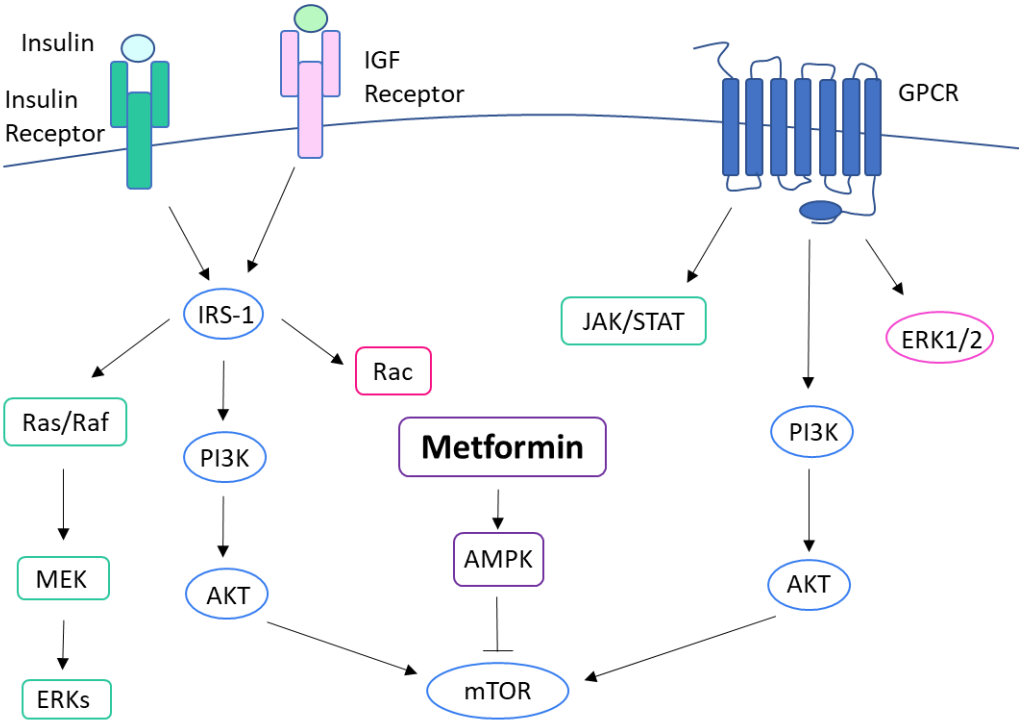


Figure 1.13: Metformin disrupts crosstalk between IGF pathway and G protein coupled receptors. Crosstalk between the IGF pathway and GPCRs lead to mTOR activation and increased protein synthesis, cell proliferation and cell survival. Metformin has been shown to disrupt this crosstalk in a potential anti-cancer mechanism.

Metformin also inhibits proliferation via downregulation of the androgen receptor. In a study which examined the effect of metformin in prostate cancer cell lines it was seen that metformin decreased prostate cell viability and increased apoptosis by targeting androgen receptor signaling (170). Metformin decreased androgen receptor mRNA rather than causing androgen receptor protein destabilization or degradation, however the mechanism was unknown. In another study, metformin decreased androgen receptor expression and did this via disruption of the translational MID1-α4 regulator complex which caused the androgen receptor mRNA to dissociate (171). This downregulation of the androgen receptor could be mimicked by knockdown of MID1 or α4 and resulted in reduced growth. Metformin also possessed androgen receptor independent effects as it caused inhibition of proliferation in androgen receptor negative cell lines, but to a lesser extent than in androgen positive cell lines (172).

Triple negative breast cancers are often more aggressive than other types of breast cancer and frequently express mutated p53 and an upregulation of STAT3 and IL-6. It has also been noted that these cancers are particularly sensitive to metformin, indicating that this drug might target the STAT3 pathway. In a study which examined the effect of metformin in triple negative breast cancer it was seen that metformin inhibited STAT3 activation at two residues, Tyr705 and Ser727, and this led to growth inhibition and the induction of apoptosis (173). Overexpression of STAT3 reversed the effect and knockdown of STAT3 increased metformins antiproliferative activity. A STAT3 inhibitor synergized with metformin while an mTOR inhibitor showed that the interaction between metformin and STAT3 was not dependent on mTOR. Metformin has also been shown to cause inhibition of STAT3 in other types of cancer including oesophageal squamous cell carcinoma (174), lung adenocarcinoma (175), pancreatic cancer (176) and bladder cancer (177).

1.9.2.2 The effect of metformin on cell migration

While AMPK activation by metformin is known to inhibit cell proliferation, it has also been shown to inhibit cell invasion. In a study which examined the effect of metformin on melanoma cell invasion it was seen that metformin inhibited cell invasion but did not affect cell migration or proliferation. This study by *Cerezo et al* examined the invasive ability of cells dosed with metformin using a number of methods including Boyden chambers, western blot analysis and 3D cell culture where 3D spheroids invasion into agar was measured (178). It was seen that when metformin was present, cells were less invasive and this correlated with expression of EMT markers such as Slug, N-cadherin SPARC, Snail and fibronectin becoming more epithelial in expression. Metformin also inhibited tumour metastasis in mouse extravasation and metastasis models. This inhibition of invasion was shown to be dependent on AMPK activation and the tumour suppressor p53.

Metformin has also been reported to inhibit cell metastasis through multiple other signaling pathways. In a paper that examined the antimigratory ability of metformin in prostate cancer cell models, metformin hindered cell motility of DU145 and PC3 prostate cancer cells *in vitro* and *in vivo* (179). This paper examined the effect of metformin on 3D cell culture, growing DU145 spheroids using the liquid overlay technique. It was seen that untreated spheroids could invade into the adjacent matrigel whereas spheroid treated with 5 mM metformin were unable to do so. Metformin also interfered with cytoskeleton organization, inhibiting Rac1 GTPase activity by interacting with its upstream regulators including P-Rex1, cAMP and CXCL12/CXCR4. Alteration of cytoskeleton organization is a key process in tumour cell migration. For cells to migrate they undergo many changes to their structural organization, leading to cell extension, formation of new adhesions and detachment from the previous site. Rho proteins are involved in all of these steps and overexpression of P-Rex, a protein which regulates Rho GTPases, has been observed in both breast and prostate cancers (180). This inhibition of Rac1 GTPase by metformin prevents alteration of the cells structural organization, decreasing cell movement.

Metformin also modulates the tumour inflammatory environment. In two recent studies, metformin was shown to both reduce tumour-infiltrating Treg (Ti-Treg) cells which act as a negative regulator for T cell mediated antitumor immunity (69) and to alleviate the fibro-inflammatory microenvironment in diabetic individuals. This reduction in inflammation was

correlated with reduced cancer progression and a reduction in extracellular matrix remodelling which is essential for tumour metastasis.

What is clear from *in vitro* and *in vivo* studies is that both aspirin and metformin are pleotropic and affect many different signalling pathways and proteins. It is thought that the accumulation of these effects causes the anti-cancer action of the drugs, primarily through inhibition of cell proliferation and cell migration which are fundamental for cancer growth and development. While *in vitro* and *in vivo* work strongly support the use of metformin as an anti-neoplastic agent most of the concentrations used in research are well above the therapeutic range that would be possible to give patients. In the studies mentioned in this chapter, metformin is used at concentrations which are well above the clinical range, with four of the studies using a concentration of 20 mM or higher *in vitro*. Most commonly concentrations of 1- 10 mM were used. While there is much controversy over translating human doses to cell culture there is also the debate about what concentration the site of interest will be exposed to. The liver and gut have been shown to experience high concentrations of metformin following oral administration due to a high concentration of the drug in the portal circulation as well as high expression of OCT1 transporters on liver cells which increase cellular uptake of the drug (181). Following administration and hepatic uptake, metformins systemic plasma concentration has been reported to be reduced to a low as 10-40 µM in animals and humans (182). However, it has also been reported that metformin accumulates in the mitochondria over time due to its positive charge and so patients might experience higher concentrations with a longer duration of treatment (183). Conversely, aspirin has a much shorter serum half-life of ~20 minutes (184) before it is metabolised to salicylate which has a half-life of 3-5 hours (185). Human plasma aspirin levels are around 1 mM (7) and 2.5 mM (8) when therapeutic doses of aspirin are given. This study aims to use concentrations of aspirin and metformin that are clinically relevant to identify the anticancer activity of the drugs in cancer treatment. While the use of aspirin and metformin has been examined in many types of cancer, information on prostate cancer is limited. More research will be beneficial in understanding the mechanism behind aspirin and metformins anti-cancer effects in the prostate.

1.10 Evidence to support the use of aspirin and metformin in combination

Both aspirin and metformin act upon many signaling networks which regulate key pathways involved in cancer proliferation, migration, invasion and cell survival. Because of the overlap between their signaling pathways it is thought that combining the drugs could increase the efficacy of their anti-cancer effects.

Several studies have looked at combining aspirin and metformin for cancer treatment, and in a study that examined their effect on the transcriptome of pancreatic cancer cells, a synergistic effect was seen when both drugs were used together (186). When each drug was used alone there was only a modest change in gene expression, 12 genes with aspirin treatment and 149 genes with metformin treatment. However, when the drugs were used in combination 1,105 genes were affected. This large change in the transcriptome profile indicated that three major pathways were altered which included pathways involved in cell cycle: G1/S checkpoint regulation, cholesterol biosynthesis and axonal guidance signaling. This suggests that when used together the drugs could have a greater effect on cell proliferation and membrane formation, both of which are important factors in cancer progression. Another study looked at the effect of combining both drugs on pancreatic cells and examined cell viability, apoptosis and cell migration (187). The combination of aspirin and metformin decreased cell viability, increased apoptosis and prevented cell migration more than when the drugs were used individually or compared with the control. Anti-apoptotic proteins were downregulated, and pro-apoptotic proteins were upregulated, indicating that used in combination the drugs induced cell death and prevented cell proliferation. Furthermore, in mouse xenograft models, there was a reduction in pancreatic tumour volume when aspirin and metformin were used together. However, while this study showed a clear synergy of the drugs, extremely high concentrations of metformin and aspirin were used which are not therapeutically achievable. The lowest concentration of metformin was 1 mM while the highest was 100 mM, much higher than the 10-40 μM concentration expected to be in the systemic circulation. For aspirin, the lowest dose used was 0.25 mM which is within the proposed therapeutic range, but doses went up to 20 mM aspirin. Currently around 2.5 mM aspirin is considered the maximum dose for *in vitro* studies. While these doses are useful to see an enhanced effect of the drug, they are unrealistic to give patients and could produce effects which don't occur at lower concentrations.

No studies to date have looked at the effect of aspirin and metformin in combination for prostate cancer, however, one study has examined the effect of metformin and the metabolite of aspirin, salicylate. In this paper which used both prostate and lung cells, salicylate activated AMPK and phosphorylated Acetyl-CoA carboxylase (ACC), an effect which was increased with metformin administration (188). Salicylate decreased clonogenic survival and together the drugs decreased cell proliferation in an additive or synergistic manner depending on the cell line and drug concentrations. Doses reached a maximum concentration of 3 mM metformin and 5 mM salicylate but were normally used at 100 µm metformin and 1 mM salicylate when in combination. This effect on clonogenic survival was thought to be through inhibition of *de novo* lipogenesis as proliferation was restored with the addition of cholesterol reserves or fatty acids in the presence of the drugs.

In summary, aspirin and metformin have been shown to affect many different signaling pathways which often overlap and interlink. While studies have started to examine the effect of using both drugs together there are still many other signaling pathways to consider. Whether the effect of the drugs is due to a combination of pleiotropic acting agents or through the enhancement of specific pathways remains to be determined.

1.11 The benefits of using aspirin and metformin for cancer treatment

Aspirin and metformin have many properties which make them attractive candidates for cancer treatment. Aspirin is commonly used to prevent cardiovascular disease, lower blood pressure and reduce inflammation and can be bought easily over the counter. Metformin is the first line agent to treat type II diabetes with 91% of people newly diagnosed with type II diabetes being prescribed metformin in the UK in 2013 (189). Since both drugs are already in use, and have been for many years, they have a well-established toxicity profile, with most of their side effects being well known and documented and making them relatively safe drugs to prescribe. The most adverse side effects of aspirin include gastrointestinal ulcers and stomach bleeding and for metformin they include gastrointestinal irritation and lactic acidosis which is produced by a build-up of lactate in the blood. However, these drugs are well tolerated by most patients which makes conducting clinical trials much easier than with a new, untested drug for which a safe dose has not yet been established. Furthermore, people tend to be more comfortable about taking part in studies where they are familiar with the drug and so this allows controlled trials with large samples sizes. As there are already many people who have been prescribed the drugs for the treatment of other illnesses it is also possible to obtain large amounts of data about their effect on cancer risk in population studies.

One of the major advantages of aspirin and metformin is that taking the drugs causes little change to a person's lifestyle unlike other forms of treatment. With surgery, the recovery time can be lengthy, and side-effects can last for years. Radiotherapy and chemotherapy can leave people feeling weak or ill during treatment and require them to attend frequent hospital visits. While aspirin and metformin aim to prevent cancer progression rather than treat cancer, prolonging the time before conventional treatment is necessary is extremely important. Aspirin and metformin allow people to carry on with their normal day to day activities without any hindrance and they can take the drugs at home. Both drugs can be self-administered orally, and this has the added benefit that it is easy to administer, with no machines, injections or medical skills needed.

One of the major problems for the drug industry is supplying drugs at a low cost whilst trying to recoup the enormous sums spent bringing a drug to market. With aspirin and metformin, they are already developed, cheap and quick to produce, making them more readily accessible to the public.

Additionally, there is much interest in the use of aspirin or metformin in combination with conventional treatments such as chemotherapy, radiotherapy and surgery with studies showing that they could enhance the efficacy of these regularly used treatments. One significant problem with chemotherapy, also seen in radiotherapy, is that there often is a population of cells that remain resistant to the treatment and cancer can recur. Accumulating evidence suggests a major role of stem cells in these recurrences with their ability to repopulate the tumour (190). Indeed in a study which examined the recurrence of breast cancer after chemotherapy it was seen that a combination of common chemotherapy drugs (5-fluorouracil, doxorubicin, and cyclophosphamide) generated an inflammatory environment that induced multidrug resistance, conferred stem-ness to non-stem cells and boosted the migratory capacity of the cells (191). However, treatment with aspirin prior to chemotherapy suppressed the acquisition of chemoresistance and sensitized stem cells to chemotherapy. This was accomplished through disruption of the NF_KB-IL6 feedback loop that generated the inflammatory environment after chemotherapy. Similarly, in a study which examined the combination of metformin and a chemotherapeutic, 5-FU, in esophageal cancer, metformin sensitized the cells to the cytotoxic effect of 5-FU (192) by targeting cancer stem cells and proteins involved in mTOR signaling. This also occurred in breast, prostate, and lung cancer cells in combination with doxorubicin where the combination was more effective at blocking tumour growth than either drug alone (193). Chemotherapy is a common form of cancer treatment but there is large variability in clinical response for patients. Currently there are more than 30 ongoing cancer trials which are examining the effect of combining metformin and chemotherapeutics (194).

Likewise, aspirin and metformin improve patient outcome when used in combination with radiotherapy. Aspirin has been shown to reduce the risk of distant metastases and improve 5-year overall survival (885 vs. 37% p= 0.03) in men who take aspirin for at least 5 years while undergoing radiation therapy for prostate cancer (195). In prostate and colorectal xenografts improved tumour oxygenation by metformin leads to improved radiation responses when metformin is given immediately before irradiation (196). Interestingly aspirin has also been shown to improve outcome after surgery. In a study which examined the risk of cancer in men who underwent a radical prostatectomy or radiotherapy while receiving anti-coagulants, including aspirin, it was seen that the risk of prostate specific mortality was significantly lower in the anti-coagulant users than in the non-user group (3% *v* 8% at 10 years; $P < 0.01$) (163) (162). Improving the effect of conventional treatments with such well-tolerated, easily administered drugs such as aspirin and metformin is a very attractive prospect and may allow

a lower dose of the drug to be effective, reducing side effects and complications. While not many studies have been conducted examining the use of aspirin or metformin with conventional treatments for prostate cancer it is hoped that the drugs can help target cells which become resistant to treatment and create environments where treatment becomes more effective to aid in controlling cancer progression, prevent recurrence and greatly improve patient prognosis.

Due to their high safety profile and widespread use there is little apprehension in prescribing either aspirin or metformin. The strong evidence indicating a positive effect of these drugs in cancer treatment and in preventing progression demonstrates that they could work well to delay the time before more aggressive treatment is necessary. Further work is needed to understand the mechanism behind how they exert their anti-cancer effects and whether combining the drugs will be beneficial.

1.12 Project hypotheses and aims

Epidemiological, *in vitro* and *in vivo* studies suggest that aspirin (NSAID) and metformin (first-line agent in type II diabetes) are negatively associated with prostate cancer risk and mortality. While many studies have been conducted examining the benefit of aspirin treatment for colon cancer, reports of its actions in prostate cancer is limited. Current literature supports the use of either aspirin or metformin in prostate cancer treatment, however most *in vitro* studies use doses which are supra pharmacological and there is a lack of information regarding the effect of combining the drugs for prostate cancer treatment. Studies suggest that both drugs may synergise through overlapping signalling pathways, with a focus on the PI3K/AKT, NF_KB, and Ras/MAPK pathways which both drugs target.

We hypothesize that aspirin and metformin will prevent prostate cancer cell growth and survival at therapeutically achievable doses if given in combination. Furthermore, we propose that the combined administration of aspirin and metformin will inhibit prostate cancer cell migration, reversing EMT to reduce the mesenchymal molecular characteristics of the tumour cells.

Specific aims:

- To examine the effect of aspirin and metformin on cell proliferation in 2D cell culture.
- To determine whether aspirin and metformin influence cell proliferation in 3D cell culture.
- To investigate the potential for aspirin and metformin to reduce prostate cancer cell migration.

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Cell lines

Cell lines were purchased from the American Type Culture Collection (ATCC) and stored at -196^{0}C in liquid nitrogen.

PNT2 cell line: This prostate epithelial cell line was established by transfection of normal adult prostate epithelium with a plasmid containing the SV40 genome with a defective replication origin to immortalize the cells. The cells possess a well differentiated morphology and are non-tumorigenic in nude mice.

LNCaP cell line: This cancerous prostate adenocarcinoma cell line was derived from the left supraventricular lymph node metastasis of a 50-year-old male in 1977. The cells have low metastatic potential and are androgen sensitive. They express prostate specific antigen (PSA) which is often used as a marker for prostate cancer diagnosis.

DU145 cell line: This cancerous prostate epithelial cell line was derived from a metastatic site in the brain of a 69-year-old Caucasian male in 1978. These cells possess moderate metastatic potential, are androgen insensitive and do not express PSA.

PC3 cell line: This cancerous prostate epithelial cell line was derived from a bone marrow metastasis of a 62-year-old Caucasian male in 1979. These cells have high metastatic potential. They do not express PSA but produce factors such as cytokines and growth factors that have been suggested to regulate the tumour microenvironment.

H642/17 primary prostate cells: These prostate cells were derived from a prostate with benign prostatic hyperplasia and were a kind gift from Professor Norman Maitland and his research group at the Cancer Research Unit, University of York.

Equipment

Cryogenic vials	(Starlab, E3110-6122)
Filters	(Starstedt, 83.1826.001)
Haemocytometer	(Fischer Scientific)
Incubator	(SANYO, MCO-18AIC)

21G microlance hypodermic needle	(BD Biosciences, 304432)
Aspiration pipette	(Greiner Bio-One, 710183)
10 and 25 ml serological pipettes	(Starstedt, 86.1254.001 and 86.1685.001)
Syringe	(VWR, 10080264)
7 ml bijou	(Greiner Bio-One, 189171)
25 ml Universal tube	(Starstedt, 60.9922.243)
50 ml Falcon tube	(Starstedt, 62.559.00)
Eppendorf, 0.5 mL	(Sigma-Aldrich, Z666521-250EA)
Muse cell analyser	(Merck)

Solutions and reagents

Growth Media (GM) – The DU145, PC3 and PNT2 cell lines were maintained in 25 mM, 4.5g glucose/L Dulbecco's modified Eagles Medium (DMEM, Sigma- Aldrich, D6429) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, 10270-106) and 5% L- Glutamine (LG, Lonza Limited, BE17-605F). The LNCaP cell line was maintained in 25 mM, 4.5 g glucose/L Roswell Park Memorial Institute (RPMI) media (ATCC, ATCC-30-2001), with 5% LG and 10% FBS. The H642/17 cells were maintained in keratinocyte serum free medium (Thermo Fisher, 17005042) supplemented with 50 µg/ml bovine pituitary extract (BPE, Thermo Fisher), 5 ng/ml epidermal growth factor 1-53 (EGF 1-53, Thermo Fisher), 5000 units/mL of penicillin and 5000 µg/mL of streptomycin (P/S, Gibco, 15070-003) and 5% FBS.

Phosphate Buffered Saline (PBS) - PBS was prepared using PBS tablets (Merck Millipore, P4417-50TAB) with 1 tablet/ 200 ml of ddH_2O (pH 7.4). Tablets were diluted in distilled water, the bottle was autoclaved and then stored at room temperature.

Trypsin-EDTA (ethylenediaminetetraacetic acid) (TE)- (Lonza, BE02-007E for PC3s and DU145s and Sigma-Aldrich, T4049 for LNCaPs and PNT2s). Trypsin was diluted from 10x to 1x with autoclaved water and was stored at -20^0.

StemPro Accutase Cell Dissociation Reagent- (Sigma-Aldrich, A6964-100ML) Stored at -20^0C.

Trypan Blue (TB)-TB stain (Sigma-Aldrich, T8154) was diluted from 0.4% to 0.165% in PBS and added in a 1:1 ratio with cell suspension to stain cells to assess viability and number. Stored at room temperature.

10% Dimethylsulfoxide (DMSO)- (Sigma-Aldrich, D4540) stored at room temperature.

CryoStor cell cryopreservation media- (Sigma, C2999-100ML) stored at 4-8^{0}C.

L-poly-lysine- (Sigma- Aldrich, P8920-500ML) stored at room temperature.

Collagen, type I solution from rat tail- (Sigma-Aldrich, C3867) stored at 4-8^{0}C.

Guava Instrument cleaning fluid (ICF)- (Merck, 4200-0140) stored at room temperature.

Muse cell viability reagent- (Merck, MCH100102) stored at 4-8^{0}C.

2.1.2 Cell maintenance

Stock cultures of DU145 and PC3 cells were grown in T175cm^2 flasks (T175, Greiner bio-one, 660175) and PNT2 and LNCaP cell lines were grown in T75 flasks (Appleton Woods Limited, BC301) to approximately 80-90% confluency before re-seeding. The H642/17 cells were grown on collagen coated petri dishes (Thermo Fisher, 172931). Cells were maintained in a 37^{0}C, 95% humidity, and 5% CO_2 atmosphere.

Resuscitation of frozen cell lines

Cells stored in cryovials were removed from liquid nitrogen and put in a water bath at 37^{0}C to thaw. 1 ml of DMEM containing 20% FBS and 2 mM L-Glutamine (LG) was added to the vial and the cells were transferred to a universal tube with the addition of 9 ml of GM. The cells were spun at 1,000 rpm for 3-5 minutes to produce a pellet. The supernatant was aspirated and the pellet was resuspended in 5 ml of GM using a 21G needle and syringe. For the LNCaP cell line, cells were first resuspended in 1 ml of GM using a p1000 pipette, rather than using a needle and syringe as this is found to increase cell death, and then 4 ml of GM were added. The media was transferred to a T25 culture flask and incubated at 37°C, 5% CO_2.

Cell passaging

Cells were split when they reached ~80-90 % confluence. Growth media was aspirated and cells were washed with PBS that was then discarded. For PC3, DU145, LNCaP and PNT2 cells 1 ml of 0.25% TE was added and the flask was incubated for 5 minutes at 37°C, 5% CO_2. For H642/17 cells 2 ml of accutase were added to the plate and left at room temperature for 5-10 minutes. Cells were detached from the bottom by firmly tapping the flask and 10 ml of GM were added to deactivate the TE or accutase. Cell suspension was collected in a universal tube and centrifuged at 1,200 rpm for 3-5 minutes and the supernatant was aspirated and discarded. The pellet was resuspended in 5 ml of GM using a needle and syringe and of this 50 µl were added to an eppendorf for cell counting. From this the number of cells in the 5 ml suspension could be determined and the subsequent volume of cell suspension that was needed to be seeded. Cells were placed into either a T25, T75 or T175 flask with varying densities depending on the cell type (Table 2-1). Media was replaced every 2-3 days.

Table 2-1: Cell densities seeded in T25, T75 and T175 flasks for each cell line.

Flask	Cell line	Cell density (x 10^6)	Company
T25	DU145/PC3	0.2	Greiner BioOne
	PNT2/LNCaP	0.4	Appleton Woods limited
T75	DU145/PC3	0.3	Greiner BioOne
	PNT2	0.8	Appleton Woods limited
	LNCaP	1.0	Appleton Woods limited
T175	DU145/PC3	0.5	Greiner BioOne

Cell counting for stocks

50 µl of cell suspension from the cell passaging were diluted at a 1:1 ratio with Trypan Blue (TB) working solution (0.165%). A 50 µl mix of cell suspension and stain were added to the top and bottom counting chambers of a haemocytometer and cells contained within the chambers were counted using a light microscope. Cells stained blue were non-viable whereas viable cells were colourless. The TB dye enters the cell when the cell membrane becomes permeable during cell death but does not penetrate viable cells. The number of viable and non-viable cells was counted for both chambers, and then multiplied by 1 x 10^4 to calculate the number of cells per ml.

Cell Freezing

A cell suspension was made consisting of 3 x 10^6 cells for DU145, PC3 and PNT2 cell lines in 500 µl of growth media and placed into a cryogenic vial. In a separate bijou 500µl of freezing mix was prepared consisting of 100 µl of 10% DMSO, 350 µl of GM, 50 µl of 5% FBS.

When the DMSO is added to growth media an exothermic reaction takes place giving out heat which would kill the cells if added immediately so it was left for a few minutes to cool. The freezing mixture was added in a drop wise fashion to the cryogenic vial, giving a 1:1 ratio of cell suspension to freezing mixture, giving a total volume of 1 ml. For LNCaP cells, 6 x 10^6 cells were spun down, resuspended in 1 ml of cryoStor cell cryopreservation media and added to the cryogenic vial. Cryogenic vials were placed in an insulating freezing container to reduce the temperature in a controlled manner and kept at -80^0C overnight. After 24 hours the cryogenic vials were moved to the liquid nitrogen tank or the -150^0C freezer and their position was recorded in a log book.

2.1.3 Subculturing

Coating plates

The LNCaP cell line required 6 well and 24 well plates to be coated with L-poly-lysine for cells to attach. L-poly-lysine was diluted x10 with autoclaved water and 24 well plates were coated with 500 µl while 6 well plates were coated with 1 ml. Plates were placed in the incubator for 1 hour and then the L-poly lysine solution was removed and plates were washed with autoclaved water and left to air-dry in the hood. Plates were used immediately.

The H642/17 required petri dishes to be coated with collagen. Collagen was diluted with autoclaved water at a 1:100 dilution with enough volume to cover the petri dish. The dishes were left in the hood overnight to air-dry. Collagen coated plates were stored at 4^0C.

Cell counting to seed for experiments

Cells were counted either using the Muse cell analyser or the trypan blue dye exclusion assay. For the Muse cell analyser, the count and viability assay was used. The cell counter was cleaned using dH_20 and Guava instrument cleaning fluid following the instructions of the Muse protocol. 20 µl of cell suspension was mixed with 180 µl of cell viability reagent and read on the cell analyser. Viable cells were gated first by viability vs size and then by viability vs nucleated cells. Viable cells/mL, % viability, and the total cells/mL was reported. For the trypan blue dye exclusion assay the cell suspension was mixed at a 1:1 ratio with trypan blue and cells were counted on a haemocytometer. The live cells appear colourless whereas in the dead cells the trypan blue can enter through the disrupted membrane, staining the cells blue. This enabled the assessment of live and dead cells in each sample.

Experiment seeding densities

All cells were seeded in growth media at suitable densities for cell culture vessel, cell type used and experiment time point (Table 2-2).

Table 2-2: Cell densities for seeding for cell counting.

Experiment	Vessel	Volume of media	Number of cells per flask/well (x 10^6)			
			PC3	DU145	LNCaP	PNT2
Cell proliferation- ^{3}H-thymidine incorporation	24 well plate	500 µl	0.015	0.015	0.017	0.016
Cell proliferation- cell counting and western blotting	T75 flask	10 ml	0.4	0.4	2.0	0.5
	T25 flask	5 ml	0.2	N/A	1.0	0.4
	6 well plate	1 ml	0.075	0.075	0.14	0.12
Cell migration- qPCR	T25 flask	5 ml	0.2	N/A	N/A	N/A
Spheroid culturing	48 well plate	250 µl	40 cells	N/A	N/A	N/A

Cells were resuspended using a needle and syringe in the volume of growth media required so that that each well or flask had the appropriate number of cells and correct final volume. The cell suspension was pipetted gently down the side of the well and the plate was moved around so that cells were evenly distributed. For a flask the cell suspension was added to the flask and then the flask was topped up with growth media to the final volume.

2.2 Reconstituting aspirin and metformin

2.2.1 Reconstituting aspirin

Solutions and reagents

Acetylsalicylic acid (aspirin)- (Sigma, A5376) stored at room temperature.

Method

Aspirin was weighed out at 2.8 mg/ml in growth media to give a stock concentration of 15.5 mM. This was chosen due to aspirins solubility at 3 mg/ml at 25^0C. The stock concentration was diluted in growth media to give the desired concentrations. Solutions were kept at 4^0C for up to 5 days.

2.2.2 Reconstituting metformin

Solutions and reagents

1-1 Dimethylbiguanide hydro-chloride (Metformin)- (Sigma, D150959) stored at room temperature.

Method

Metformin was weighed out at 10 mg/ ml in growth media to give a stock concentration of 60.4 mM. This was diluted 1:100 with growth media to give a second stock concentration of 604 µM. Both stock concentrations were diluted in growth media to give the desired concentrations. Solutions were kept at 4^0C for up to 5 days.

2.3 Cell proliferation and cell death assays

2.3.1 Tritiated Thymidine (^{3}H-thymidine) Incorporation Assay

This technique incorporates a radioactive nucleoside, ^{3}H-thymidine, into the strands of chromosomal DNA when the cell is dividing and so the output for ^{3}H-thymidine incorporation, DPM, is a measure of DNA synthesis. Therefore, it can be used as a proxy for cell proliferation. The advantage of incorporation assays such as this is that it is a direct measure of proliferation.

Equipment

β-scintillation Counter	(Beckman, LS6500)
Plate rocker platform	(Bellco Biotech)

Solutions and reagents

[^{3}H]- Thymidine: 1 µCi/ml stock: (GE Healthcare) Stored at 4^{0}C

1 M sodium hydroxide (NaOH): (Fisher Scientific 10142540) 20 g of NaOH were dissolved in 500ml of dH_2O and stored at room temperature

5% w/v Trichloroacetic acid (TCA): (Merck, 100807) 5 g of TCA were added to 1 L of dH_2O and stored at 4^{0}C.

Scintillation fluid-Ultima Gold (Packard Bioscience Limited, GSQ1)

Method

Cells were incubated with 0.1 µCi [3H] Thymidine per well for the final 4 hours of the dosing time period at 37^{0}C. After 4 hours incubation, supernatant was removed and cells were incubated with 500 µl of 5% w/v TCA at 4^{0}C for 10 minutes. TCA was aspirated and 500µl of 1 M sodium hydroxide were added to each well. Cells were incubated for 1 hour at room temperature on a plate rocker. The resulting suspension was placed into individual scintillation vials and 2 mls of scintillation fluid were added. Vials were shaken and samples were analysed using a Beckman Scintillation Counter. Data were recorded as disintegrations per minute (DPM).

2.3.2 Cell counting

Equipment

Muse analyser	(Merck)
6 well plates	(CellStar, 657160)
25 ml universal tube	(Starstedt, 60.9922.243)
Eppendorf, 0.5 mL	(Sigma-Aldrich, Z666521-250EA)

Solutions and reagents

Trypsin-EDTA (TE)	(Lonza, BE02-007E or Sigma-Aldrich, T4049)
Muse count and viability reagent	(Merck, MCH100102)

Method

Cells were trypsinized from the plate and added to the supernatant. The cell suspension was spun down and resuspended in the required volume of media to give 1 x 10^6 to 1 x 10^7 cells/ml. 20 µl of cell suspension was mixed with 180 µl of Muse count and viability reagent and left for 5 minutes before being read on the Muse analyser.

The number of live and dead cells for each condition was recorded. Cell counting was used as a proxy for cell proliferation while the percentage of non-viable cells determined if the drugs induced cell death.

2.3.3 Annexin V and Dead Cell assay

Equipment

Muse analyser	(Merck)
6 well plates	(CellStar, 657160)
25 ml universal tube	(Starstedt, 60.9922.243)
Eppendorf, 0.5 mL	(Sigma-Aldrich, Z666521-250EA)

Solutions and reagents

Trypsin-EDTA (TE)	(Sigma-Aldrich, T4049)
Muse Annexin V & Dead Cell Reagent	(Merck, MCH100105)

At the end of the experiment the cells were trypsinised from the plate and added to the supernatant. The cell suspension was spun down and resuspended in the required volume of media to give 1 x 10^5 to 5 x 10^5 cells/ml. 100 µl of annexin V and dead cell reagent was mixed with 100 µl of cell suspension and left in the dark for 20 minutes before being read on the Muse analyser. Cells were gated as live (Annexin V negative and 7-AAD negative), early apoptotic cells (Annexin V positive and 7-AAD negative), late stage apoptotic and dead cells (annexin V positive and 7-AAD positive) and mostly nuclear debris (Annexin V negative and 7-AAD positive). When cells enter apoptosis, they externalize phosphatidylserine (PS) to the cell surface which is bound by annexin V. In this assay Annexin V is fluorescently labelled allowing identification of when it binds to cells. The membrane-impermeant dye 7-AAD is a dead cell dye which is used to distinguish dead cells from early apoptotic cells.

2.3.4 Q-VD-OPh assay

Equipment

Muse analyser	(Merck)
6 well plates	(CellStar, 657160)
25 ml universal tube	(Starstedt, 60.9922.243)
Eppendorf, 0.5 mL	(Sigma-Aldrich, Z666521-250EA)

Solutions and reagents

Trypsin-EDTA (TE)	(Sigma-Aldrich, T4049)
In solution Q-VD- OPh Non-O-methylated-Calbiochem	(Merck, 551476)
Muse count and viability reagent	(Merck, MCH100102)

Method

Q-VD-OPh is a cell permeable, irreversible broad-spectrum caspase inhibitor, inhibiting caspases 1, 8, 9 and 3 (IC_{50} = 50, 100, 430, and <25 nM respectively). It prevents all major caspase-mediated cellular apoptosis pathways and does not exhibit any cytotoxic effects, even when used at extremely high concentrations. After 96 hours of dosing, the cells were trypsinized from the plate and added to the supernatant. The cell suspension was spun down and resuspended in growth media. 20 µl of cell suspension was mixed with 180 µl of Muse Count & Viability Reagent and left for 5 minutes before being read on the muse analyser. The number of alive and dead cells for each condition was recorded.

2.4 Western Immunoblotting - Protein analysis

2.4.1 Cell lysis

Solutions and reagents

Lysis buffer: consisted of 50 mM Sodium Chloride (Fisher, S/13120/63), 50 mM Sodium Fluoride (Sigma-Aldrich, S-6521) (2 g/L) to inhibit serine/threonine proteases, 15 mM Sodium Pyrophosphate (Sigma-Aldrich S-9515) to inhibit phosphatases (4 g/L), 10 mM Tris HCL (Sigma-Aldrich, T3253-500G) (1.57 g/L), 5mM Ethylenediaminetetraacetic acid (EDTA, Acros Organics, 147850010) to inhibit metalloproteases, 100 µM Sodium Orthovanadate (Sigma-Aldrich, 205330500) to inhibit ATPases and phosphate transferring enzymes and 1% Triton x -100 (Sigma-Aldrich, X100) which is a mild non-denaturing detergent that replaces fat within the membrane. The lysis buffer was adjusted to pH 7.6 and was stored at 4^0C.

To make up the 100 µM Sodium Orthovanadate, 36.8 mg Sodium Orthovanadate was added to 20 ml dH_2O to make a 10 mM solution. The pH was adjusted until it reached 10 and became active, the solution turned yellow. The solution was boiled until it became clear and was adjusted to pH 10 again.

Phosphatase inhibitor cocktail: (Sigma-Aldrich, P5726-1ML) Kept at 4^0C.

Protease inhibitor cocktail: (Sigma-Aldrich, P8340-1ML) Kept at -20^0C.

Method

Lysis buffer was prepared for use by adding 10 µl/ml of phosphatase inhibitor cocktail and 10 µl/ml of protease inhibitor cocktail to 1 ml of lysis buffer solution. Lysis buffer was added to the cell pellet at a volume that was dependent on pellet size and the pellet was resuspended with a pipette on ice. The eppendorf was left at 4^0C for 20 minutes and then kept at -20^0C for short term use or -80^0C for long term.

2.4.2 Protein Quantification

To calculate total protein concentration within the cell lysates to ensure the samples are loaded at the same concentration for western blot analysis.

Equipment

iMark Microplate reader (Biorad)

Solutions and reagents

For protein estimation the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit Thermo Fisher Scientific, 23225) was used according to manufactures instructions.

Lysis buffer- As described in section 2.3.8

BCA reagent A- contains bicinchoninic acid, sodium carbonate and sodium titrate in 0.1 M sodium hydroxide. Stored at room temperature.

BCA reagent B- contains 4% cupric sulphate. Stored at room temperature.

Albumin Standard Ampules, 2 mg/ml- contains bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide.

Standards were prepared using Albumin Standards (2 mg/ml) in Lysis Buffer (Table 2-3). Diluted standards were stored at -20^{0}C.

Method

Cell lysates were centrifuged at 13,000 rpm for 3 minutes at 4^{0}C and the supernatant was transferred to a new eppendorf. The cell debris was discarded. The protein samples were diluted with lysis buffer depending on the number of cells lysed to achieve a protein concentration within the standards range. 5 µl of standards (Table 2-3) and diluted lysates were placed into 96 well plates in duplicate. Reagent A was mixed with reagent B at a 50:1 ratio and 200 µl were added to each well. The plate was incubated at room temperature for 30 minutes and then read via a spectrophotometer using a 540 nm filter and microplate manager software.

Table 2-3: Albumin standards preparation.

Vial	Standard Concentration (µg/ml)	BSA (µl)	Lysis Buffer (µl)
A	2000	300 µl of stock	0
B	1500	375 µl of stock	125
C	1000	325 µl of stock	325
D	750	175 µl of vial B	175
E	500	325 µl of vial C	325
F	250	325 µl of vial E	325
G	125	325 µl of vial F	324
H	25	100 µl of vial G	400

In this assay, the reduction of Cu^{+2} to $Cu^{+}1$ by the protein in the sample can be measured by the colorimetric detection of Cu^{+1} using a unique reagent containing bicinchoninic acid. The more protein that is present the stronger the purple colour of the reaction product. The complex exhibits absorption at 562 nm which can be detected by wavelengths from 540- 590 nm. The protein concentration can be determined by reading a standard curve, which plots each BSA standard vs. its concentration in µg/ml.

2.4.3 Protein Analysis by Electrophoresis

Sample preparation

Equipment

AccuBlock Heating Block	(Labnet International Inc.)
Micro centrifuge	(MSE)

Solutions and reagents

2x Laemmli sample buffer- (Sigma-Aldrich, S3401) Stored at -20^{0}C.

Method

Protein concentration was determined for each cell lysate from the protein assay and the volume required to give 30 µg protein was placed into an eppendorf. PBS was added to samples to ensure all samples were of equal volume. 2x sample buffer was added to each at a 1:1 ratio of sample buffer to lysate. Samples were boiled at 95^{0}C for 5-8 minutes to unfold the protein and then left to stand for a minute. Samples were centrifuged at 13,000 x g for 1 minute.

2.4.3.1 SDS - Polyacrylamide Gel Electrophoresis (SDS-PAGE) Preparation of Gels

SDS-PAGE identifies proteins by separating them according to molecular weight. The polyacrylamide gel is split into two parts; the stacking and resolving sections which have different pore sizes and pH values. The stacking gel is less extensively polymerised than the resolving gel resulting in bigger pore sizes which causes the proteins to all concentrate in one band and enter the resolving section at the same time. The resolving gel separates the proteins according to molecular weight. Altering the percentage of acrylamide within the gel alters the resolution of proteins depending on their size i.e. decreasing the percentage of acrylamide allows for the resolution of larger proteins.

Equipment

1.5 mm glass plates	(Biorad)
1.5 mm comb	(Biorad)
Casting Base	(Biorad)
Clamps	(Biorad)

Solutions and reagents

Ethanol- (Sigma, 34852-2.5L-M) Stored at room temperature.

40% Acrylamide/ BIS (N, N-methylene bis acrylamide): (Geneflow limited, EC-891) Stored at room temperature.

10% (w/v) Sodium dodecyl sulphate, pH 7.2 (SDS): (Fisher Scientific, BP166-500) 50 g electrophoresis grade SDS was dissolved in 400 mls distilled water. The pH was adjusted to 7.2 before the solution was made up to 500 mls. It was filter sterilized and stored at room temperature.

10% (w/v) Ammonium persulphate (APS): (Acros Organics, 201530010) 1 g was dissolved in 10 mls of distilled water and stored at -20^{0}C.

TEMED (N,N,N,N-tetramethyethylenediamine): (Fisher Scientific, T/P190/04) Stored at room temperature.

Resolving gel buffer (1.5 M Tris-HCl pH 8.8, 0.4% SDS)**:** 181.71g Tris Base (Fisher Scientific, BP152-1) was dissolved in 800 ml distilled water. Hydrochloric acid (HCl) (Fisher Scientific, 10467640) was used to adjust the pH to 8.8 and then the total volume was brought to 1 L with distilled water. Stored at 4^0C.

Stacking gel buffer 0.5 M Tris-HCl pH 6.8, 0.4% SDS)**:** 78.8 g Tris-HCL (Sigma, T3253-500G) was dissolved in 800 ml distilled water. Sodium hydroxide (NaOH) (Fisher Scientific, 10528240) was used to adjust the pH to 6.8 and then the total volume was brought to 1 L with distilled water. Stored at 4^0C.

Method

The glass plates were washed and dried to remove debris and then wiped with ethanol. Then they were inserted into a clamp, secured, and placed onto a casting base. A percentage for the gel was selected depending on the size of the proteins of interest (Table 2-4). The gel solutions were prepared (Table 2-5) omitting the APS and TEMED until just before pouring as both agents in combination act to polymerise the gel. The APS and TEMED were added to the resolving gel solution and this was poured between the glass plates until about a centimetre below to allow space for the well comb. Ethanol was pipetted on top of the resolving solution to level out the surface of the gel and allow the gel to set quickly by preventing its exposure to air. The gels were left to polymerize for approximately 30 minutes. Once set the ethanol was removed and the gel was washed with distilled water three times and then dried with filter paper. The APS and TEMED were added to the stacking gel solution and this was poured onto the resolving gel. A well comb was placed into the top of the gel and the gel was left to set for approximately 30 minutes. Once set, the gels were either stored, wrapped in wet paper towel at 4^0C for up to 3 days or used immediately.

Table 2-4: Percentage of gel for optimum separation.

Gel Percentage	Optimum Separation Range (kDa)
7.5%	40- 200
10%	30- 150

Table 2-5: Reagents for making hand cast gels.

Solutions	8% Resolving	10% Resolving	5% Stacking
Distilled water	10.6 ml	9.6 ml	6 ml
40% Acrylamide	4 ml	5 ml	1.2 ml
Tris Buffer	5 ml (1.5 M, pH8.8)	5 ml (1.5 M, pH8.8)	2.5 ml (0.5 M, pH6.8)
10% SDS	200 µl	200 µl	100 µl
10% APS	200 µl	200 µl	100 µl
THEMED	20 µl	20 µl	15 µl

2.4.3.2 SDS - Polyacrylamide Gel Electrophoresis (SDS-PAGE) Running Gels

Equipment

Gel Tank	(Biorad)
Gel Cassette	(Biorad)
Power Pack	(Biorad)

Solutions and reagents

10 x Running buffer- 30.3 g of 25 mM Tris Base (Fisher Scientific, BP152-1), 144 g of 190 mM glycine (Fisher Scientific BP381-5), 10 g of 0.1% SDS (Fisher Scientific, BP166-500) and dH_2O to make the final volume up to 1 litre. The pH was adjusted to 8.3 if necessary. 1x running buffer was prepared by diluting this buffer by 10 times with dH_2O and this dilution was used when running gels. Stored at room temperature.

SeeBlue Plus 2 Pre-stained protein standard- (Geneflow limited, S6-0024) Stored at 4^0C.

Method:

The well comb was removed from the glass plates and the wells were rinsed with running buffer. The gels were inserted into a gel cassette and placed into a gel tank. Once the apparatus was set up the reservoir between the gel cassettes was filled with running buffer and checked to ensure there were no leaks. Then the main tank was filled with running buffer, making sure the top of the gel was fully covered. 4 µl of SeeBlue Plus 2 Pre-stained protein standard were loaded into

the first well and samples were added to the following wells. The tank was attached to the power pack and the gel was run at 140 volts for 80 minutes.

2.4.3.3 Gel Transfer

Equipment

Transfer Cassette	(Biorad)
Transfer Tank	(Biorad)
Power Pack	(Biorad)
Sponges	(Biorad)
Filter Paper	(Biorad)
Thick blotting paper	(Biorad)
Nitrocellulose membrane	(Biorad)

Solutions and reagents

10x Transfer buffer, pH 8.3**:** 30.3 g Tris Base (Fisher Scientific, BP 152-1) and 144 g Glycine (Fisher Scientific, BP381-5) was made up to 1 litre with dH_2O and dissolved. For 1 x transfer buffer 200 ml of methanol (Fisher Scientific, M/4000/17) and 100 µl of 10 x transfer buffer was added to 700 ml of dH_2O to make 1 litre. Both transfer buffers were kept at 4^0C.

Method

The glass slides were opened up and the stacking portion of the gel was cut off and discarded. The resolving gel was placed into 1x transfer buffer to ensure it did not dry out. The filter paper, thick blotting paper, nitrocellulose membrane, and sponges were left to soak in the transfer buffer. The transfer cassette was laid down on the bench with the red side at the bottom, black side at the top. A wet sponge was placed on top of the red side followed by 2 pieces of filter paper, and the membrane. The gel was placed onto the membrane and rolled to remove any bubbles. A piece of thick blotting paper was placed on top of the gel, followed by a sponge and the cassette was closed. The transfer cassette was placed into a transfer tank with the black face nearest the cathode (-ve, black) and an ice pack was added. 1 x transfer buffer was used to fill the transfer tank and the tank was placed into a cooler box, surrounded by ice. The tank was connected to the power pack and the gel was transferred at 100 volts for 90 minutes.

2.4.3.4 Immunoblotting

In this process the membrane is blocked to prevent any nonspecific binding of antibodies to the membrane. Then it is probed with enzyme labelled antibodies which recognise the protein of interest.

Equipment

Agitator

Solutions and reagents

10 x Tris-buffered saline with Tween 20 (TBST) buffer: One tablet (Calbiochem, 524753) was dissolved in 500 mL of deionized water to yield 150 mM NaCl, 50mM Tris-HCl buffer, pH 7.6.

Blocking buffer**:** Made of either 3- 5% bovine serum albumin (BSA) (Fisher Scientific, 11403164) in TBST or 3- 5% skimmed milk powder in TBST. Blocking buffer was kept at 4^0C for a maximum of a week.

Method

Blocking- After transfer the cassette was opened, the gel discarded and the membrane was cut depending on the location of the proteins of interest. The membrane sections were blocked in 5% BSA for 1-2 hours at room temperature or overnight at 4^0C on an agitator.

Primary Antibody- Antibody solutions were prepared depending on the protein of interest (Table 2-6). The membrane was covered in primary antibody solution and left at 4^0C overnight.

Secondary Antibody- The next day the membrane was rinsed 3 times in TBST for 5 minutes each time. The membrane was incubated with the HRP-conjugated secondary antibody solution for 1-2 hours at room temperature and then rinsed 3-5 times in TBST for 15 minutes.

Table 2-6: Antibody solutions.

Protein	Source	MW (KDa)	Primary Antibody	Secondary Antibody	Condition	Company, product number
α-Tubulin	Mouse	50	1:5000	1:5000	5% BSA	Millipore, 05-829
Cyclin D1	Rabbit	36	1:2000	1:2000	5% BSA	Abcam, ab134175
E-cadherin	Rabbit	135	1:1000	1:2000	5% BSA	Cell Signaling, 3195
GAPDH	Mouse	35	1:5000	1:5000	5% BSA	Millipore, MAB374
N-cadherin	Mouse	140	1:1000	1:2000	5% BSA	Cell Signaling, 4061
p-53 (DO-1)	Mouse	53	1:1000	1:2000	5% BSA	Santa Cruz, sc-126
Phosphorylated p53 (Ser15)	Rabbit	53	1:1000	1:2000	5% BSA	Cell Signaling, 9284
PCNA	Mouse	35	1:2000	1:2000	5% BSA	Millipore, MAB424

2.4.3.5 Detection

This process allows for the detection of the proteins which have been labelled with the antibodies. The enzyme peroxidase which is bound to the secondary antibody acts upon the hydrogen peroxide formed by the decay of peroxide and in the presence of luminol produces light: this can be detected by an imager.

Equipment

ChemiDoc-It Imaging System (Biorad)

Solutions and reagents

Western ECL clarity substrate kit (Biorad, 1705061): Consisting of luminol and peroxidase. Stored at room temperature.

Method

The chemiluminescent substrates, peroxidase and luminal, were applied to the membrane at a 1:1 ratio and used to continually wash the membrane for 5 minutes. Chemiluminescent signal was detected using the ChemiDoc-It Imaging System.

2.5 Wound healing assay

Equipment

2 well cell culture inserts	(Thistle Scientific Limited, IB-80286)
24 well plates	(Thermo Fisher Scientific, 930186)
Tweezers	

Solutions and reagents

Mitomycin C: 2 mg were resuspended in 200 ml of growth media and filter sterilized. It was kept in the dark at 4^0C (Sigma, M4287).

Method

At the end of 96 hours of dosing, cells were trypsinized from the flasks and counted. Ibidi 2 well silicone culture inserts with a defined cell free gap of 500 µm were placed into a 24 well plate using tweezers. The cells were seeded either side the 2 well ibidi insert at 0.04 x 10^6 in 70 µl per well in growth media with their respective concentration of drug. The cells were left to attach for 22 hours and then the media was removed and media containing mitomycin C was added for a further 2 hours to inhibit cell proliferation. After this, the insert was removed using tweezers, the wells were washed with PBS, and media containing the appropriate concentration of drug was added. The scratch was imaged for the first time at 0 hours and then was imaged every 12 hours after this point in the same location. ImageJ was used to calculate the area of the gap (Figure 2.1: **ImageJ calculated gap area.**.

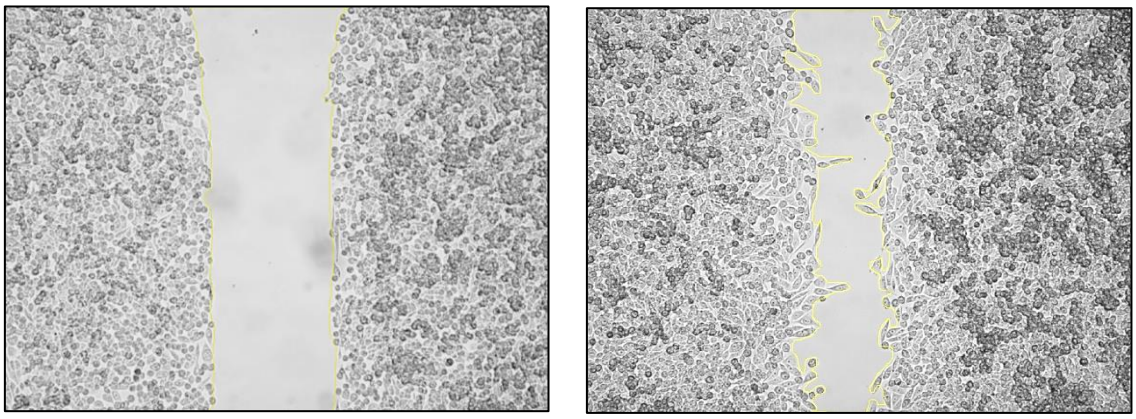


Figure 2.1: ImageJ calculated gap area.

2.6 qPCR

Equipment

Microcentrifuge	
Hot block	
NanoPhotometer	(Geneflow)
ThermoCycler	(Biorad)
ABI StepOne Plus PCR system	(Applied Biosystems)
PCR machine	(Applied Biosystems)
PCR tubes	(Alpha Laboratories, LW2340)

Solutions and reagents

Ribozol RNA extraction reagent	(VWR life sciences, N580)
Chloroform	(Fisher Scientific, BP1145-1)
Isopropanol	(Fisher Scientific, 19515-500 ml)
Ethanol	(Sigma, 34852-2.5L-M)
DEPC Treated Water	(Ambion, AM9906)
High capacity RNA to cDNA kit	(Thermo Fisher, 4387406)
SYBR Green Jumpstart Taq Readymix (20 mM Tris-HCL, pH 8.3, 100 mM KCL, 7 mM $MgCl_2$, 0.4 mM each dNTP (dATP, dCTP, dGTP, TTP), stabilizers, 0.05 unit/µl Taq DNA polymerase, JumpStart Taq antibody, and SYBR Green)	(Sigma- Aldrich, S4438-500RXN)
Forward and reverse primers for GAPDH, E-cadherin, N-cadherin and Slug (10 µM stock)	(Sigma- Aldrich)
QuantiTect Primer Assay-MMP9	(Qiagen, QT00040040)

2.6.1 RNA extraction

At the end of 96 hours of dosing the media was removed and the cells were washed with PBS. In a fume hood, 1 ml of Ribozol RNA extraction reagent was added and pipetted up and down to remove all the cells from the flask and then transferred to clean RNase/DNase free microfuge tubes and incubated for 5 minutes at room temperature to completely dissociate nucleoprotein complexes. Chloroform was added at 0.2 ml per 1 ml of Ribozol and then shaken for 15 seconds before incubation at room temperature for 10 minutes. The eppendorf was spun at 4^0C, 12,000 rpm for 15 minutes to separate the mixture into 3 phases: a red organic phase which contained

protein, an interphase which contains DNS and a colourless aqueous phase which contains RNA. The top, aqueous layer was transferred to a new eppendorf and 500 µl of isopropanol was added and then left at room temperature for 10 minutes to precipitate the RNA. The eppendorf was spun for 10-15 minutes at 12,000 rpm, 4^0C and the supernatant was removed by pouring into a waste bottle, leaving a white RNA precipitate on the side of the tube. 1 ml of 70% ethanol was added and the eppendorf was vortexed. It was then spun for 10 minutes, 4^0C at 7,500 rpm and the supernatant was removed. 1 ml of ethanol was added to the pellet and the eppendorf was spun for 10 minutes, 4^0C at 7,500 rpm. The supernatant was removed and 20 µl hyclone water were added and the eppendorf was placed in a hot block at 58^0C for 5 minutes. This was stored at -80^0C.

2.6.2 RNA quantification

RNA was quantified using a Geneflow Implen NanoPhotometer using Micro Applications and then RNA quantification settings. The LabelGuardTM Microliter Cell was inserted into the cell holder and 1 µl of hyclone water was pipetted onto the measuring window for a blank. Each sample was measured in the same way and the concentration (ng/µl), the A260/A280, and the A260/A230 was recorded. The ratio of A260/A280 was used as an indicator of protein/phenol contamination while A260/A230 ration served as an indicator of organic solvents/isothiocyanate contamination. A ratio of 1.6-2 for the absorbance at 260/280 nm indicated that the RNA was of a good quality.

2.6.3 DNase treatment

DNase treatment was performed to remove contaminating genomic DNA from the RNA extraction. The DNase digestion mix was prepared by combining 1 µl of 10x Reaction Buffer with 1µl of DNase. The 2 µl of the DNase digestion mix was mixed with 8µl of RNA samples in RNase-free water and incubated for 15 minutes at room temperature. 1 µl of DNase Stop Solution was added to terminate the reaction. To inactivate the DNase the samples were incubated at 70^0C for 10 minutes and then chilled on ice before the reverse transcription step.

2.6.4 Converting RNA to cDNA

RNA was converted to cDNA using the high capacity RNA to cDNA kit. For each sample 1 µl of enzyme and 10 µl of buffer were added to an eppendorf and kept on ice. 2000 ng of RNA and hyclone water were added to make the total volume up to 20 µl. The eppendorfs were placed into a ThermoCycler and run at 37^0C for 60 minutes, 95^0C for 5 minutes and then held at 4^0C infinitely. The cDNA was stored at -20^0C for up to 6 months.

2.6.5 qPCR

qPCR was performed using a SYBR Green Jumpstart Taq ReadyMix. The master mix was made in qPCR tubes consisting of SYBR Green, internal reference dye, forward primer, reverse primer and Hyclone water for each gene following the volumes in Table 2-7. Primer sequences are shown in Table 2-8.

Table 2-7: **Mastermix components for qPCR.**

Reagents	Stock concentration	Final concentration
SYBR Green JumpStart Taq ReadyMix	2x	1 x
Internal Reference Dye	100 x	1 x
Forward Primer	10 µM	0.4 µM
Reverse Primer	10 µM	0.4 µM
Hyclone water		To make final volume 9 µl

In a 96 well plate 9 µl of master mix for each gene were added per well along with 1 µl of cDNA in duplicate. 1 µl of hyclone water was added to one well to use as a blank to check for possible contamination. The plate was covered with optically clear sealing tape and centrifuged for a few seconds to mix the cDNA and master mix. The plate was inserted into the qPCR machine and using ABI StepOne Plus Real-Time PCR system the programme was set:

94^0C- 2 minutes

94^0C- 15 seconds

62^0C- 30 seconds

The programme was run for 50 cycles and a melting curve at every 0.3^0C from 60 to 95^0C was measured to confirm the PCR products were specific. Double stranded products are generated as the reaction progresses which the SYBR Green dye intercalates into and fluoresces. Once enough products have accumulated to cause the fluorescence to rise above the background the

threshold cycle or CT is reached. mRNA levels were normalized to the housekeeping gene, GAPDH, using the 2-ΔΔCT method (Pfaffl, 2001) to determine fold change in gene expression.

Table 2-8: Primer sequences for qPCR.

Protein (Gene)	Forward primer	Reverse primer
Snail (SNAI1)	GAAAGGCCTTCAACTGCAAA	TGACATCTGAGTGGGTCTGG
Slug (SNAI2)	CTTCCTGGTCAAGAAGCA	GGGAAATAATCACTGTATGTGTG
Twist (TWIST1)	CAAGTCTGCAGCTCTCGCCA	CCAACGGCTGGCGCACAC
E-cadherin (CDH1)	TTGAACGAATGGGGCAATCG	ACCAGCAACGTGATTTCTGC
N-cadherin (CDH2)	TCGCCATCCAGACCGACCCA	TGAGGCGGGTGCTGAATTCCC
Matrix metalloproteinase 9 (MMP-9, CLG4B)	Primer sequence not provided by Qiagen	Primer sequence not provided by Qiagen
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	GATCATCAGCAATGCCTCCT	TGTGGTCATGAGTCCTTCCA

2.7 3D Cell Culture- Spheroids

2.7.1 Seeding and maintaining spheroid cultures

Cells cultured in 2D grow as flat monolayers while cells cultured in 3D form spheroids, aggregates of cells which form solid or hollow spheres. 3D cell culture better represents the cell to cell interactions and cell to extracellular matrix signalling that is essential for differentiation, proliferation, and cell survival.

Equipment

Syringe

21G Microlance hypodermic needle (BD Biosciences, 304432)

40-µm cell strainer

48 well plates

Solutions and reagents

Advanced DMEM/F12 (ADF): 2 ml of 0.1% Sodium Bicarbonate (Sigma Aldrich, S6751-500G) 5 mM LG, 2.5 ml of 10 mM HEPES buffer (Sigma-Aldrich, 83264) P/S was added to a 500 ml bottle of Advanced DMEM/F12 (Thermo Fisher scientific, 12634028). From this 48.5 ml was aliquoted into 50 ml tubes and stored at -20^{0}C. Before use the ADF was thawed and 500 µl of 1:100 N2 supplement (Thermo Fisher scientific, 17502048), 1 ml of 1:50 B27 supplement (Thermo Fisher scientific, 17504044) and 100 µl of 1:500 N-acetyl-cysteine (Sigma-Aldrich, A9165) added. This media was made fresh at least every 2 weeks and stored at 4^{0}C.

Keratinocyte medium: 25 mg of bovine pituitary extract (BPE, Thermo Fisher scientific), 2.5 µg of epidermal growth factor 1-53 (EGF) (Thermo Fisher scientific), P/S and 10 ml of FBS were added to a 500 ml bottle of keratinocyte serum free medium. Stored at 4^{0}C.

Matrigel: Matrigel Basement membrane matrix phenol red free (Corning, 356321). Stored at -20^{0}C.

Calcein AM and ethidium homodimer-1 dyes: (Thermo Fisher Scientific, L3224). Calcein AM: 4 mM in anhydrous DMSO. Ethidium homodimer-1: 2 mM in DMSO/H2 O 1:4 (v/v). Both stored at -20^{0}C.

4% Paraformaldehyde (PFA) in PBS- (Alfa Aesar, J61899) Stored at 4^0C.

0.5% TritonX-100- 0.5 ml triton X-100 (Sigma-Aldrich, X100) in 100 ml PBS. Stored at room temperature.

Glycine rinse- 100 mM glycine (Fisher Scientific, BP381-5) in PBS, filter and store at room temperature.

IF buffer- 0.1% BSA (Fisher Scientific, 11403164), 0.2% TritonX-100 (Sigma-Aldrich, X100), 0.05% Tween 20 (Sigma-Aldrich, P7949), PBS. pH to 7.4 and store at room temperature.

AlexaFluor 594 anti-mouse- (Fisher Scientific, A-11005) Stored at 4-8^0C. 1:50 ratio in IF buffer.

AlexaFluor 488 anti-rabbit- (Fisher Scientific, A-11008) Stored at 4-8^0C. 1:50 ratio in IF buffer

Method

Cell preparation- Prior to starting work the required number of plates were placed into the incubator to warm. Cells cultured for at least 2 weeks were trypsinized from the flask, spun down and resuspended in PBS using a syringe and a 21G needle for PC3 and PNT2 cells and a p1000 pipette for the H642/17 cells as use of a needle has been shown to increase cell death in this cell type. The cell suspension was passed through a 40 µm cell strainer into a 50 ml falcon tube and cells were counted.

Seeding spheroids- On ice, cells were resuspended in Matrigel using pre-frozen tips. 20 µl of Matrigel and cells was reverse pipetted into each well of a pre-warmed 48-well plate as a blob in the centre. Cells were seeded in quadruplets in the centre of the plate, ensuring that there was a boarder of wells left empty around the edge of the plate. The plate was placed upside-down in the incubator for the matrigel to solidify. Placing the plate upside-down reduced the number of cells that stuck to the bottom of the plate instead of remaining suspended in the matrigel and formed 2D culture rather than 3D. After 15 minutes 250 µl of Advanced DMEM F12 (ADF) culture medium supplemented with N2, B27 and N-acetyl cysteine were added to each well for PC3 and PNT2 cells. 250 µl of keratinocyte medium supplemented with BPE, EGF, P/S and FBS were added to each well for the H642/17 cells. 500 µl of sterile PBS were added to all empty wells to help maintain internal humidity.

Spheroid maintenance- The plate was placed into a sandwich box containing a damp towel to maintain humidity. The media was replaced twice a week and the towel once a week for the rest of the culturing period.

2.7.2 Imaging spheroids

After 4 days spheroid area was assessed using Leica Application Suite X software on a widefield microscope. In this program spheroids were imaged on 5x magnification, taking images in the x,y and z planes to ensure all spheroids were picked up throughout the matrigel. 35 images were taken in the z axis, working from where the matrigel was attached to the plate upwards. These 35 images formed a stack for each position in the well. 5 different positions were imaged per well (Figure 2-2). This was repeated at days 7, 11 and 14 for seeding density experiments. For dosing experiments to examine spheroid formation, cells were imaged at day 14 and for dosing experiments to examine spheroid growth cells were imaged at day 21.

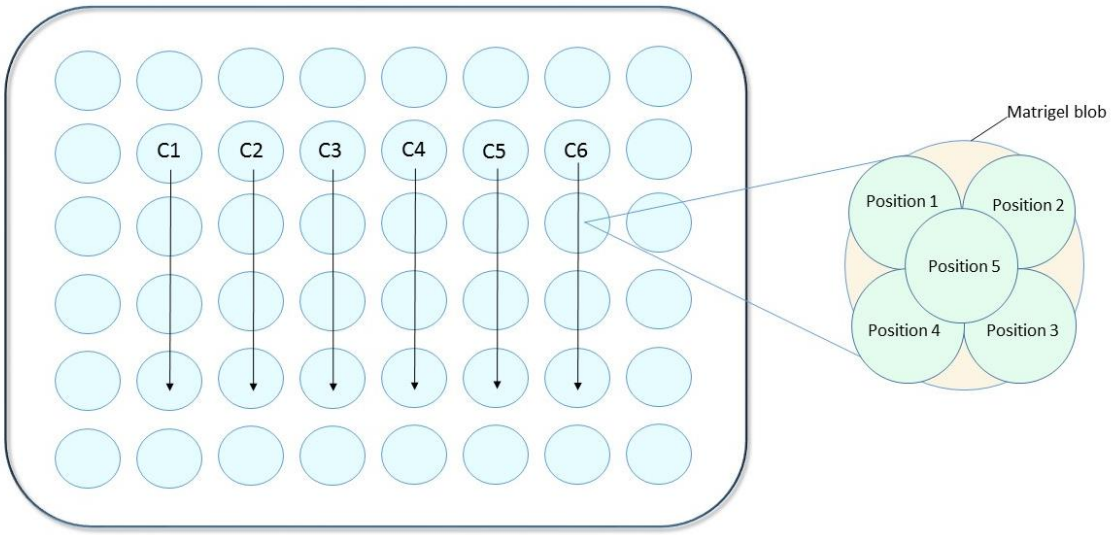


Figure 2.2: Plate layout for seeding spheroids and imaging
C1-C6 represent the six different conditions, performed in quadruplet. For imaging 5 different positions within the matrigel blob were imaged per well, each containing multiple spheroids. This gave 20 images per condition.

2.7.3 Analysis of spheroids

The stack of 35 images taken per position were compressed into 2D using Matlab and average spheroid area was calculated automatically on Matlab. This Matlab program was created by Dr Adam Chambers in collaboration with Dominic Alibhai at the Wolfson Bioimaging Centre, The University of Bristol. The spheroid area was checked manually to ensure spheroids were identified correctly. Matlab did not pick up spheroids which were partially cut out of frame and could distinguish when spheroids were growing into each other and separate them. Any spheroids that were not picked up correctly were excluded so as not to skew the data. For the PC3 spheroid they grew with long projections around the edge which were not always picked up by the Matlab software. As this occurred across all the conditions it was not considered a problem.

Parameters on the Matlab program were altered depending on the cell line being imaged as it depended on the size and shape of the spheroid. Spheroids which had many protrusions such as the PC3s had an increased circular threshold compared to the rounded spheroids.

The spheroid areas for each of the 5 positions per well were put into an Excel spreadsheet. All four duplicates were combined, giving 20 images per condition with multiple spheroids in each image. Data was plotted using Graphpad prism as box and whisker plots to show the spread of the data with the whiskers including the 5-95% percentiles. A Kruskal Wallis one-way ANOVA and Duns post hoc test was used to calculate pairwise significance.

2.7.4 Staining spheroids with calcein AM and ethidium homodimer-1

A live/dead viability/cytotoxicity kit, for mammalian cells (Thermo Fisher Scientific, L3224) was used to stain spheroids for live and dead cells. Spheroids were grown as stated in Chapter 2, section 2.7.1 and imaged. Media was removed from wells containing spheroids to be stained and 200 µl of fresh ADF media were added to each well. In the dark 0.5 µl of both the calcein AM and ethidium homodimer-1 dyes were added and the plate was incubated at room temperature for 30 minutes. Stained spheroids were imaged using the Leica Application Suite X software on a widefield microscope.

2.7.5 Immunofluorescence with spheroid cultures

The immunofluorescent protocol for 2D cells was modified with the help of Professor Norman Maitland and his research group at the Cancer Research Unit, University of York. Media was removed from spheroids and spheroids were fixed in 4% PFA for 30 minutes at room temperature. Spheroids were rinsed twice with PBS and permeabilized with 0.5% Triton-X-100 in PBS for 30 minutes. Spheroids were washed with glycine rinse three times for 15 minutes and then blocked with goat serum diluted 1:50 in IF buffer for 1 hour at room temperature in the dark on a tilting table. They were then incubated overnight at room temperature with the primary antibody in IF buffer. The next day, spheroids were washed 3 times with IF buffer for 15 minutes and then incubated in the dark for four hours with the secondary antibody in IF buffer, either AlexaFluor 594 anti-mouse or AlexaFluor 488 anti-rabbit depending on the primary antibody. Spheroids were rinsed with IF buffer 3 times for 15 minutes and then stained with DAPI for four hours. Spheroids were washed with PBS and then imaged on a confocal microscope using the HCX PL Fluotar 10x 0.3 Dry objective. They were kept in PBS for up to a week.
For the negative control spheroids followed the same immunofluorescence protocol but were not incubated overnight with the primary antibody.

2.8 Statistical analysis

Graphpad prism software version 7.03 (Graph Pad, San Diego, California, USA) was used for statistical analysis. If the data was normally distributed a two-way ANOVA was used with either Dunnets post hoc test to compare the control to each condition or Tukeys post hoc test to compare each condition with each other. If the data was not normally distributed a Kruskal Wallis one-way ANOVA was used with Duns post hoc test. p-values below 0.05 were determined statistically significant.

Chapter 3

The effect of aspirin and metformin on cell proliferation and cell death in 2D cell culture

3.1 Introduction

3.1.1 Cell proliferation, the cell cycle and cancer

Regulation of cell division is critical in human development and growth with the balance between cell proliferation and apoptosis essential for tissue homeostasis (198). The cell cycle is initiated by the presence of growth factors, however, once the cell passes a restriction point in G1 phase the cells are committed to the cycle and will continue even during growth factor deprivation (199) (Figure 3.1). The entire process requires an extensive cell cycle toolkit and proteins involved in cell proliferation are often used to assess where cells are in the cycle and whether drugs or other treatments impact their transition through it. While there are many markers of proliferation, the most commonly used include ki-67, p120, proliferating cell nuclear antigen (PCNA), minichromosome maintenance complex component-2 (MCM-2), cyclins, and cyclin dependent kinases (CDKs) (200). Expression of ki67, PCNA and p120 have been correlated with Gleason score, tumour differentiation, stage and prostatic specific antigen (PSA) levels in prostate carcinoma smears and are useful as indicators of tumour aggressiveness (201). Ki67 and PCNA are now routinely used as prognostic makers in cancer diagnosis (202). Because of their role in cell cycle progression in this thesis PCNA and cyclin D1 are used to assess cell proliferation. PCNA is a protein which is highly expressed during G1 and S phases, and plays a major role in DNA replication, DNA repair, chromatin remodelling and cell cycle control (203). During DNA replication it plays an essential role in the DNA replication machinery, acting as an accessory protein for DNA polymerase δ and ε (204). PCNA also associates with a D-type cyclin and a CDC2 kinase inhibitor to control the cell cycle by preventing premature DNA synthesis during the G1 phase. Cyclin D1 levels vary throughout the cell cycle depending on which stage the cell is in. During G1 cyclin D1 levels are elevated due to growth factor exposure which consequently initiates DNA synthesis (205). In S phase cyclin D1 is suppressed, allowing efficient DNA synthesis and then in G2 phase cyclin D1 levels are elevated again. This fluctuation of cyclin D1 expression ensures that cells only proliferate when the extracellular environment is conducive for growth, with quiescent cells expressing extremely low levels of cyclin D1 (206). Indeed, in a study which assessed cyclin D1 expression in 85 patients who underwent radical prostatectomy for prostate cancer and 10 normal prostate tissue samples retrieved from autopsies it was seen that normal prostate tissues were negative for cyclin D1 and high-grade Gleason score (≥7) tumours had higher cyclin D1 expression than low-grade Gleason score tumours (207).

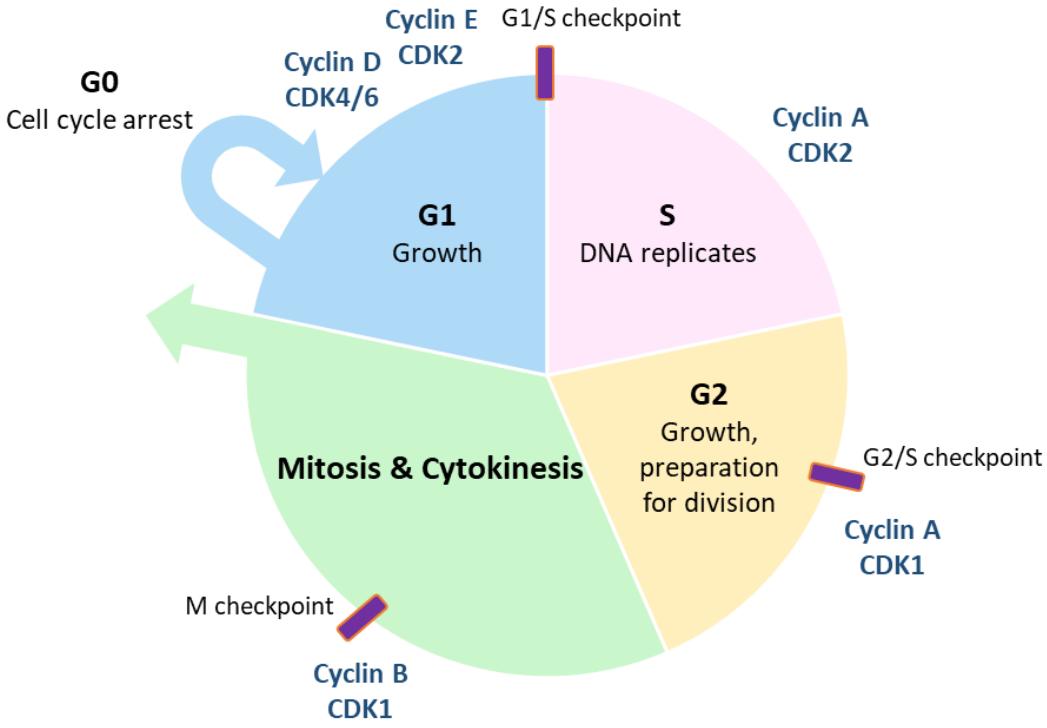


Figure 3.1: The stages of the cell cycle
Cell proliferation is mediated by growth factors which activate different signalling pathways and direct cells which are in zero growth (G0) to enter the cell cycle. The cell cycle is regulated by a number of checkpoints and proteins such as cyclins and cyclin-dependent kinases that are expressed at different levels throughout the cycle.

Evidence that aspirin and metformin affect cancer cell proliferation is extremely compelling with many studies showing that each drug inhibits proliferation pathways and causes cell cycle arrest (208) (209). However, the effect of both drugs used in combination on cell proliferation is less well understood and the mechanism by which they could enhance each other's anti-proliferative effects is yet to be explored.

3.2 Hypotheses and aims

In this chapter, the effects of aspirin or metformin alone and in combination on cell proliferation and cell death in 2D cell culture are described.

Hypothesis: The literature suggests that aspirin and metformin reduce the proliferative capacity of cancer cells. In this study, it is hypothesised that low dose aspirin and metformin will reduce proliferation of the prostate cancer cell lines; PC3, DU145 and LNCaP. It is hypothesized that the combination of aspirin and metformin will have the greatest effect as both drugs target similar proliferation pathways.

Aims and objectives:

1. To examine the effect of aspirin and metformin individually on cell proliferation and cell death.
2. To determine whether the drugs have synergistic effects.

3.3 Materials and Methods

3.3.1 Cell seeding and dosing for experiments

For the ^{3}H-thymidine incorporation assay, cell counting, annexin V/7-AAD, Q-VD-OPh and western blotting experiments the cells were seeded as described in Chapter 2, Section 2.1.3. Aspirin and metformin were reconstituted as described in Chapter 2, Section 2.2. The cells were dosed for a total of 72 hours for the ^{3}H-thymidine incorporation assay and western blotting with p53 and phosphorylated p53 (Ser15). In all other experiments cells were dosed for 96 hours. The media was collected and replaced every 24 hours for all experiments. Statistical analysis was performed as described in Chapter 2, Section 2.8.

3.3.2 Dosing protocol for ^{3}H-thymidine incorporation assay

PC3, DU145, LNCaP and PNT2 cells were dosed with 0, 10 and 30 µM metformin and 0, 0.5, 1, 1.5 and 2mM aspirin. This ensured that doses were kept within the therapeutic range, 0-2 mM aspirin and 0-40 µM metformin, with a few higher concentrations to see an enhanced effect. Cells were dosed with each drug alone as well as a combination of the two drugs at each of the concentrations, resulting in 16 conditions. The ^{3}H-thymidine incorporation assay was performed as described in Chapter 2, Section 2.3.1**.**

3.3.3 Dosing protocol for PC3 and PNT2 cell counting to determine drug concentrations for combination experiments

PC3 and PNT2 cells were dosed with 0, 10, 20, 30 and 5000 µM metformin and 0, 0.5, 1, 2, 4 and 10 mM aspirin. Cells were counted following the protocol described in Chapter 2, Section 2.3.2.

3.3.4 Dosing protocol for combination cell counting and annexin V, 7AAD staining

Cells were dosed with 0, 10 and 30 µM metformin and 0, 0.5 and 2 mM aspirin alone and in combination, resulting in 9 conditions. The number of live and dead cells were counted following the protocol described in Chapter 2 section 2.3.2. Cells were stained with annexin V and 7-AAD and analysed on a Muse cell analyser as described in Chapter 2 section 2.3.3.

3.3.5 Dosing protocol for Q-VD-OPh and aspirin treatment

LNCaP cells were dosed with either 20 µM Q-VD-OPh, 2 mM aspirin or a combination of 20 µM Q-VD-OPh and 2 mM aspirin as described in Chapter 2, Section 2.3.4. Cell counting was performed as described in Chapter 2, Section 2.3.2.

3.3.6 Dosing protocol for western blotting

For Western blotting with p53 and phosphorylated p53 (Ser 15) cells were dosed with 2 mM aspirin for 72 hours. After 72 hours the cells were dosed again and then were scraped off the flasks 0, 15, 30, 60 and 120 minutes after dosing and lysed. For western blotting with PCNA and cyclin D1 the cells were dosed with 0, 10, 20, 30 and 1000 µM metformin and 0, 0.25, 0.5, 1, 2 and 4 mM aspirin and lysed after 96 hours. Western blotting was performed as described in Chapter 2, Section 2.4.

3.4 Results

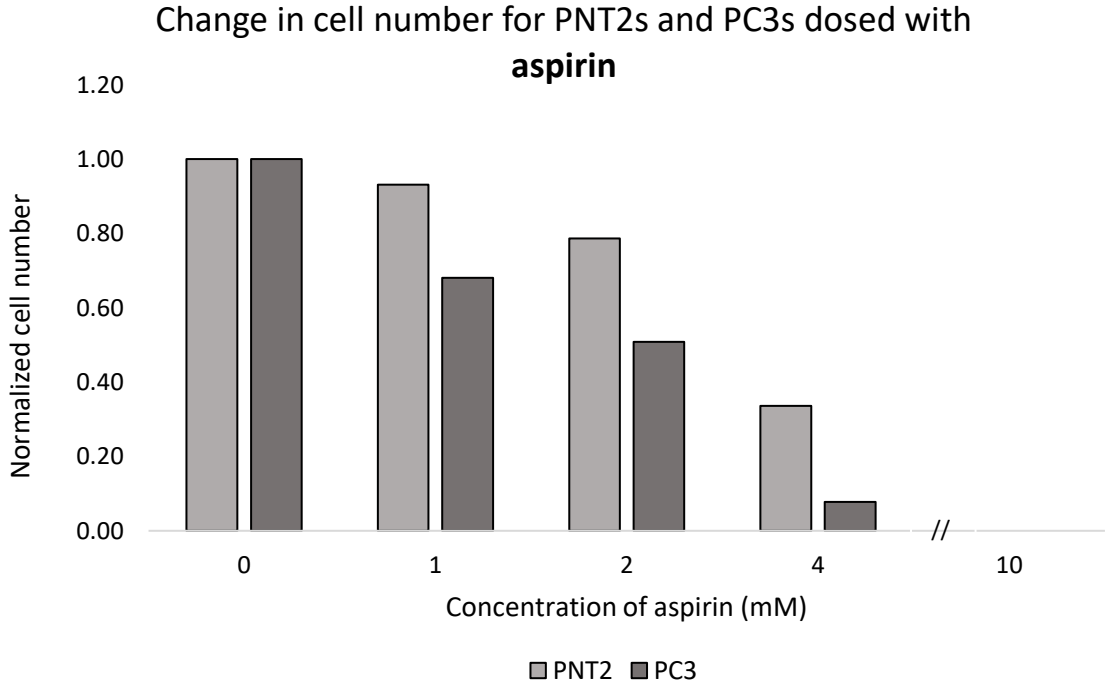
3.4.1 Optimisation of dosing concentrations for aspirin and metformin

3.4.1.1 Proliferative response of PC3 and PNT2 cells to aspirin

A preliminary dose response experiment was performed to characterize the effect of the drugs on cell proliferation and to select drug concentrations for dosing with a combination of aspirin and metformin. Concentrations to be selected were those that reduce cell proliferation compared to the control, but by less than 50% so that an enhanced effect could still be seen with the combination. For this experiment, a normal prostate cell line, PNT2, and a cancerous cell line, PC3, were selected to examine the effect of the drugs on a non-tumorigenic cell line and the most advanced cancerous cell line.

When cells were dosed with either of the drugs there was a dose-dependent decrease in cell number (Figure 3.2 and Figure 3.3). The PC3s were more affected by aspirin than the non-cancerous PNT2s with a 40% decrease in cell number at the highest clinically relevant concentration, 2 mM, whereas the same concentration only reduced cell number by 22% for the PNT2s. For both cell lines aspirin was toxic at 10 mM, killing all the cells as determined by trypan blue counting.

a.



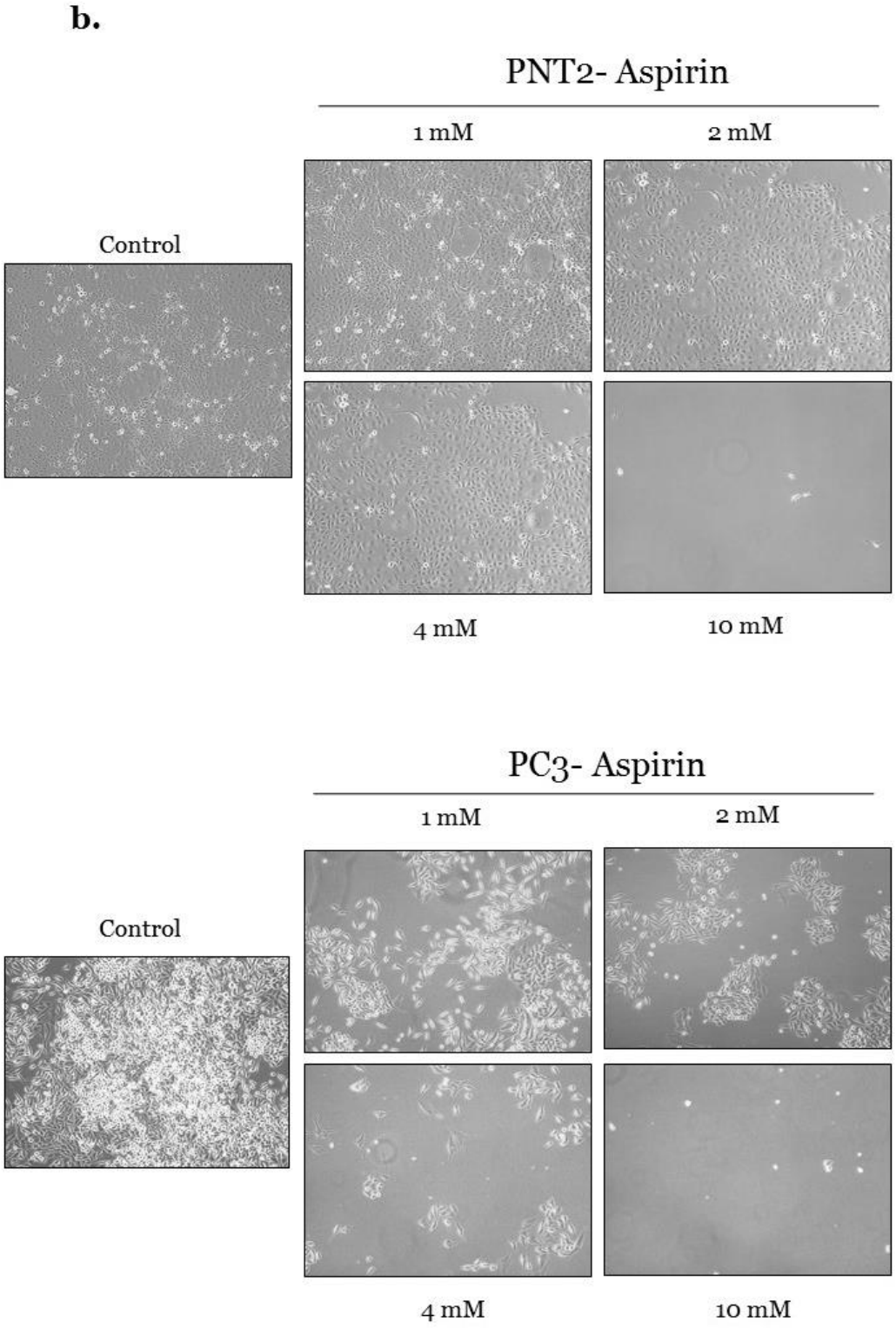
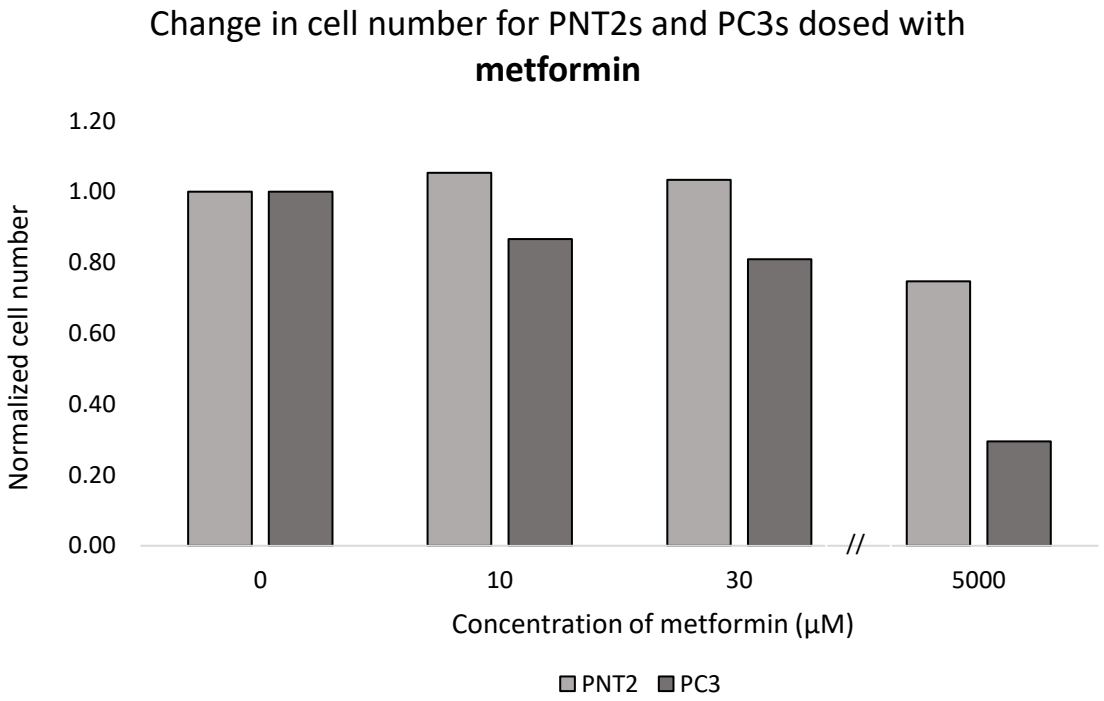


Figure 3.2: Dose response to aspirin for PNT2 and PC3 cells.
(a) Change in PC3 and PNT2 cell number after dosing with aspirin every 24 hours for a total of 96 hours. Data represents one independent experiment. **(b)** Images of PNT2 and PC3 cells after dosing with aspirin for 96 hours. The field of view was randomly selected. Images were captured using a phase contrast microscope, 10x objective.

3.4.1.2 Proliferative response of PC3 and PNT2 cells to metformin

Similar to aspirin, the PC3s displayed a dose-dependent decrease in cell number when dosed with metformin (Figure 3.3). They were also more affected than the non-cancerous PNT2s with a 19% decrease in cell number when dosed with 30 µM metformin compared to a 0% decrease for PNT2s. Even at 5000 µM metformin, which is well above the considered maximum therapeutic concentration of 30 µM, there were still some cells which remained alive. For the PNT2s this was the only concentration at which a decrease in cell number was observed.

a.



b.

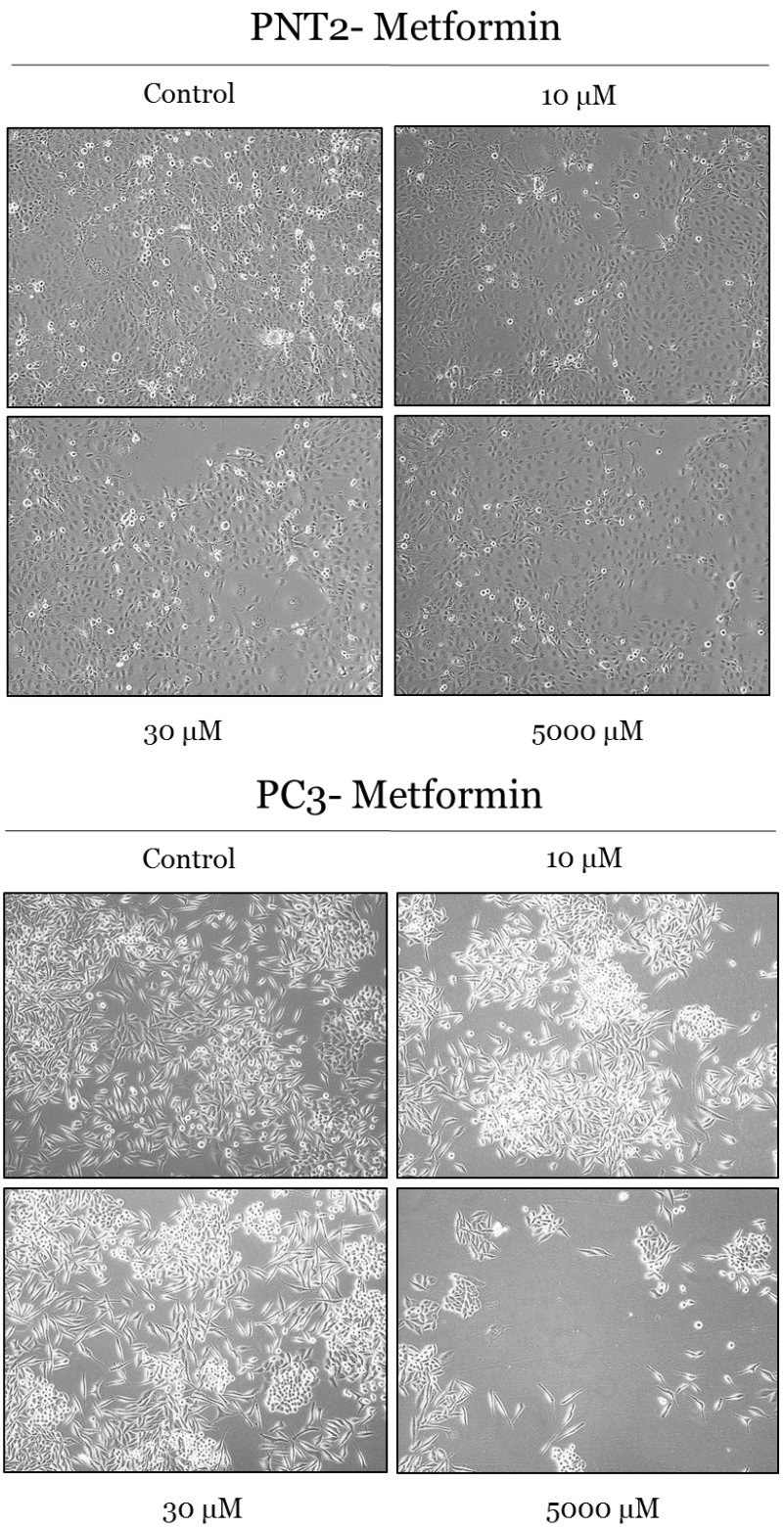


Figure 3.3: Dose response to metformin for PNT2 and PC3 cells.
(a) Change in PC3s and PNT2 cell number after dosing with metformin every 24 hours for a total of 96 hours. Data represents one independent experiment. **(b)** Images of PNT2 and PC3 cells after dosing with metformin for 96 hours. The field of view was randomly selected. Images were captured using a phase contrast microscope, 10x objective.

3.4.2 Proliferative response of PC3, DU145, LNCaP and PNT2 cells to aspirin and metformin: tritiated thymidine (^{3}H- thymidine) incorporation

From the previous experiment concentrations of up to 2 mM aspirin and 30 µM metformin were selected and the cancerous PC3, DU145 and LNCaP cells and non-cancerous PNT2 prostate cell lines were dosed with 0, 0.5, 1 and 2 mM aspirin and 10 or 30 µM metformin for 72 hours before performing a tritiated thymidine incorporation assay.

For the PC3 cell line dosing with aspirin alone significantly reduced ^{3}H-thymidine incorporation from 1 mM aspirin onwards ($p= 0.001$, <0.001 and <0.001 for 1, 1.5 and 2 mM aspirin) and dosing with metformin reduced DPM for both 10 and 30 µM ($p =0.005$ and 0.02 respectively) (Figure 3.4 a). While the combinations of aspirin and metformin had the greatest effect on cell proliferation for all conditions, it was only significant when the cells were dosed with either 0.5, 1 or 1.5 mM in combination with 30 µM metformin ($p= 0.01$, <0.001 and 0.03 respectively). This was the only cell line in which an additive effect of the drugs was seen with ^{3}H-thymidine incorporation.

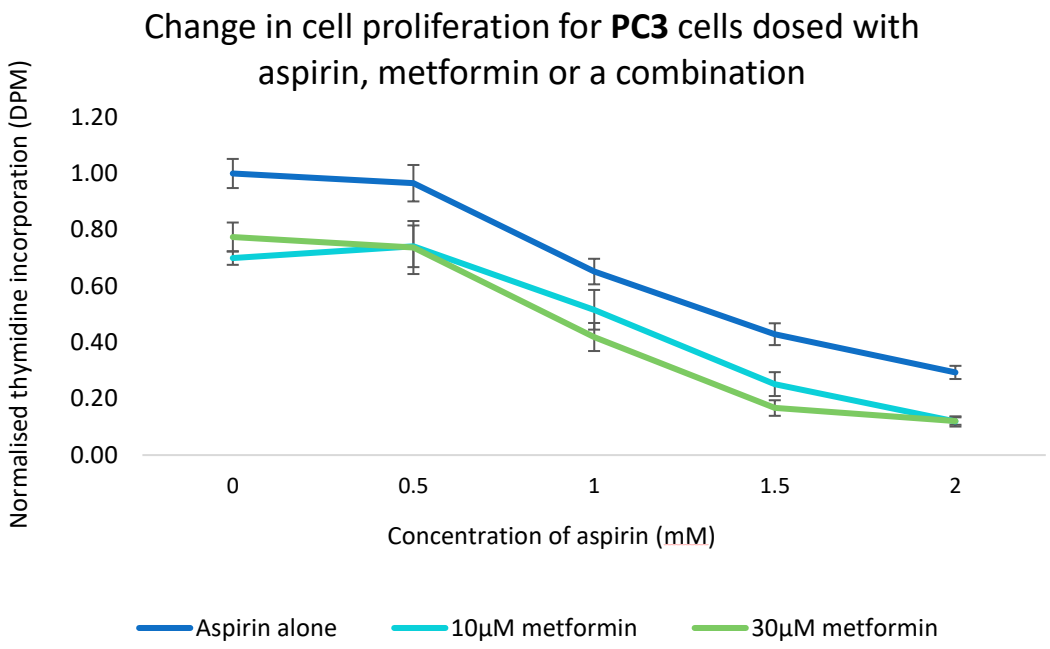
As with the PC3 cell line, the DU145s experienced a significant decrease in cell proliferation with aspirin treatment from 1 mM onwards ($p= 0.001$, <0.001 and <0.001 for 1, 1.5 and 2 mM aspirin) but were not affected by dosing with metformin alone (Figure 3.4 b). There was no additive effect when the drugs were used in combination, indicating that the decrease in proliferation was solely due to aspirin.

The LNCaP cell line was the most responsive to aspirin, with a sharp decrease in DPM of 46% ± SEM compared to the control (100%) when cells were dosed with 0.5 mM. There was a reduction in cell proliferation at all concentrations of aspirin tested ($p<0.001$ for 0.5-2 mM) (Figure 3.4 c). Similar to the DU145s, LNCaP cell proliferation was not affected by metformin and the decrease observed when the drugs were used in combination was solely due to aspirin.

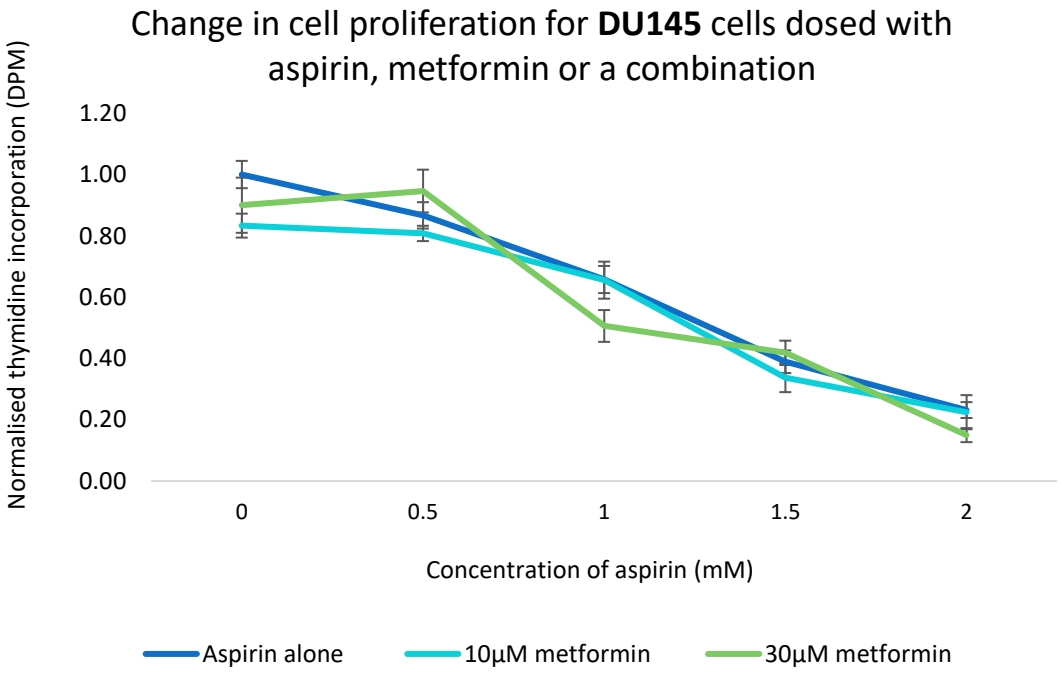
The PNT2 cell line was less sensitive to aspirin than the other cell lines, however there was an effect at high drug concentrations with a significant decrease in proliferation when dosing with 1 mM aspirin onwards ($p= 0.001$, <0.001 and <0.001 for 1, 1.5 and 2 mM aspirin) (Figure 3.4 d). There was a slight decrease in DPM when cells were dosed with metformin, however this was not significant. The PNT2s were the least affected cell line by the combination of the aspirin and metformin and an additive effect was not observed.

The decrease in proliferation was similar for each of the cell lines when dosed with a combination of the highest concentrations of the drugs, 2 mM aspirin and 30 µM metformin, with only around 20% of the cells continuing to proliferate.

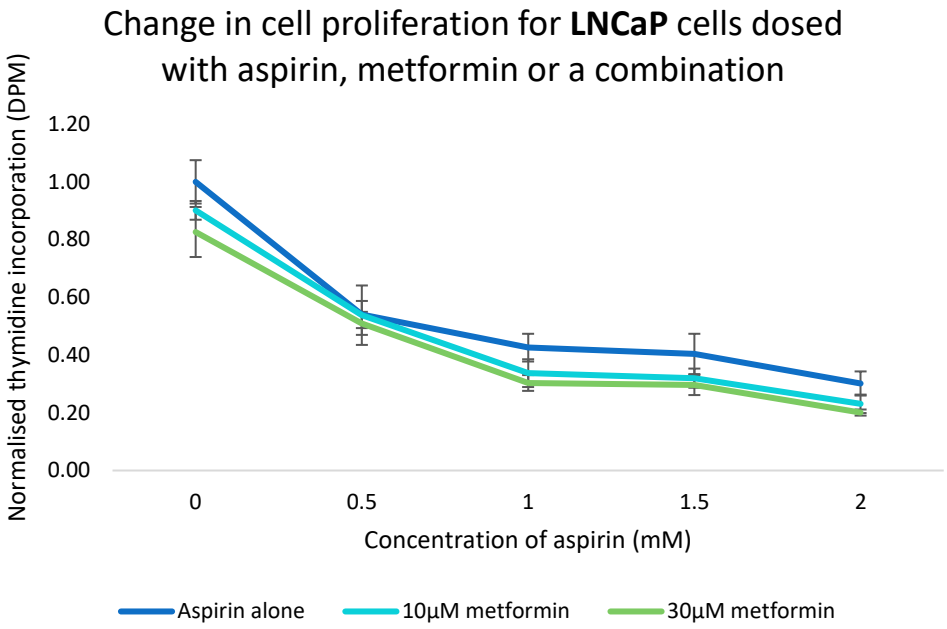
a.


Change in cell proliferation for **PC3** cells dosed with aspirin, metformin or a combination
Normalised thymidine incorporation (DPM)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
0.5
1
1.5
2
Concentration of aspirin (mM)
Aspirin alone
10µM metformin
30µM metformin

b.


Change in cell proliferation for **DU145** cells dosed with aspirin, metformin or a combination
Normalised thymidine incorporation (DPM)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
0.5
1
1.5
2
Concentration of aspirin (mM)
Aspirin alone
10µM metformin
30µM metformin

c.



d.

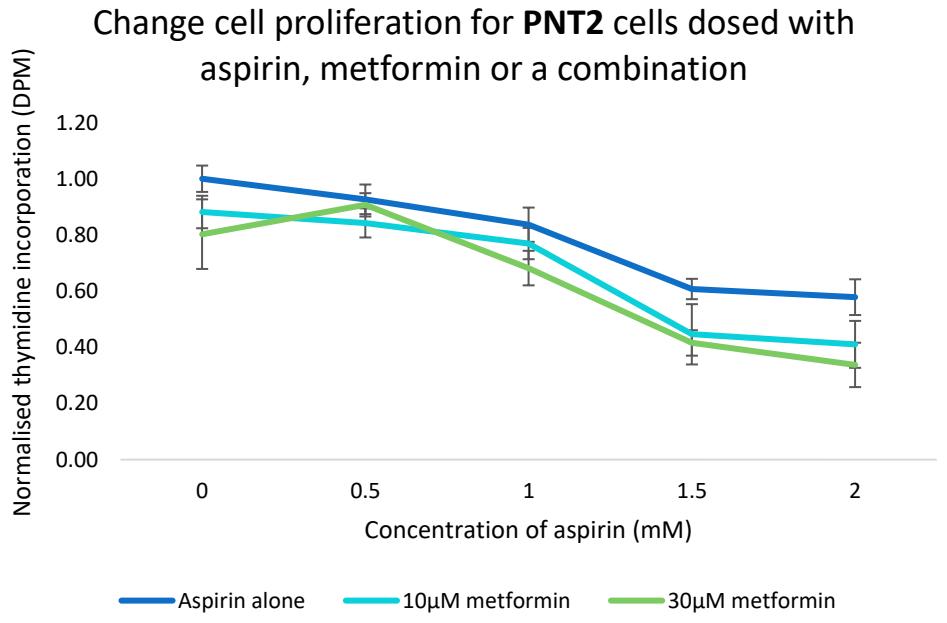
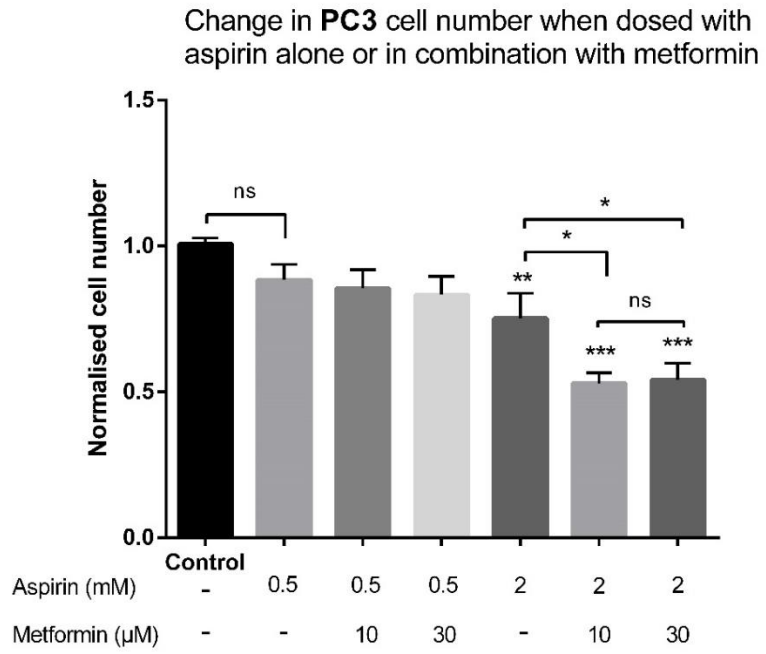


Figure 3.4: The effect of aspirin and metformin on cell proliferation using ^{3}H-thymidine incorporation. **(a)** PC3, **(b)** DU145, **(c)** LNCaP and **(d)** PNT2 cells were dosed with either aspirin and metformin alone or in combination every 24 hours for a total of 72 hours. Error bars represent the standard error of mean. Data were analysed using a one-way ANOVA and Tukeys test for multiple comparisons. Data represents three independent experiments each performed in triplicate.

3.4.3 Proliferative response of PC3, DU145, LNCaP and PNT2 cells to aspirin and metformin: cell yield

To further examine the effect of combining the drugs on cell proliferation two concentrations of both aspirin and metformin were selected and all four cell lines were dosed for 96 hours and counted. The concentrations selected were 0.5 mM and 2 mM for aspirin and 10 μM and 30 μM for metformin. For the PC3 cell line, cell number was unaffected in response to 0.5 mM aspirin but was decreased when cells were dosed with 2 mM aspirin ($p < 0.008$) (Figure 3.5). Neither concentration of metformin alone significantly altered cell number. When 2 mM aspirin was used in combination with either 10 or 30 μM metformin there was a significant reduction in cell number which was different to dosing with each drug alone, indicating that aspirin and metformin have an additive effect, $p < 0.001$ and $p < 0.001$ for 2 mM aspirin with 10 and 30 μM metformin respectively. There was no difference between using 2 mM aspirin with 10 or 30 μM metformin demonstrating that low metformin concentrations were able to exert the additive effect. As seen with ^{3}H-thymidine incorporation, the PC3 cell line was the only one of the four where the drugs had an additive effect.



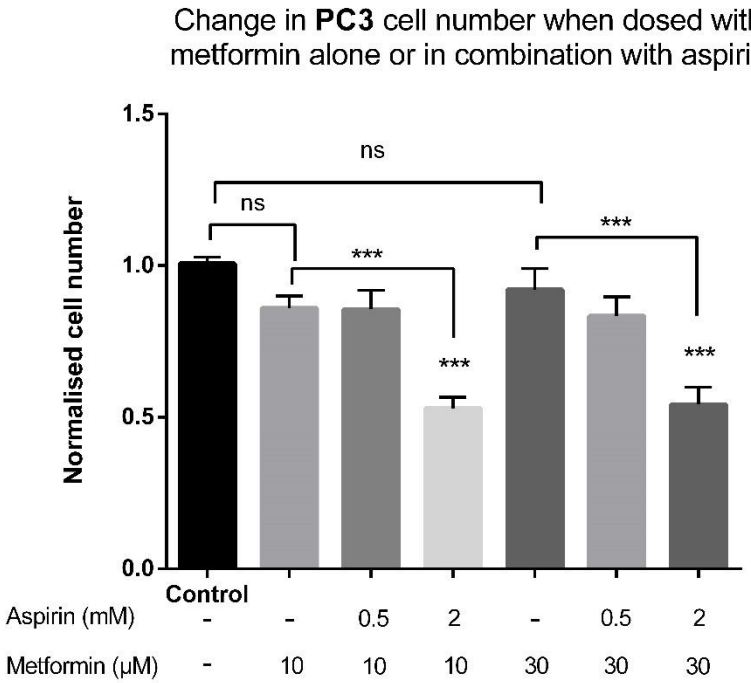


Figure 3.5: Cell counting following dosing with metformin and aspirin alone and the combination of the two in the PC3 cell line.

PC3s were dosed with aspirin, metformin or a combination of the two drugs every 24 hours for a total of 96 hours. Error bars represent the standard error of mean. Data were analysed using a one-way ANOVA and Tukey's test for multiple comparisons. Where there is an asterisk but no significance bar the condition is compared to the control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data represents three independent experiments each performed in triplicate.

Similar to the PC3s, DU145 cell number was unaffected by either 10 or 30 µM metformin and 0.5 mM aspirin but was reduced when cells were dosed with 2 mM aspirin (p= 0.002) (Figure 3.6). Unlike the PC3s, the combination of 2 mM aspirin and either 10 or 30 metformin did not significantly reduce cell number compared to dosing with 2 mM aspirin alone (p≤ 0.05). However, the combination of 2 mM aspirin and 30 µM metformin did cause the greatest reduction in cell number, a decrease of 53% ± SEM compared with the control.

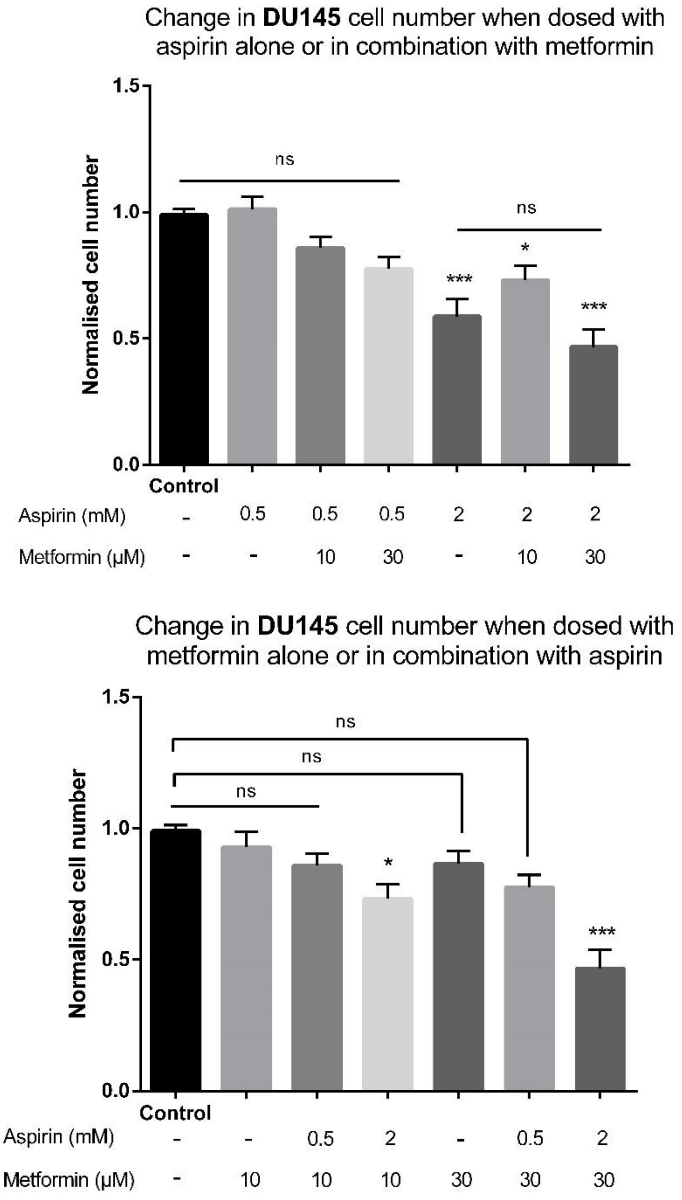
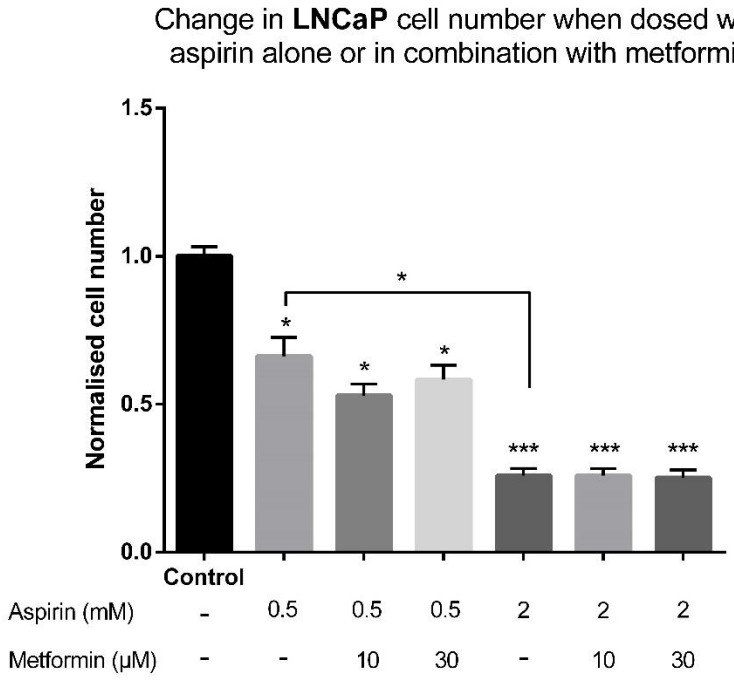


Figure 3.6: Cell counting following dosing with metformin and aspirin alone and the combination of the two in the DU145 cell line.

DU145s were dosed with aspirin, metformin or a combination of the two drugs every 24 hours for a total of 96 hours. Error bars represent the standard error of mean. Data were analysed using a one-way ANOVA and Tukey's test. Where there is an asterisk but no significance bar the condition is compared to the control. * p <0.05, ** p <0.01, *** p < 0.001. Data represents three independent experiments each performed in triplicate.

When the LNCaP cells were dosed with metformin no significant change in cell number was observed (Figure 3.7). Dosing the cells with aspirin caused a reduction of 34% ± SEM and 74% ± SEM in cell number for 0.5 and 2 mM aspirin respectively ($p= 0.022$ and $p <0.001$). This was the only cell line to exhibit a significant change in cell number at 0.5 mM aspirin, suggesting that the LNCaPs respond to lower concentrations of aspirin. It was also observed that the LNCaPs were the most sensitive to aspirin as when cells were dosed with 2 mM aspirin cell number was decreased the most compared to the other cell lines. This was a reduction of 74% (± 0.02), compared to 41% (± 0.07) for DU145s, 32% (± 0.09) for PC3s and 16% (± 0.06) for the non-cancerous PNT2s. The combination of aspirin and metformin did not significantly decrease cell number compared to dosing with aspirin alone indicating that like the DU145s the effects observed were solely due to aspirin.



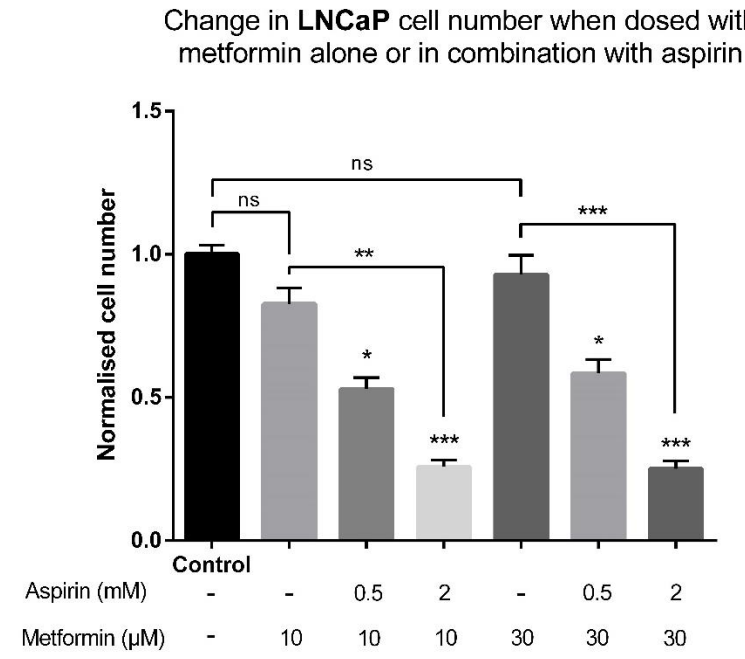


Figure 3.7: Cell counting following dosing with metformin and aspirin alone and the combination of the two in the LNCaP cell line.

LNCaPs were dosed with aspirin, metformin or a combination of the two drugs every 24 hours for a total of 96 hours. Error bars represent the standard error of mean. Data were analysed using a one-way ANOVA and Tukey's test. Where there is an asterisk but no significance bar the condition is compared to the control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data represents three independent experiments each performed in triplicate.

The non-tumorigenic PNT2s were the least affected of all the cell lines with neither aspirin nor metformin alone or the combination causing a significant change in cell number (Figure 3.8).

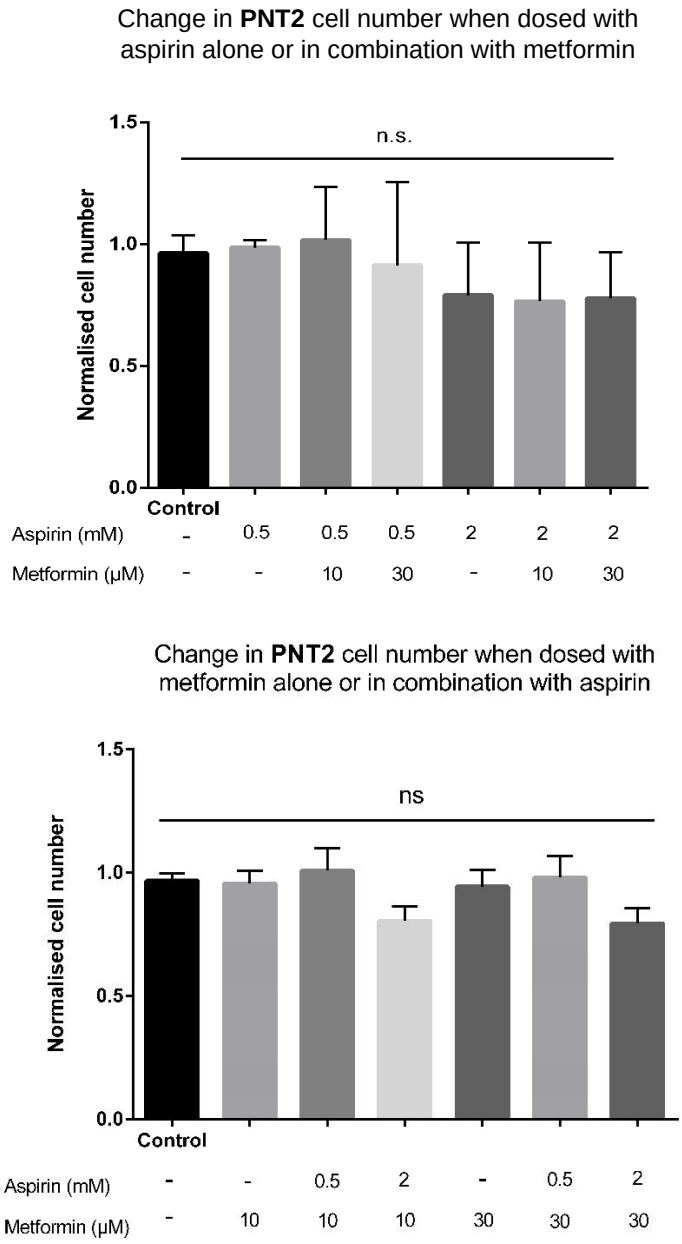


Figure 3.8: Cell counting following dosing with metformin and aspirin alone and the combination of the two in the PNT2 cell line.
PNT2s were dosed with aspirin, metformin or a combination of the two drugs every 24 hours for a total of 96 hours. Error bars represent the standard error of mean. Data were analysed using a one-way ANOVA and Tukeys's test. Data represents three independent experiments each performed in triplicate.

For all four cell lines the combination of aspirin and metformin caused the greatest decrease in cell number. For 2 mM aspirin in combination with 30 μM metformin this was a decrease of 75%, 53%, 46% and 21% for LNCaP, DU145, PC3 and PNT2 cells respectively.

3.4.4 Alterations in proliferation marker expression following dosing with aspirin and metformin

Western blots were performed to examine basal protein abundance of markers of cell proliferation that included cyclin D1 and PCNA. Cyclin D1 is an important regulator of the G1 to S phase transition and is expressed in the G1 phase and then rapidly declines at the G1/S phase boundary (210). PCNA is a critical component of the DNA replication machinery and is often expressed in the nuclei of cells during the DNA synthesis (S) phase of the cell cycle. Examining expression of these proteins allows analysis of different phases of the cell cycle to determine whether drugs induce an anti-proliferative effect.

A panel of prostate cell lines was used to examine PCNA and cyclin D1 abundance (Figure). PCNA and cyclin D1 were expressed in all cell lines and as expected the non-tumorigenic epithelial prostate cell line, PNT2s had the lowest abundance of each. Surprisingly basal cyclin D1 expression was also low in the DU145 cells.

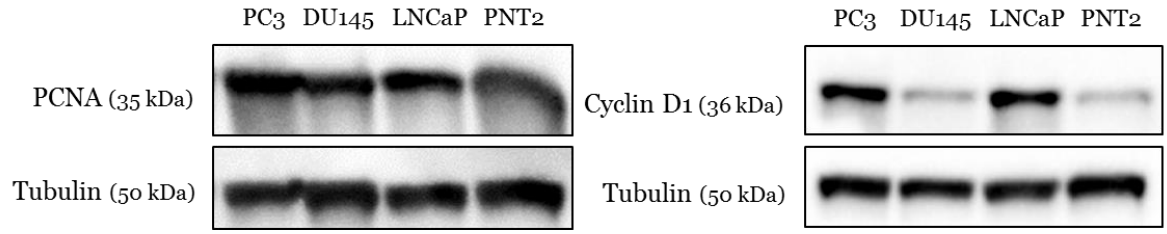


Figure 3.9: PCNA and cyclin D1 levels in prostate cancer and epithelial cell lines.
Expression of PCNA (30 kDa) and cyclin D1 (35 kDa) in PC3, DU145, LNCaP and PNT2 cell lines. The housekeeping gene tubulin was used as a loading control.

For the PC3 cell line, dosing with aspirin (0.25- 4 mM) caused a slight decrease in PCNA and cyclin D1 levels (Figure 3.10), however the decrease was only significant for cyclin D1 when cells were dosed with 4 mM aspirin (p=0.01). This was different to cell counting in which a decrease in cell proliferation was observed when cells were dosed with 2 mM aspirin (Figure 3.5).

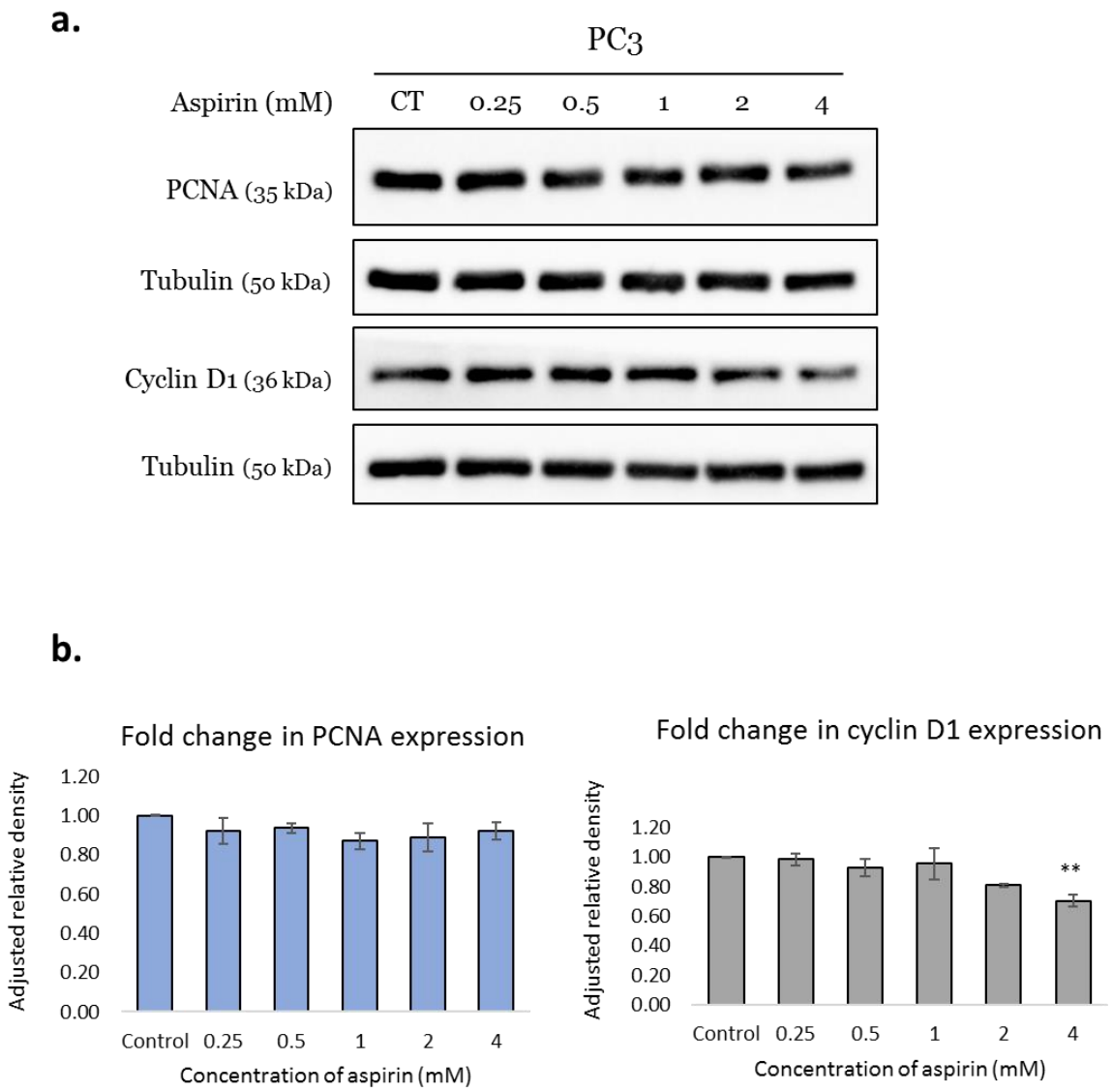


Figure 3.10: Western blot analysis of markers of cell proliferation for PC3 cells dosed with aspirin. **(a)** Levels of markers of cell proliferation, PCNA and cyclin D1, in PC3 cells dosed with aspirin every 24 hours for a total of 96 hours. **(b)** Densitometry was performed to quantify PCNA and cyclin D1 protein levels. Error bars represent the standard error of mean. Data were analysed using GraphPad Prism with a one-way ANOVA and Dunnett's post hoc test. Representative of experiments repeated 3 times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Dosing PC3 cells with metformin (10-1000 µM) caused a significant decrease in PCNA and cyclin D1 at 1000 µM ($p=0.01$ and $p<0.001$ respectively) (Figure 3.11). This indicates that in this cell line, metformin does not affect cell proliferation at clinically relevant concentrations (up to 30 µM metformin) as was observed in cell counting.

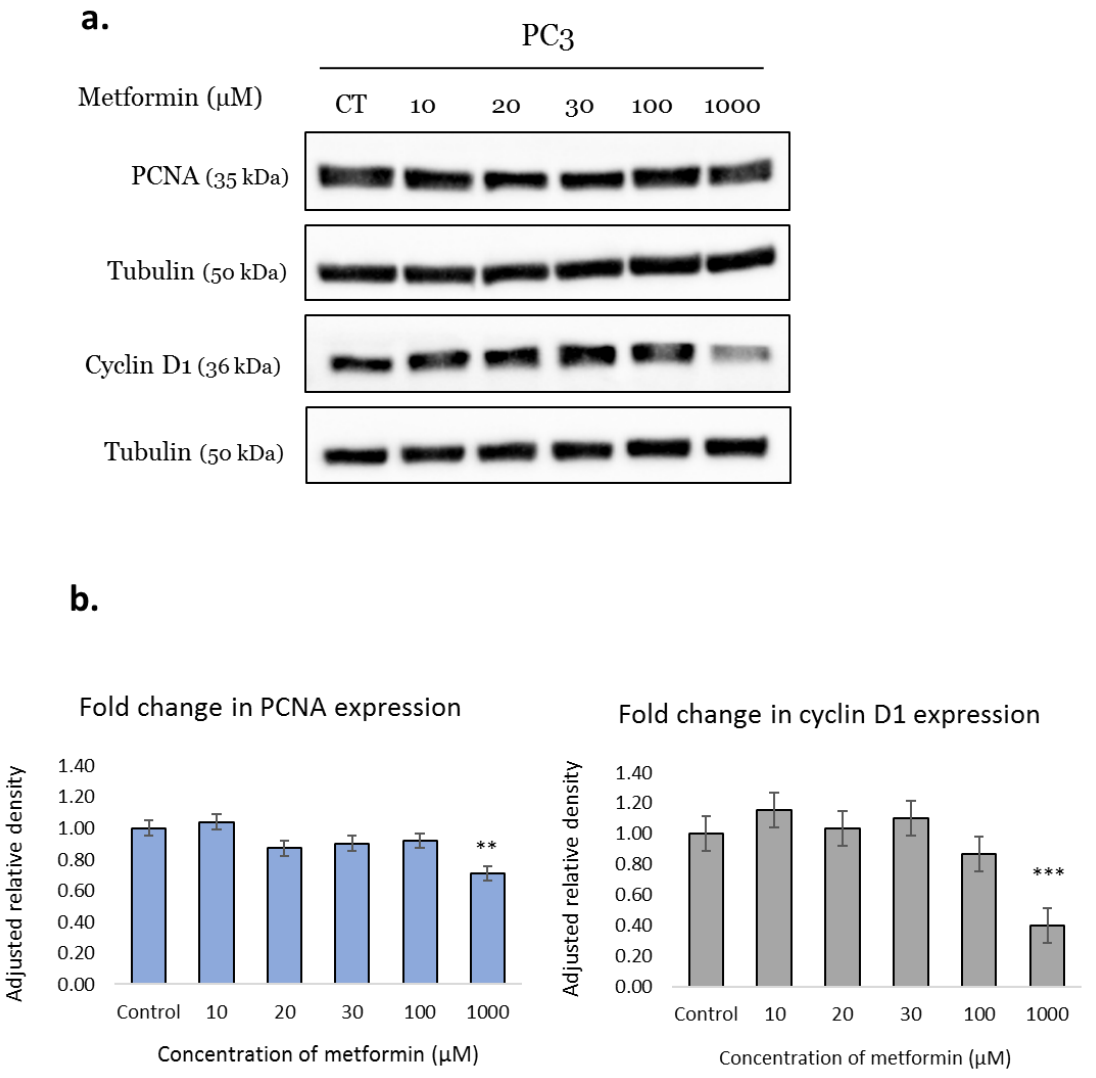


Figure 3.11: Western blot analysis of markers of cell proliferation for PC3 cells dosed with metformin. **(a)** Levels of markers of cell proliferation, PCNA and cyclin D1, in PC3 cells dosed with metformin every 24 hours for a total of 96 hours. Blot is a representative of experiment repeated three times **(b)** Densitometry was performed to quantify protein levels. Error bars represent the standard error of mean. Data were analysed using Graphpad Prism with a one-way ANOVA and Dunnett's post hoc test. Data for three independent experiments combined. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

When the PC3 cell line was dosed with a combination of aspirin (2 mM) and metformin (30 µM) both PCNA and cyclin D1 decreased, p=0.04 and p=0.049 respectively (Figure 3.12).

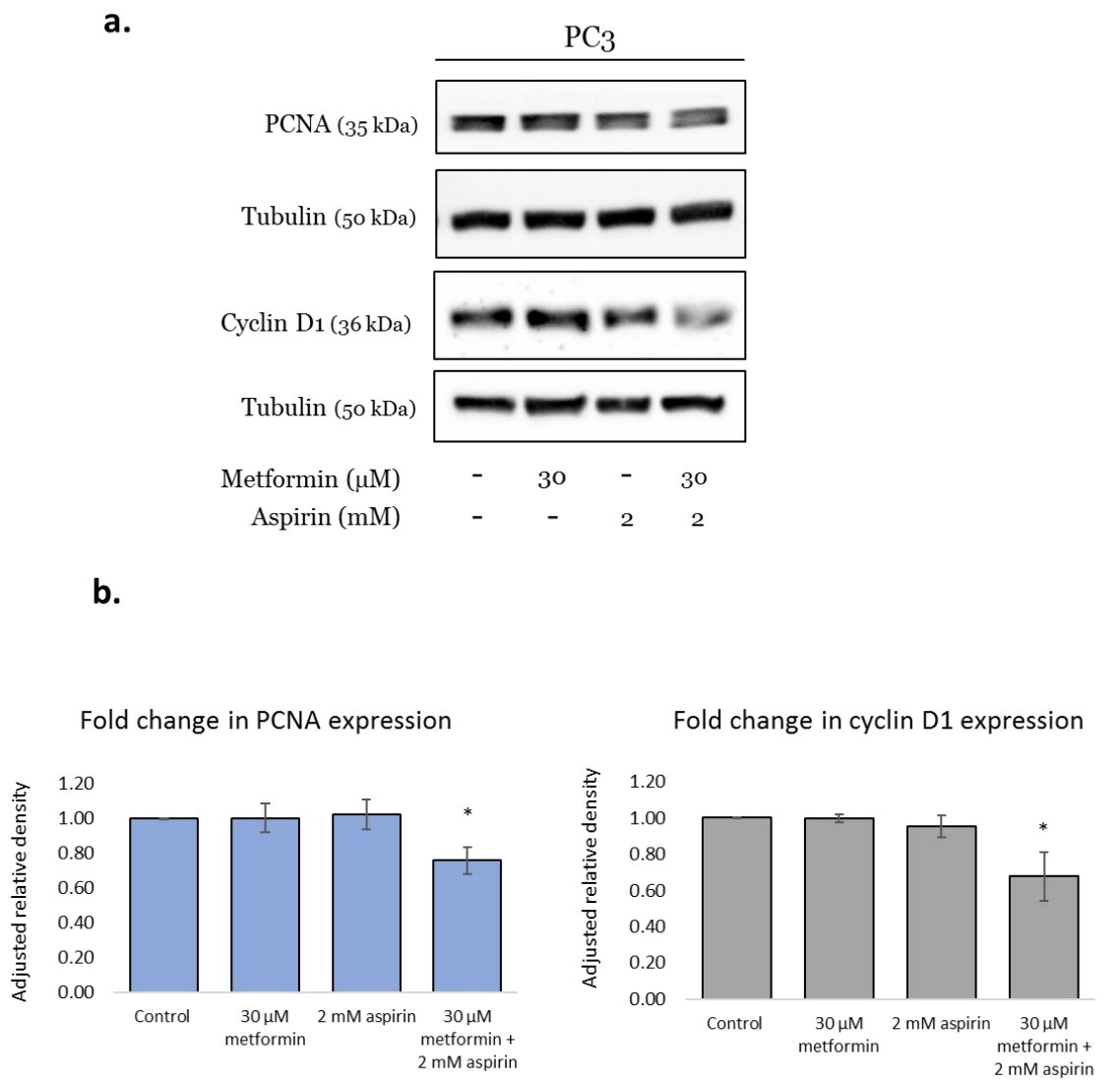


Figure 3.12: Western blot analysis of markers of cell proliferation for PC3 cells dosed with aspirin and metformin alone and in combination.
(a) Levels of markers of cell proliferation, PCNA and cyclin D1, in PC3 cells dosed with metformin every 24 hours for a total of 96 hours. Blot is a representative of experiment repeated three times. **(b)** Densitometry was performed to quantify protein levels. Error bars represent the standard error of mean. Data were analysed using Graphpad Prism with a one-way ANOVA and Tukey's post hoc test. Representative of experiments repeated 3 times. Data for three independent experiments combined. * p <0.05, ** p <0.01, *** p < 0.001.

The LNCaP cell line was dosed with 0-4 mM aspirin as were the other cell lines, however, western blotting could not be conducted for cells dosed with 4 mM aspirin as at this concentration aspirin was toxic, killing most of the cells and not producing enough lysate. The cell counting experiments indicated that aspirin inhibited cell proliferation at 0.5 and 2 mM. In western blotting there was a decrease in both PCNA and cyclin D1 which was significant at 1 and 2 mM aspirin for PCNA, p= 0.44 and 0.001, and at 2 mM for cyclin D1, p <0.001 (Figure 3.13).

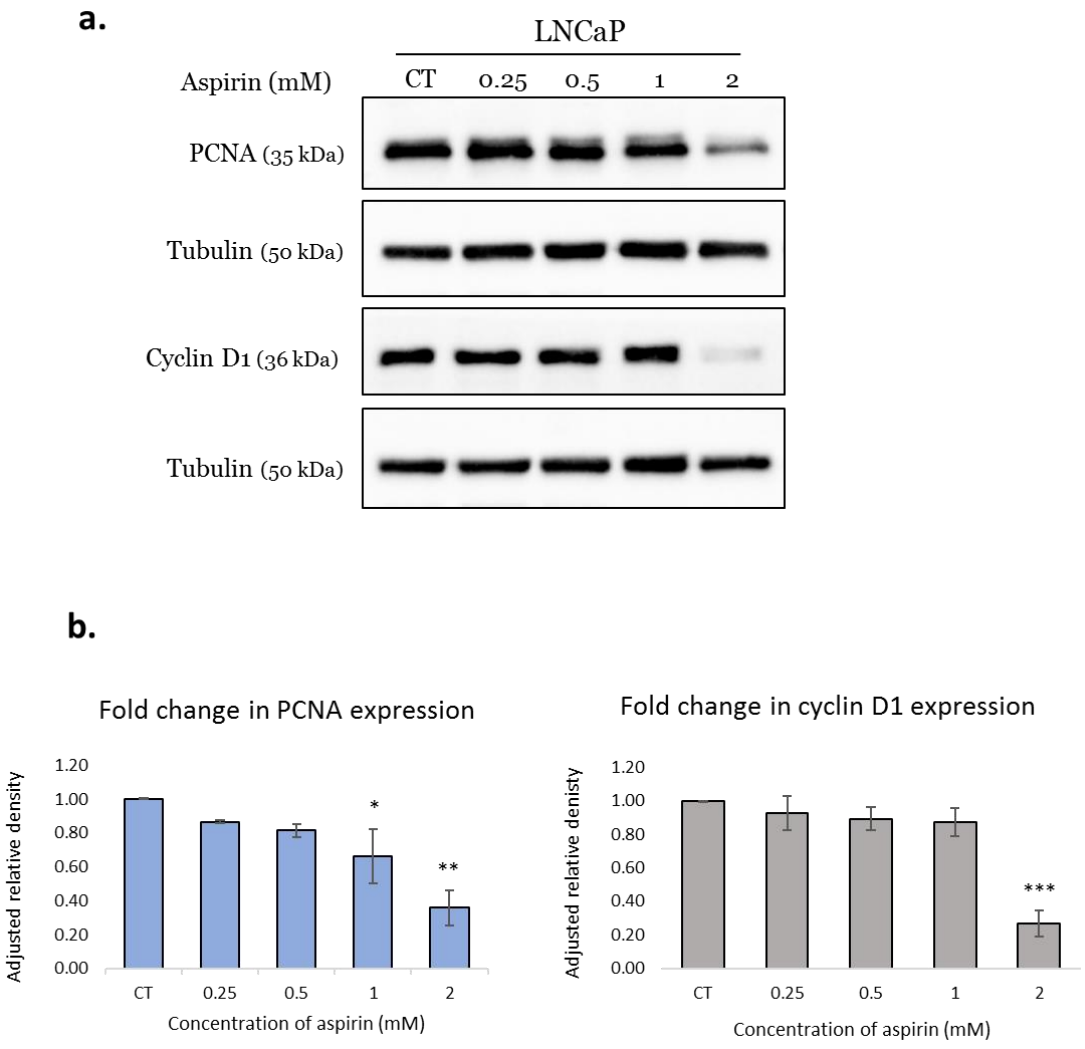


Figure 3.13: Western blot analysis of markers of cell proliferation for LNCaP cells dosed with aspirin. **(a)** Levels of markers of cell proliferation, PCNA and cyclin D1, in LNCaP cells dosed with aspirin every 24 hours for a total of 96 hours. Blot is a representative of experiment repeated three times. **(b)** Densitometry was performed to quantify protein levels. Error bars represent the standard error of mean. Data were analysed using GraphPad Prism with a one-way ANOVA and Dunnett's post hoc test. Representative of experiments repeated 3 times. Data for three independent experiments combined. * p <0.05, ** p <0.01, *** p < 0.001.

Metformin had no significant effect on either marker of proliferation at clinically relevant concentrations of metformin (<30 µM) in the LNCaP cell line (Figure3.14). This corresponds to no effect of these concentrations on cell number (Figure 3.7). However, when dosed with 1000 µM metformin there was a significant decrease in PCNA p= 0.04.

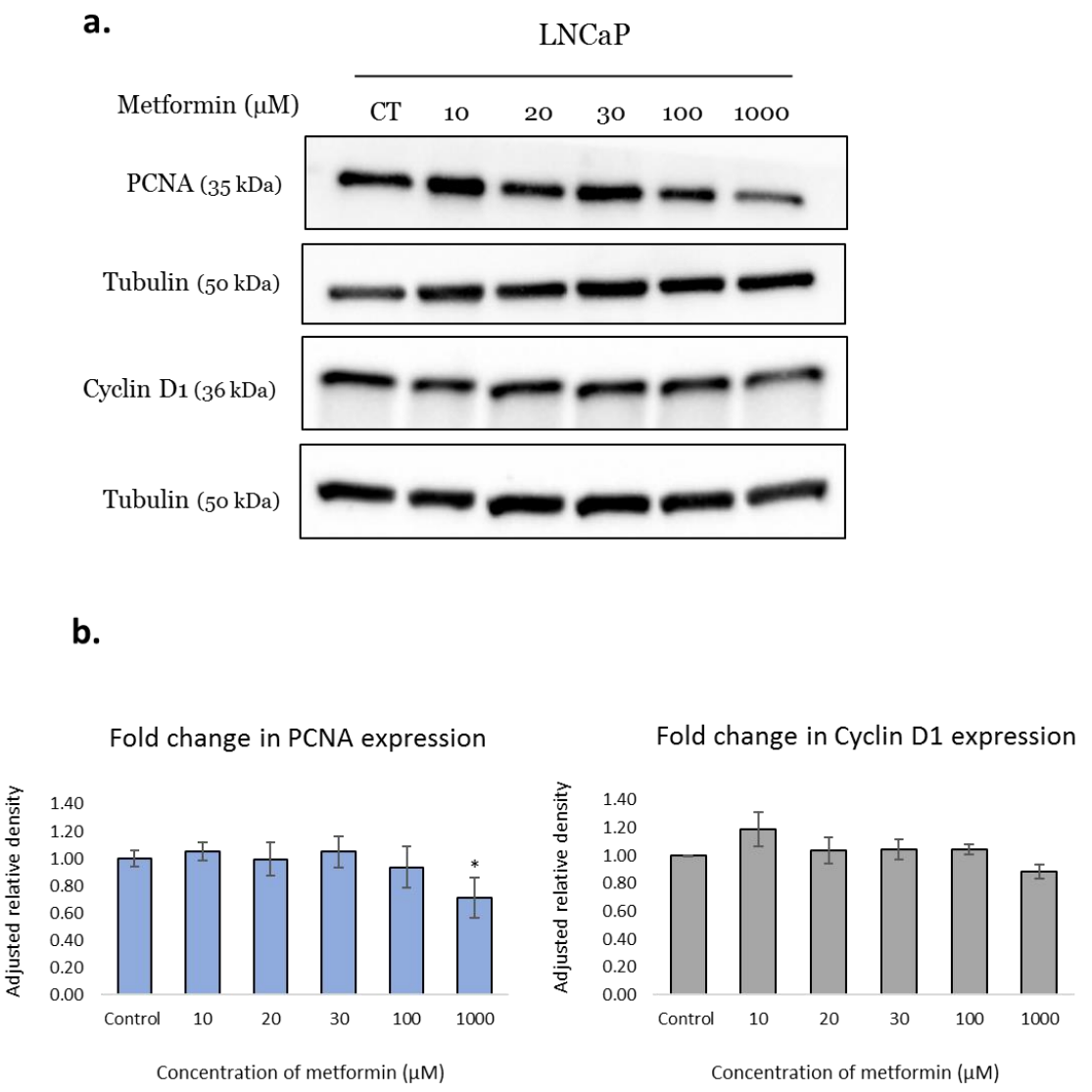


Figure 3.14: Western blot analysis of markers of cell proliferation for LNCaP cells dosed with metformin. (a) Levels of markers of cell proliferation, PCNA and cyclin D1, in LNCaP cells dosed with metformin every 24 hours for a total of 96 hours. Blot is a representative of experiment repeated three times. **(b)** Densitometry was performed to quantify protein levels. Error bars represent the standard error of mean. Data were analysed using GraphPad Prism with a one-way ANOVA and Dunnett's post hoc test. Representative of experiments repeated 3 times. Data for three independent experiments combined. * p <0.05, ** p <0.01, *** p < 0.001.

When LNCaP cells were dosed with both aspirin (2 mM) and metformin (30 µM), there was a significant decrease in both PCNA ($p < 0.001$) and cyclin D1 ($p= 0.004$) abundance (Figure 3.15). However, this was not significantly different from the decrease observed when cells were dosed with 2 mM aspirin alone, indicating that the effect was solely due to aspirin.

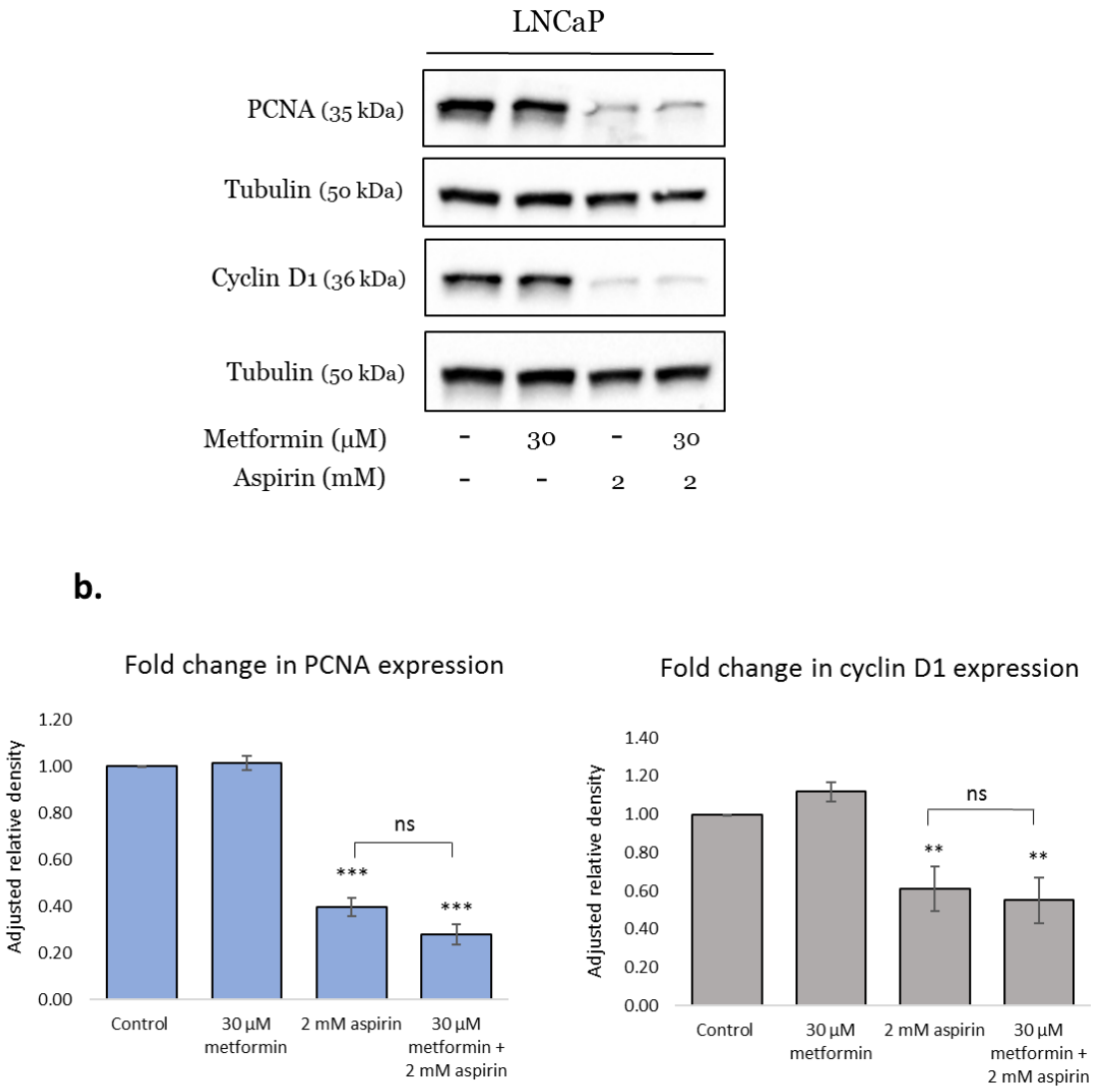


Figure 3.15: Western blot analysis of markers of cell proliferation for LNCaP cells dosed with aspirin and metformin alone and in combination.
(a) Levels of markers of cell proliferation, PCNA and cyclin D1, in LNCaP cells dosed with metformin every 24 hours for a total of 96 hours. Blot is a representative of experiment repeated three times. **(b)** Densitometry was performed to quantify protein levels. Error bars represent the standard error of mean. Data were analysed using GraphPad Prism with a one-way ANOVA and Tukey's post hoc test. Representative of experiments repeated 3 times. Data for three independent experiments combined. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

There was no change in markers of cell proliferation in the non-cancerous PNT2 cell line ($p > 0.05$) when cells were dosed with either aspirin or metformin (Figure 3.16 and Figure 3.17). This was anticipated as there was also no change in cell yield when cell counting (Figure 3.8).

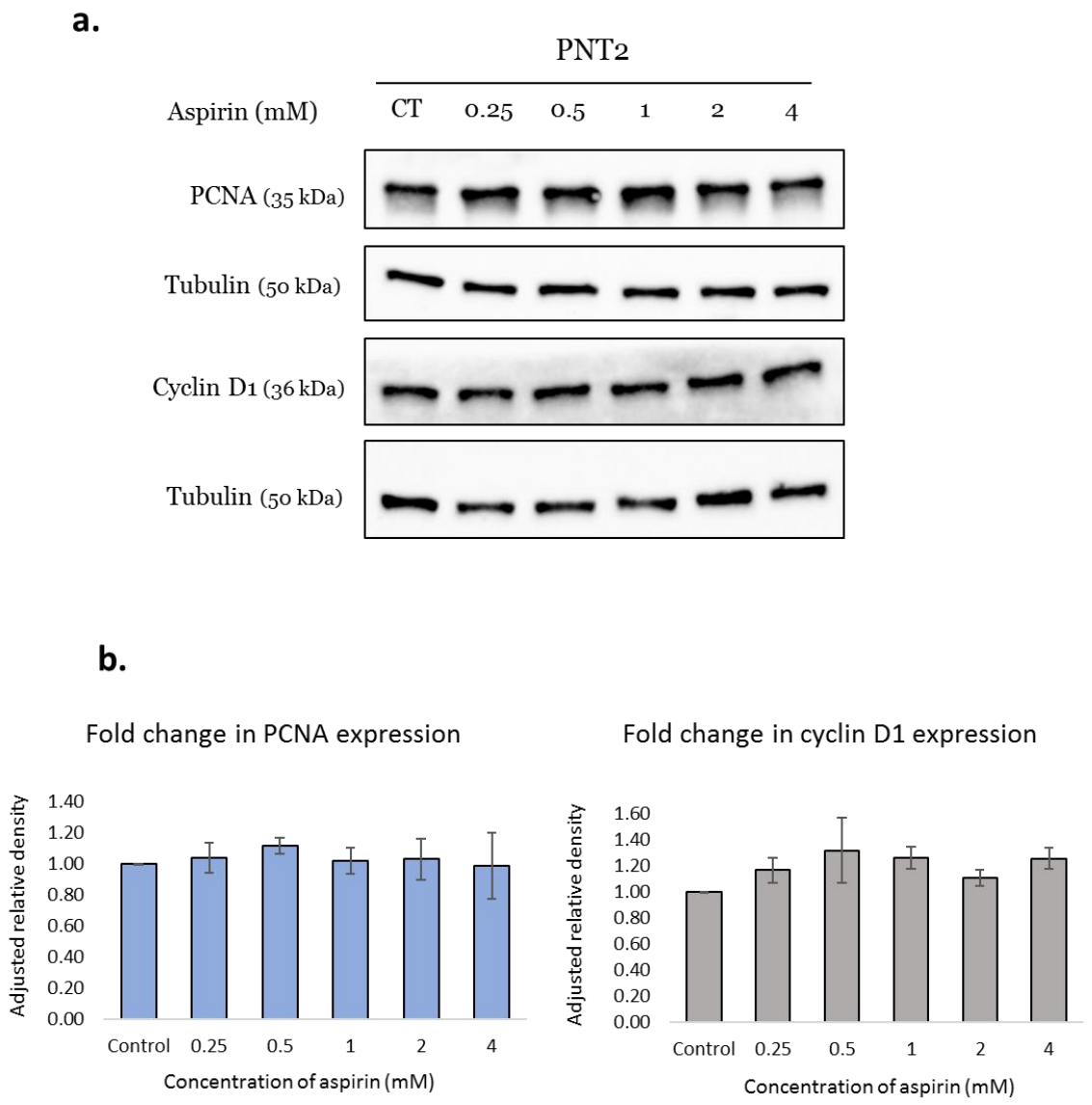


Figure 3.16: Western blot analysis of markers of cell proliferation for PNT2 cells dosed with aspirin. **(a)** Levels of markers of cell proliferation, PCNA and cyclin D1, in PNT2 cells dosed with aspirin every 24 hours for a total of 96 hours. Blot is a representative of experiment repeated three times. **(b)** Densitometry was performed to quantify protein levels. Error bars represent the standard error of mean. Data were analysed using GraphPad Prism with a one-way ANOVA and Dunnett's post hoc test. Representative of experiments repeated 3 times. Data for three independent experiments combined. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

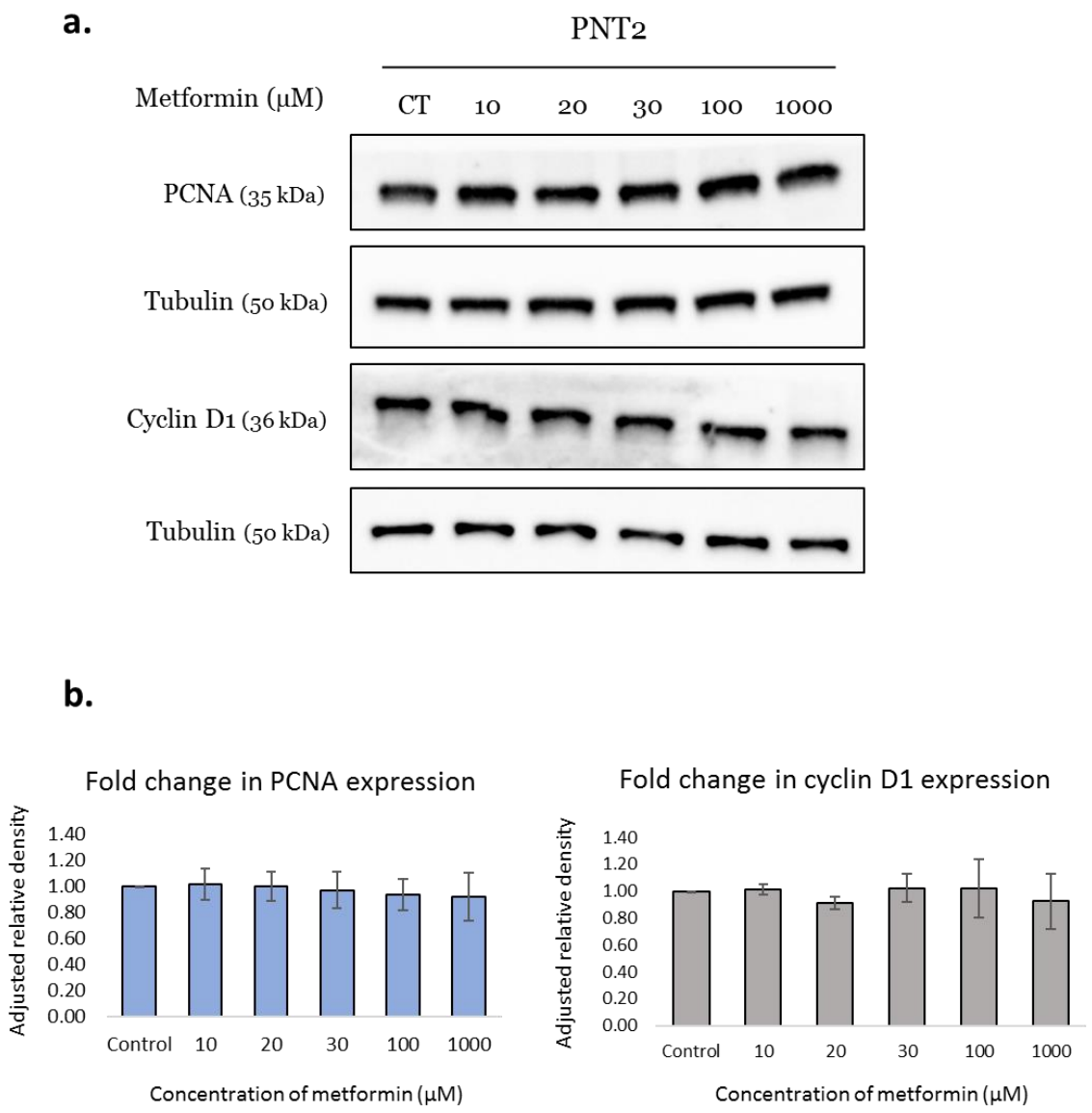


Figure 3.17: Western blot analysis of markers of cell proliferation for PNT2 cells dosed with metformin. **(a)** Levels of markers of cell proliferation, PCNA and cyclin D1, in PNT2 cells dosed with aspirin every 24 hours for a total of 96 hours. Blot is a representative of experiment repeated three times. **(b)** Densitometry was performed to quantify protein levels. Error bars represent the standard error of mean. Data were analysed using GraphPad Prism with a one-way ANOVA and Dunnett's post hoc test. Representative of experiments repeated 3 times. Data for three independent experiments combined. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

As expected from cell counting, combining aspirin and metformin also did not affect PCNA or cyclin D1 levels in PNT2 cells (p >0.05) (Figure 3.18).

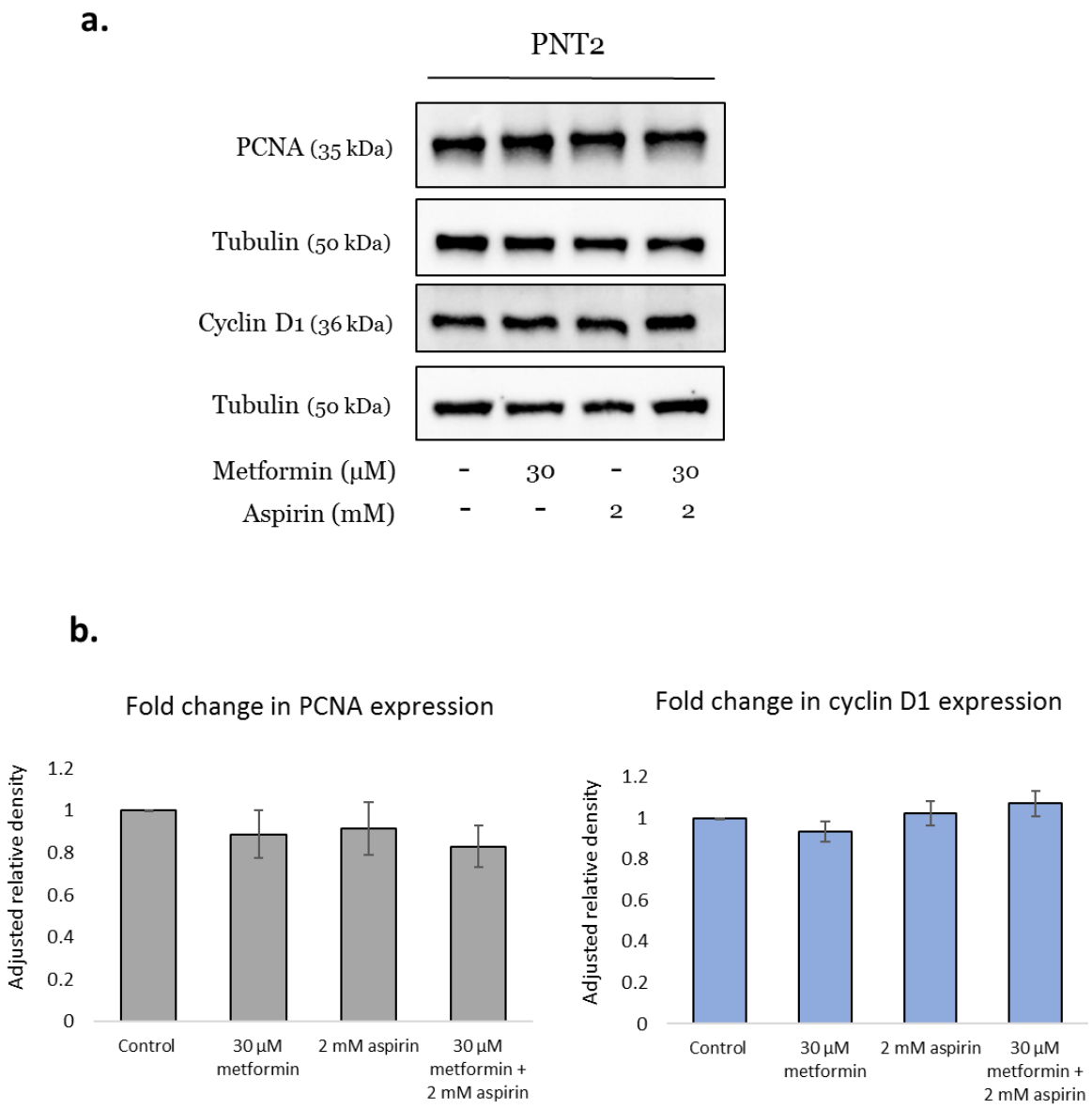


Figure 3.18: Western blot analysis of markers of cell proliferation for PNT2 cells dosed with aspirin and metformin alone and in combination.
(a) Levels of markers of cell proliferation, PCNA and cyclin D1, in PNT2 cells dosed with metformin every 24 hours for a total of 96 hours. Blot is a representative of experiment repeated three times. **(b)** Densitometry was performed to quantify protein levels. Error bars represent the standard error of mean. Data were analysed using GraphPad Prism with a one-way ANOVA and Tukey's post hoc test. Representative of experiments repeated 3 times. Data for three independent experiments combined. * p <0.05, ** p <0.01, *** p < 0.001.

3.4.5 Cell death in PC3, DU145, LNCaP and PNT2 cells following dosing with aspirin and metformin

To investigate whether the decrease in cell number was due a decrease in proliferation or an induction of death, the number of dead cells was assessed after treatment. For the PC3s, DU145s and PNT2s there was not a significant change in cell death when dosed with aspirin, metformin or a combination of the two drugs ($p<0.05$) (Figure 3.19). For the PC3 and DU145 cell lines this confirms that the decrease in cell number (Figure 3.5 and Figure 3.6) was due to the drugs inhibiting proliferation rather than promoting cell death. For the PNT2s there was no significant change in cell number when the cells were dosed with either the drugs alone or in combination (Figure 3.8), therefore as anticipated there was also no change in cell death.

For the LNCaP cell line, there was no significant change in the percentage of cell death when dosed with metformin alone (Figure 3.20). There was also no change in cell death when dosed with 0.5 mM aspirin alone or in combination with metformin even though there was a reduced cell number in the previous experiment. In contrast, there was large increase in cell death when cells were dosed with 2 mM aspirin ($p= 0.005$) which was the same level of cell death when cells were treated with the combination of 2 mM aspirin with 10 or 30 µM metformin ($p<0.001$ for both conditions). This indicates that aspirin has dose-dependent effects, inhibiting cell proliferation at 0.5 mM and causing cell death at 2 mM. A summary of the drug induced effects on cell counting, which were dependent on cell type, are displayed in Table 3-1.

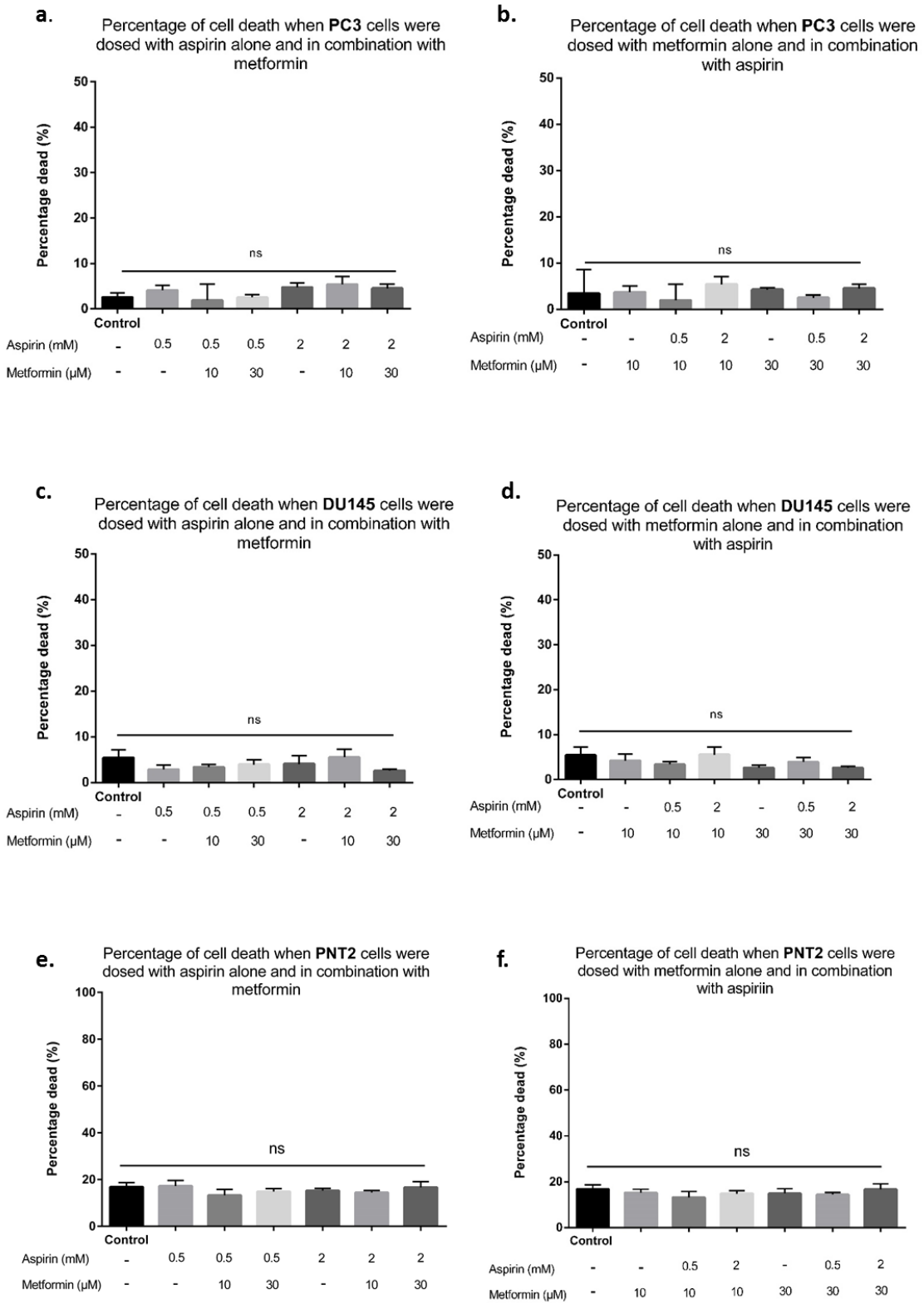
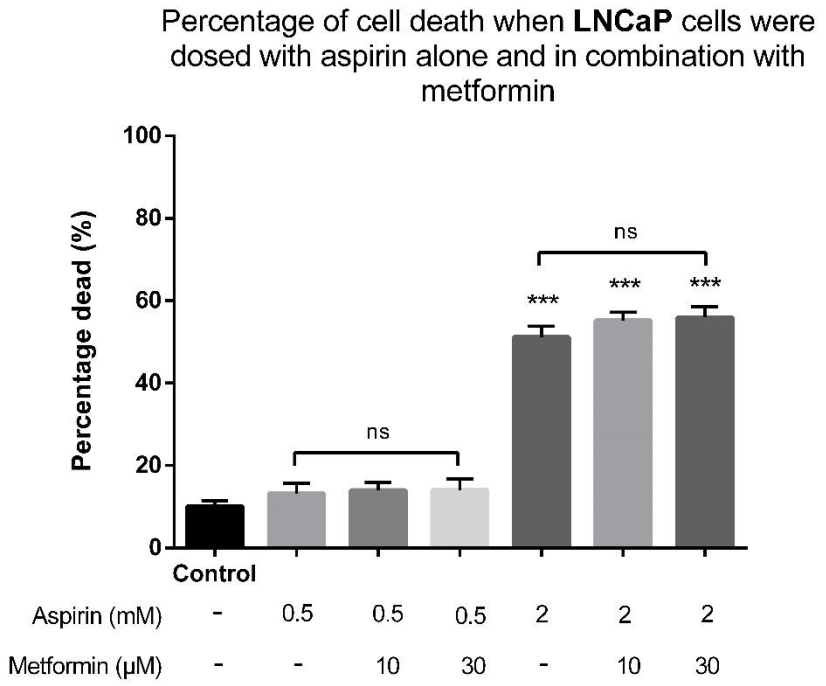


Figure 3.19: Percentage of cell death as determined by cell counting in the PC3, DU145 and PNT2 cell lines when dosed with aspirin, metformin or a combination of the two drugs.
(a, b) PC3 **(c, d)** DU145, and **(e, f)** PNT2s were dosed with aspirin, metformin or a combination of the two drugs every 24 hours for a total of 96 hours. Error bars represent the standard error of mean. Data were analysed using a one-way ANOVA and Tukey's test. Data represents three independent experiments each performed in triplicate.



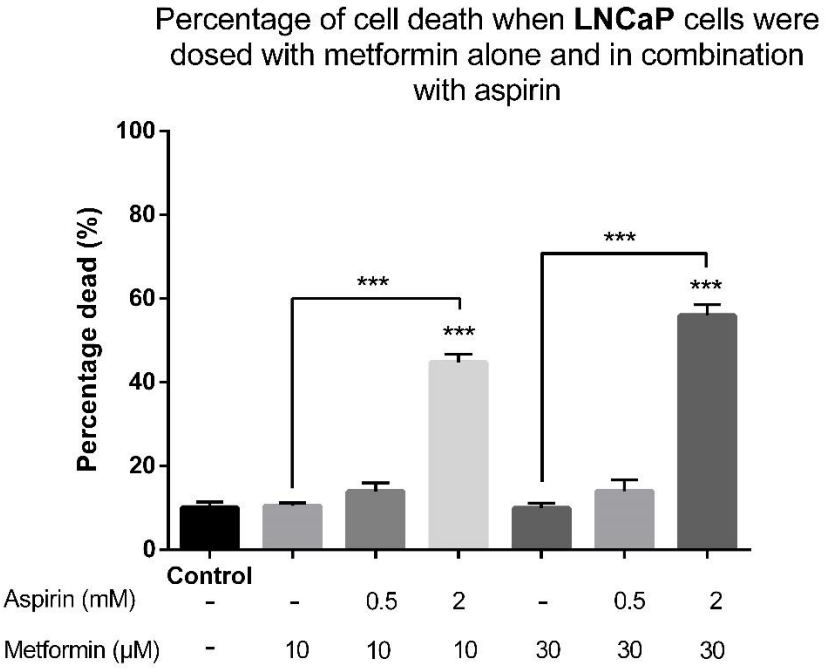


Figure 3.20: Percentage of cell death following dosing with metformin and aspirin alone and in combination in the LNCaP cell line.

LNCaPs were dosed with aspirin, metformin or a combination of the two drugs every 24 hours for a total of 96 hours. Error bars represent the standard error of mean. Data were analysed using a one-way ANOVA and Tukey's test for multiple comparisons. Where there is an asterisk but no significance bar the condition is compared to the control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data represents three independent experiments each performed in triplicate.

Table 3-1: Summary of the effect of aspirin or metformin alone and in combination on the cancerous cell lines from cell counting.

Cell line	10 or 30 µM metformin	0.5 mM aspirin	2 mM aspirin	Either combination of aspirin and metformin
LNCaP	No significant effect	Inhibits cell proliferation	Inhibits cell proliferation and causes cell death.	Inhibits cell proliferation and causes cell death solely due to aspirin.
DU145	No significant effect	No significant effect	Inhibits cell proliferation	Inhibits cell proliferation solely due to aspirin.
PC3	No significant effect	No significant effect	Inhibits cell proliferation	Additive effect

3.4.6 Aspirin induces apoptosis in LNCaP cells

To further investigate whether the cell death was apoptosis, LNCaP cells were stained with annexin V and 7-aminoactinomycin (7-AAD) and treated with 2 mM aspirin for 96 hours. During apoptosis cells undergo characteristic changes in morphology. Among these alterations phosphatidylserine (PS) is externalized to the cell surface and is readily bound by Annexin V. A dead cell marker, 7-AAD, identifies cells in the later stages of apoptosis as it is an indicator of structural integrity. Cells that were not apoptotic were negative for both markers, early apoptotic cells were Annexin V positive and 7-AAD negative, late stage apoptotic and dead cells were positive for both markers and nuclear debris was Annexin V negative and 7-AAD positive.

Cells were gated according to cell viability and Annexin V staining, producing an apoptosis profile (Figure 3.21). Analysis of the data showed no change in the percentage of live cells or cells undergoing apoptosis when the cells were dosed with 0.5 mM aspirin alone but a large increase in annexin V, 7-AAD positive apoptotic cells when dosed with 2 mM aspirin (Figure 3.22 a). When the cells were dosed with metformin, either 10 or 30 µM, there was no change in the percentage of live cells or dead cells (Figure 3.22 b). When dosing the cells with a combination of aspirin and metformin, 2 mM aspirin with either 10 or 30 µM metformin induced apoptosis, however this was the same as dosing with 2 mM aspirin alone. These results agreed with the previous data from the cell counting (Figure 3.7)

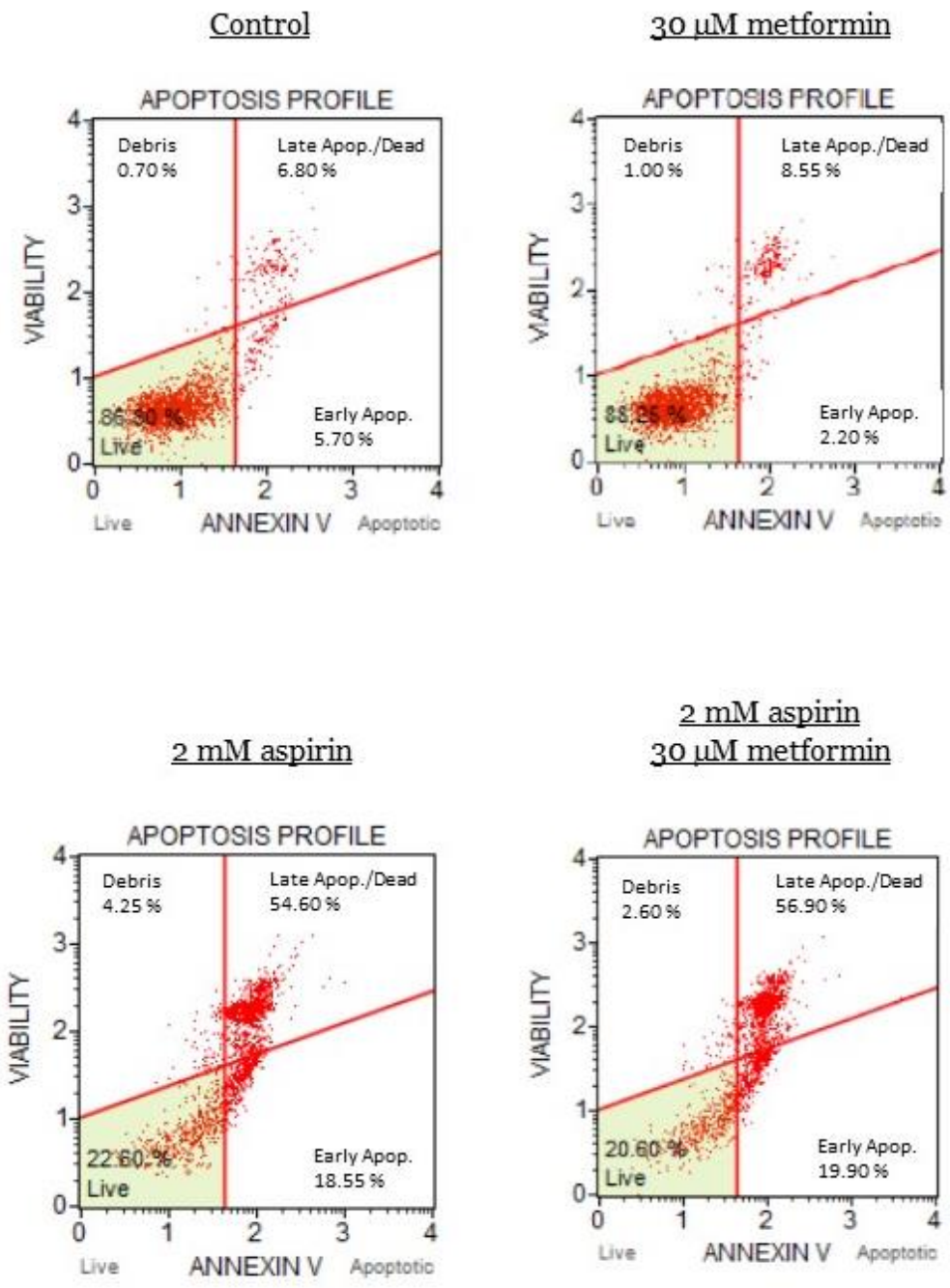
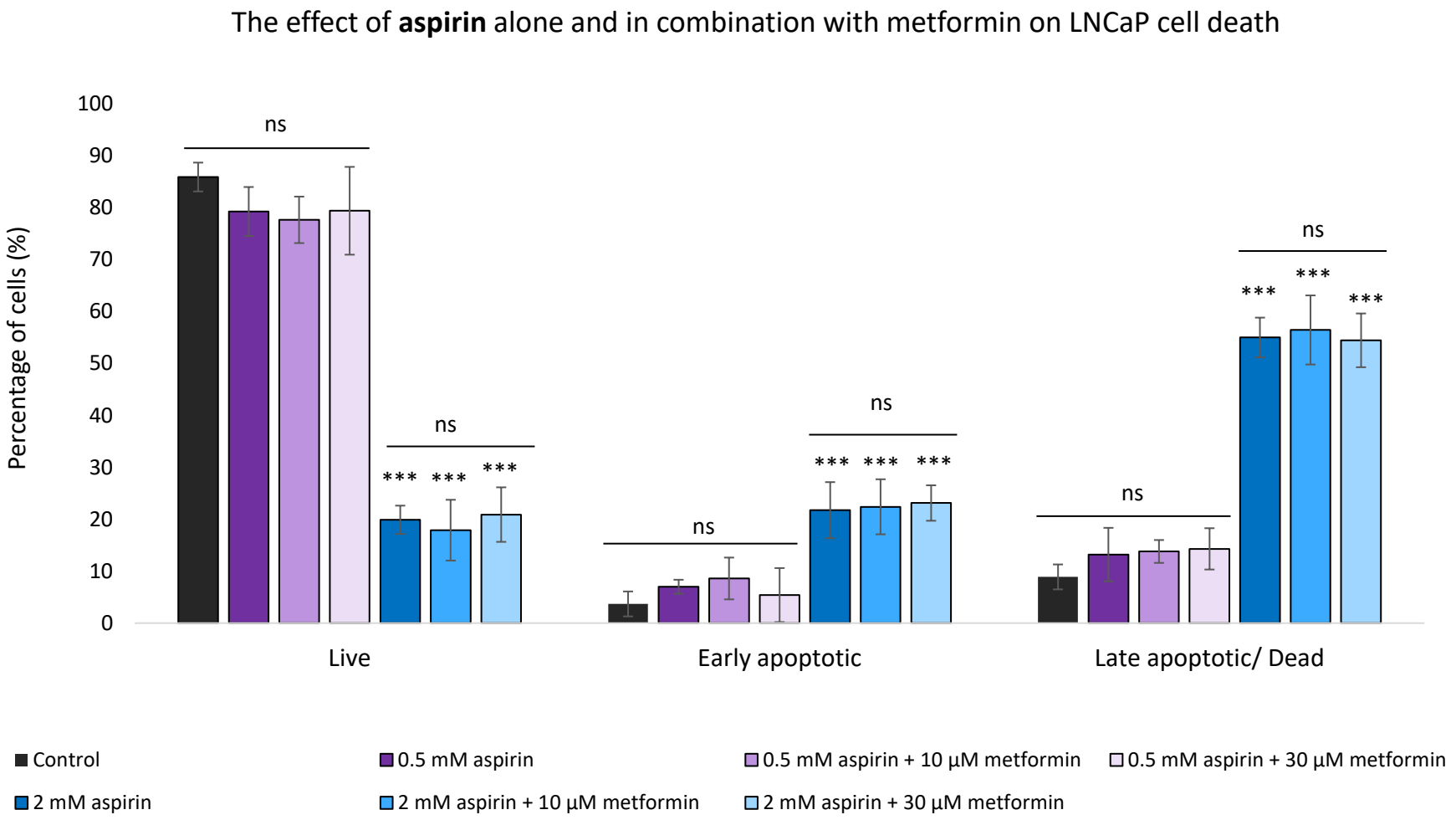


Figure 3.21: Plots representing the apoptosis profile of LNCaP cells stained with the Annexin V and 7-AAD following dosing with aspirin, metformin or a combination of both drugs.
LNCaP cells were dosed with aspirin, metformin or a combination of the two drugs every 24 hours for a total of 96 hours. Annexin V and 7-AAD staining indicated the percentage of live, early apoptotic, late apoptotic and dead cells.

a.


The effect of **aspirin** alone and in combination with metformin on LNCaP cell death
Percentage of cells (%)
100
90
80
70
60
50
40
30
20
10
0
ns
ns
***
***
***
ns
ns
***
***
***
ns
ns
***
***
***
Live
Early apoptotic
Late apoptotic/ Dead
Control
0.5 mM aspirin
0.5 mM aspirin + 10 µM metformin
0.5 mM aspirin + 30 µM metformin
2 mM aspirin
2 mM aspirin + 10 µM metformin
2 mM aspirin + 30 µM metformin

b.

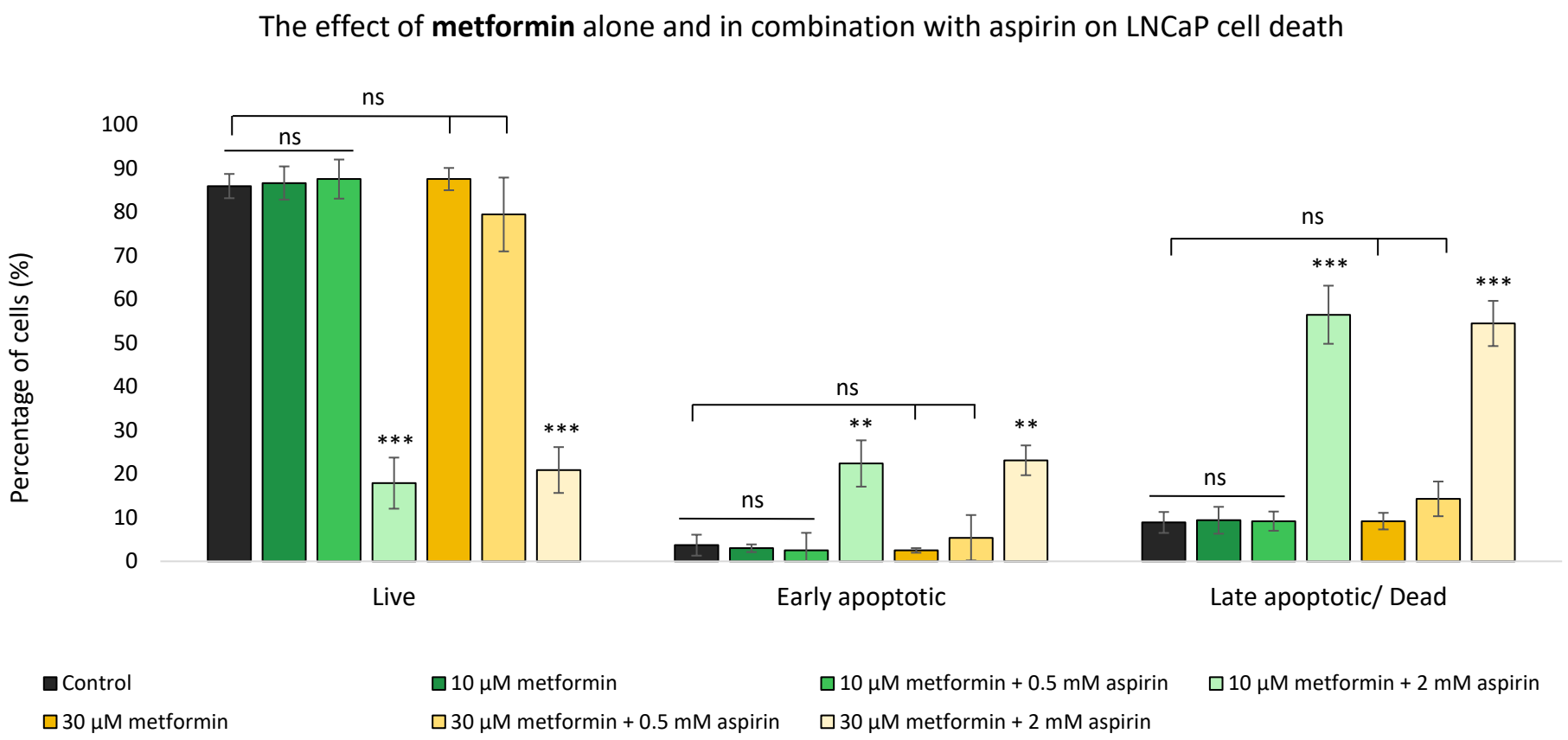


Figure 3.22: Percent of LNCaP cells which are live, early apoptotic, late apoptotic or dead when stained with Annexin V and 7-AAD.
Cells were dosed with **(a)** aspirin alone or in combination with metformin **(b)** metformin alone or in combination with aspirin. Data represents three independent experiments performed in triplicate. Where there is an asterisk but no significance bar the condition is compared to the control. Error bars represent the standard error of mean. Data was analysed using a one-way ANOVA and Tukey's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4.7 Caspase inhibitor Q-VD-OPh rescues LNCaP cells from aspirin induced apoptosis

Q-VD-OPh is a broad-spectrum caspase inhibitor and was used to assess whether aspirin induces caspase dependent apoptosis in the LNCaP cell line. When the LNCaP cells were dosed with 2 mM aspirin cell viability was reduced to around ~30% ($p < 0.0001$) (Figure 3.23). However, with the addition of 20 µM Q-VD-OPh to aspirin treated cells, cell viability was rescued, increasing cell viability to ~76% of control Q-VD-OPh treated cells. However, while cell viability was increased, it was not a full rescue, indicating that in this cell line aspirin induces apoptosis through caspase-dependent mechanisms but there may also be caspase-independent cell death.

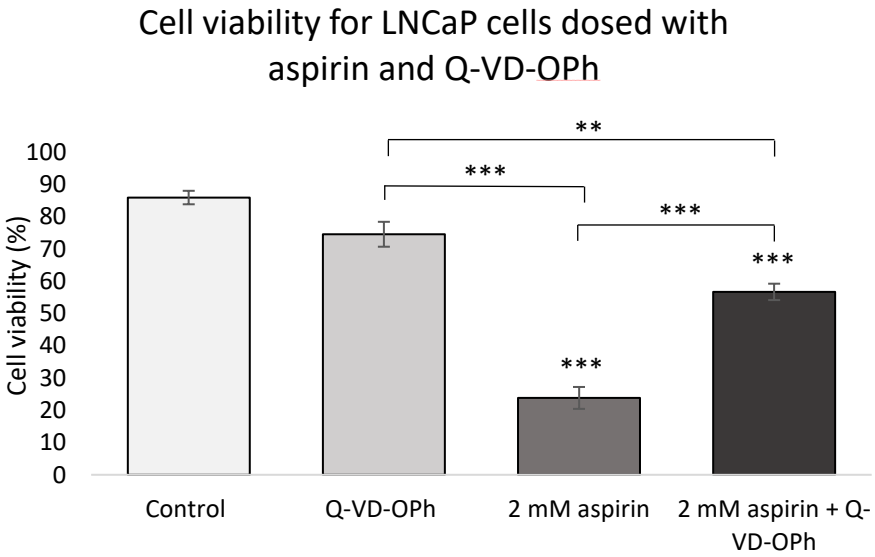


Figure 3.23: Cell viability as determined by cell counting for LNCaP cells dosed with aspirin and Q-VD-OPh.
Cells were dosed for 96 hours with 20 µM Q-VD-OPh, 2 mM aspirin and a combination of 20 µM Q-VD-OPh and 2 mM aspirin with the media being replaced and collected every 24 hours. Data represents three independent experiments performed in triplicate. Error bars represent the standard error of mean. Data was analysed using a one-way ANOVA and Tukey's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Where there is an * but no bar the condition is compared to the control.

3.4.8 p53 is activated in LNCaP cells following aspirin treatment

As the LNCaP cell line was the only one in which aspirin induced apoptosis it was suggested that this may be because it is the only cell line to express functional p53. p53 is a tumour suppressor and an important regulator of genome stability (211). Under stress the p53 protein can be stabilized and accumulates in the nucleus where it can be phosphorylated, acetylated or sumoylated (212). Phosphorylation on serine 15 leads to activation of p53 as a transcription factor where it activates expression of downstream genes which can cause apoptosis (213). Therefore, p53 and phosphorylated p53 (Ser15) serve as markers for apoptosis, with increased expression during programmed cell death.

Through western blotting it was seen that the abundance of p53 and phosphorylated p53 (Ser15) were increased, reaching maximum levels at around 30 minutes after dosing with 2 mM aspirin (Figure 3.24). Expression then decreased, returning to approximately control levels after 2 hours. The increase in phosphorylated p53 was greater than that of total p53.

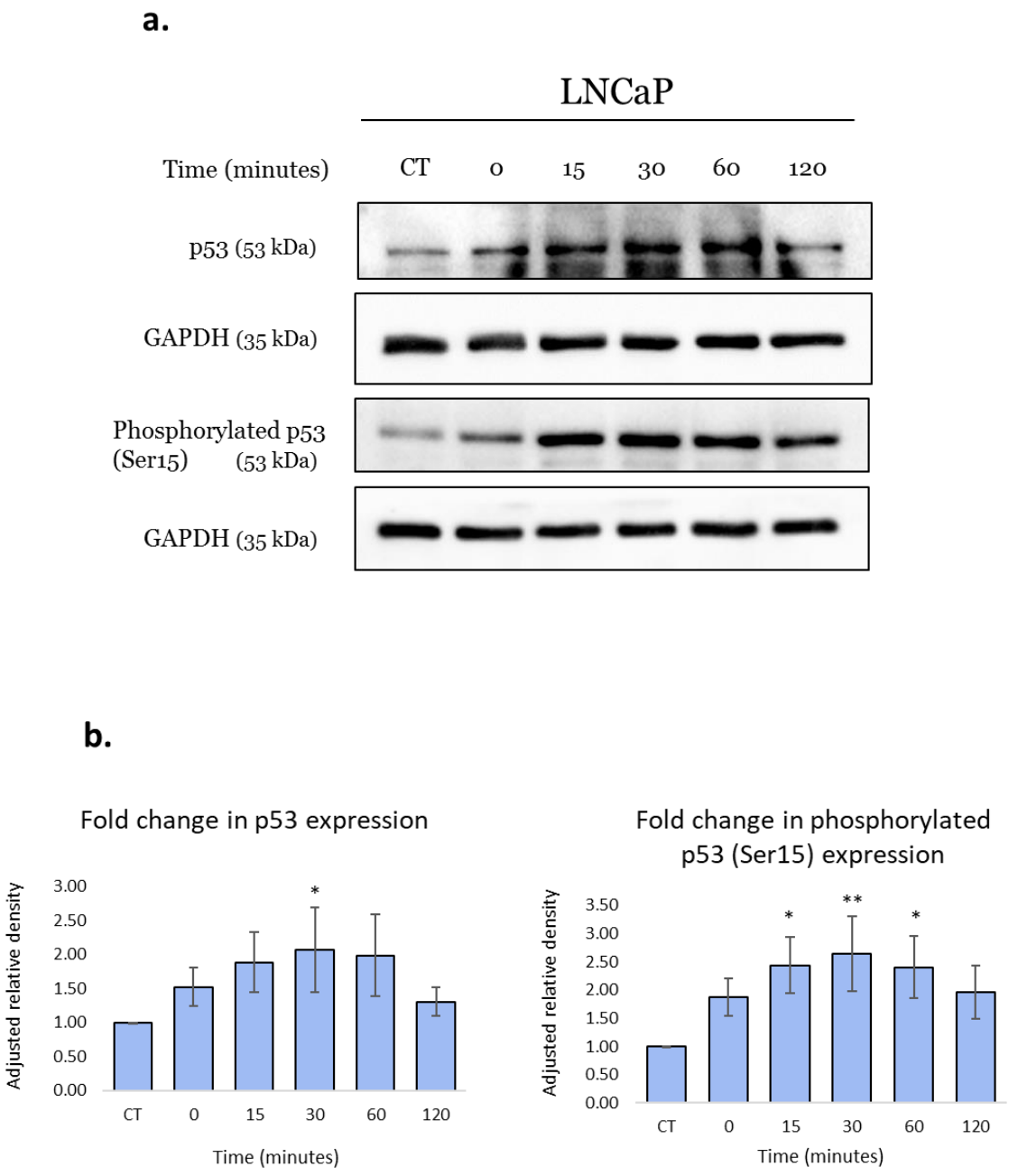


Figure 3.24: Expression of p53 and phosphorylated p53 (Ser15) in LNCaP cells dosed with aspirin. **(a)** Cells were dosed with aspirin for 72 hours and then dosed with aspirin again and lysed at 0, 15, 30, 60 and 120 minutes after dosing. The housekeeping gene GAPDH represents a loading control. Blot is representative of experiment repeated three times. **(b)** Densitometry from the three independent experiments combined. Error bars represent the standard error of mean. Data was analysed using a one-way ANOVA and Dunnet's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5 Discussion

Upregulated and uncontrolled cell proliferation is a feature common to all types of cancer and has been a key focus in treatment regimens. In this chapter, the effects of aspirin and metformin alone and in combination on prostate cancer cell proliferation were examined. For all four cell lines a decrease in proliferation with each of the drugs was observed in the ^{3}H- thymidine incorporation assay. The greatest decrease was when the drugs were used in combination, however the PC3 cell line, the most advanced of the four, was the only one in which aspirin and metformin had an additive effect. The non-tumorigenic PNT2s were the least affected which indicates that the drugs more readily target the rapidly dividing cancer cells, an observation also reported in other tissues (164) (214). In all cell lines and at all doses tested there remained some proliferating cells. Cancer recurrence is a major problem in treatment as even a few remaining cells can repopulate a tumour. It would be interesting to continue the experiment to see if long term treatment changes the phenotype.

The results from cell counting were similar to those observed with the ^{3}H-thymidine incorporation assay with a decrease in proliferation for the PC3s, DU145s and LNCaP cell lines. As with the ^{3}H- thymidine incorporation assay, the non-tumorigenic prostate cell line, the PNT2s, were the least affected by the drugs and experienced a non-significant reduction in cell number. Prostate cancer normally occurs in men over the age of 50 and whilst ideally the drugs would have no effect on non-cancerous cells, at this age the prostate is fully developed and cells enter a more quiescent, G0, stage. In G0 cells do not proliferate except when they need to re-enter the cell cycle to repair damaged tissue (215). Because of this, the drugs suppression of proliferation in normal cells would not have an enormous impact on the fully developed prostate. However, it was noted that aspirin appeared to have a greater effect on the less aggressive cancer cells than the ones in the more advanced stages, inducing growth arrest and apoptosis in LNCaP cells but only inhibiting cell proliferation in the DU145s and PC3s. This may suggest that in terms of the effect of the drug on cell proliferation, people who are in the earlier stages of prostate cancer development will receive the most benefit from aspirin treatment.
Interestingly, no significant change in cell number was observed in the PC3, DU145 and LNCaP cell lines when dosed with 10 or 30 μM metformin. In the DU145 and LNCaP cells cell number was significantly reduced when dosed with aspirin and the LNCaP cells were the most responsive, with a change in cell number at both 0.5 and 2 mM aspirin rather than just at 2 mM as seen with the DU145s.

When examining markers of cell proliferation, PCNA and cyclin D1, a decrease in the PC3 cell line was only observed at the highest concentrations of the two drugs, 4 mM aspirin and 1000 µM metformin, concentrations that are above the realistic therapeutic range. For all the cell lines the drug induced changes in western blotting were less obvious than the effect of the drugs on cell yield. Western blotting determines generalized protein expression for the entire population of cells which will include cells at different stages of the cell cycle as they are continually cycling through it. Therefore, it is often a less sensitive assay than cell counting.

In the LNCaP cell line, aspirin had dose-dependent effects, causing an inhibition of cell proliferation at 0.5 mM whilst both inhibiting cell proliferation and inducing cell death at 2 mM as seen with the cell counting and annexin V/7-AAD staining. Incubation of LNCaP cells which were treated with aspirin and Q-VD-OPh, a caspase inhibitor, returned cell viability almost to normal levels indicating that aspirin causes apoptosis through caspase mediated mechanisms. As aspirin has been shown to target cell proliferation the difference between the control and the cells dosed with aspirin and Q-VD-OPh could be due to a reduction in cell number but also could be through a caspase independent pathway. Indeed, aspirin has activated caspase independent cell death in other cancer types (216) (132).

It is known that LNCaP cells carry wild type p53 protein while PC3s are p53 negative due to a frameshift mutation which leads to a premature stop codon (217) and the DU145s have a non-functional mutated version as a result of two point mutations (218). p53 is the protein product of the TP53 gene and functions as a tumour suppressor, holding the cell in the cell cycle during cellular stress or initiating apoptosis if the damage is irreparable (219). Loss of this protein means that cells do not undergo apoptosis during cellular damage and survive, incorporating new mutations and propagating the cancer. It is thought that the TP53 gene is mutated in more than half of all cancers (220). In this study the LNCaP cell line was the only cell line to undergo apoptosis when dosed with 2mM aspirin. Due to the mutation status of the cells it was thought that this induction of apoptosis may be through the p53 pathway and so western blotting was performed to examine expression of p53 and a phosphorylated version of the protein. p53 is known to have a short half-life of around 5-20 minutes in most cell types, including those of the prostate (221). Mouse double minute 2 homolog (MDM2) is a negative regulator of p53, quickly ubiquitinating p53 and sending p53 to the proteasome to be degraded. However, upon cellular stress MDM2 is phosphorylated, blocking its binding to p53 and p53 accumulates in the nucleus where its half-life is increased by several fold (222). It then undergoes phosphorylation on three residues, Serine 15, Serine 20 and Serine 46 as well as acetylation and this promotes its

stabilization and activation of downstream targets for cell cycle arrest, senescence and apoptosis. Western blotting confirmed that in LNCaP cells both p53 and phosphorylated p53 (Ser15) increased after dosing with 2 mM aspirin, reaching maximum expression after 30 minutes and remaining elevated for over two hours. This suggests that aspirin causes p53 stabilization and activation of its downstream pathways (223). In liver, colon and breast cancer cell lines aspirin was shown to increase the binding of ribosomal protein to MDM2 which decreased MDM2s binding to p53 and led to its stabilization (224). Aspirin has also been shown to acetylate p53, activating its downstream targets (225). It could be that aspirin increases p53 in prostate cells in the same manner.

When aspirin and metformin were used in combination an additive effect of the drugs was observed for the PC3 cell line in all the assays; ^{3}H- thymidine incorporation, cell counting and western blotting. An important consideration in these cells is their phosphatase and tensin homolog (PTEN) status. PTEN is a tumour suppressor gene that inhibits cell proliferation and growth via inactivation of the PI3K pathway (226). It is one of the most commonly lost tumour suppressors in cancer and is negative in both the PC3 and LNCaP cell lines (227). The DU145s however are heterozygous for this gene (228) and the PNT2s are PTEN positive (229). Mutations in PTEN lead to constitutive activation of AKT and mTOR (230), a downstream target of both aspirin and metformin. Since aspirin and metformin both target the PI3K pathway it was thought that they might be more effective to treat cancers where PTEN is lost. Indeed, in this study the ^{3}H- thymidine incorporation and cell counting data showed that the PC3 and LNCaP cell lines were more responsive to aspirin and metformin than the DU145s and PNT2s. It may be that the blockade of the PI3K pathway by aspirin and metformin is the reason an additive effect was observed in the PC3 cell line and should be examined in future experiments.

Together the data from this chapter suggests that androgen dependent, p53 positive cells such as LNCaP cells are sensitive to aspirin treatment and experience inhibition of cell proliferation with low dose aspirin (0.5 mM) and induction of cell death with high dose aspirin (2 mM). The more advanced, androgen independent, p53 negative, PTEN negative cells such as the PC3s experienced a decreased in cell proliferation at higher aspirin concentrations but were not affected by treatment with metformin alone. When the drugs were used in combination, metformin enhances efficacy of aspirin potentially through inhibition of the PI3K pathway.

Chapter 4

The effect of aspirin and metformin on cell proliferation and cell death in 3D cell culture

4.1 Introduction

4.1.1 3D cell culture systems

One of the major difficulties with *in vitro* experiments is modelling the cellular and organizational complexity that is seen with tumour masses *in vivo*. While 2D cell culture has informed our understanding of biological processes for years, 3D culture provides a model which better represents the cell to cell interactions and surrounding environment which is essential for cell differentiation, proliferation and survival (231). Cells grown in 3D form spheres composed of aggregates of cells with growth in all directions.

There are multiple techniques used to grow cells in 3D culture. These include the hanging drop method, the liquid overlay method, magnetic levitation, the use of scaffolds, spinner flasks, hydrogels or other extracellular component matrixes such as collagen, agarose or matrigel (Figure 4.1) (232). In this study, cells were seeded in matrigel, where they invade outwards to form spheroids over time. This technique was chosen as it allows the formation of spheroids from single cells, enabling the effect of the drugs on both spheroid formation and spheroid growth to be monitored.

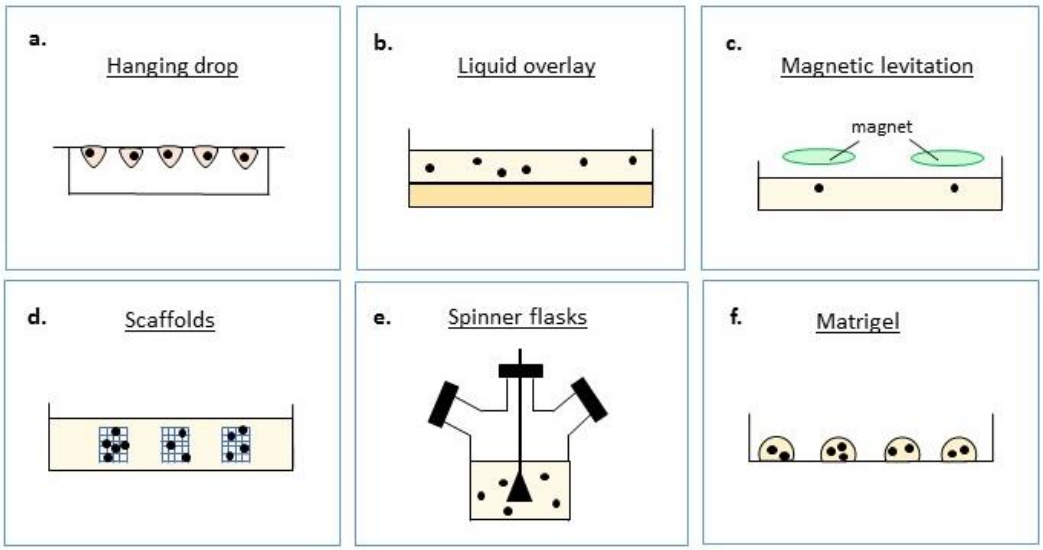


Figure 4.1: Techniques used to create 3D cell culture.
(a) The hanging drop method uses gravity to bring the cells together and form a 3D aggregate. **(b)** The liquid overlay technique inhibits cell attachment to plates by using low adhesive surfaces such as agarose. **(c)** In magnetic levitation, cells are treated with a magnetic nanoparticle assembly, which causes them to become magnetic. A magnetic field causes the cells to aggregate. **(d)** Cells proliferate and adhere to scaffolds. Over time they interact with each other and form spheroids. **(e)** In spinner flasks cells aggregate due to centrifugal forces. **(f)** Matrigel and other hydrogels are used to seed single cells within an extracellular matrix which form spheroids over time.

4.1.2 Advantages and disadvantages of 3D cell culture compared to 2D

3D cell culture does have advantages and disadvantages to consider and both 2D and 3D culture is useful depending on the question of interest.

4.1.2.1 Advantages of 3D cell culture

In 2D culture, the cells are grown as a monolayer, which results in the cells being limited to horizontal growth and signalling. For cells grown in 3D, the cells are suspended within the matrigel, allowing cell-cell signalling throughout the spheroid as well as cell-ECM signalling with the extracellular components of the surrounding matrigel (233).This establishes a signalling network that is essential for spheroid homeostasis and survival. In addition, the structure of the spheroid creates diffusion gradients, with gradients for oxygen, drugs, waste, and nutrients present (234). This is considered more representative of a solid tumour where there are central hypoxic regions and the concentration of drugs is highest at the outer layers of the tumour (235). Furthermore, gene and protein expression are more comparable with the genetic profile seen in tumour masses *in vivo* than in 2D cell culture (236). In a study which examined biomarker expression in prostate cells cultured in matrigel there was a significant difference in morphology, biomarker expression, and proliferation rates between non-cancerous epithelial cells, non-invasive and metastatic prostate cancer cells lines. Cells grown in 3D culture more precisely mimicked the cancer progression reported *in vivo* (237). In addition, this model can be adapted to incorporate other cell types and scaffolding to create a more tissue like microenvironment. Many studies have shown that the tumour microenvironment (TME) is important for cancer progression and response to treatments. The TME consists of the extracellular matrix (ECM), different non-cancer cell types, their stroma which includes mesenchymal supporting cells, fibroblasts, immune cells and cells of the surrounding vasculature (238). Together the TME has a specific role in the function, structure and physiology of the tumour. Therefore, many 3D culture models attempt to encompass the surrounding environment, incorporating tumour cells, cancer associated fibroblasts and macrophages (239) to create a dynamic environment with cell to cell interactions and the secretion of signalling molecules such as cytokines and pro-inflammatory factors.

4.1.2.2 Disadvantages of 3D cell culture

Despite the advantages in providing a more representative tumour model, 3D cell culture does have some disadvantages. It can be expensive, with the added cost of gels or scaffolding as well as more specific media requirements to induce differentiation or development of structures such as crypt formation from primary colorectal cultures. Furthermore, as 3D cell culture has only started to be used relatively recently, the protocols are quite labour intensive and are still being optimized and developed. Consequently, the downstream assays are not as reproducible as those for 2D cell culture and are continually improving. Quantifying the effect of drugs, metabolites and chemicals on 3D culture can also be difficult as the cells aren't exposed to uniform concentrations of a substance which leads to variable effects within the spheroid. However, as time goes on the techniques will become less expensive and more robust, increasing outputs from these models and making them more widely available.

4.1.2.3 3D cell culture in drug discovery and testing

Drug discovery and development is one of the main goals of clinical research. Identifying drugs that work well in humans is difficult and often drug induced effects seen in the laboratory are not translated to clinical practice. While animal studies help to bridge this gap, the use of animals is very expensive and must be ethical which means there is less high content screening. The use of 3D cell culture before animal testing assesses the drug could help determine whether animal tests are necessary (240) (241). Indeed, a study which compared the effect of chemotherapeutics on 2D and 3D models observed that the response of the 3D prostate cell culture to docetaxel was more consistent with *in vivo* results than those cultured in 2D (242).

To date there are no studies examining the effect of aspirin or metformin in 3D culture for prostate cancer and no studies that have examined combining the drugs in 3D cell culture for any type of cancer. It is thought that testing aspirin and metformin in 3D culture will give a better indication of what effect the drugs will have in humans.

4.2 Hypotheses and aims

In this chapter, the effects of aspirin and metformin on cell proliferation and apoptosis in 3D cell culture are described.

Hypothesis: As aspirin was shown to reduce proliferation of prostate cancer cells in 2D cell culture in Chapter 3 it is expected that aspirin will also cause growth inhibition of 3D prostate spheroids. Metformin will have no effect on cell proliferation in 3D culture when administered alone but may enhance the efficacy of aspirin as seen in 2D cell culture.

Aims and objectives:

1. To optimise 3D cell culture for PC3, PNT2 and primary prostate cells.
2. To examine the effect of aspirin and metformin alone and in combination on 3D spheroid formation and growth. These experiments will be designed to address if administering the drugs before cancer development is beneficial and if the drugs have the capacity to reduce the growth of an established tumour.

4.3 Materials and methods

Spheroid cultures were seeded and maintained as described in Chapter 2, Section 2.7.1. They were imaged and analysed as described in Chapter 2, Sections 2.7.2 and 2.7.3. Statistical analysis was performed as described in Chapter 2, Section 2.8.

4.3.1 Examining the effect of aspirin and metformin on spheroid formation

Cells were seeded in a 48 well plate with 40 cells per well in 20 µl of matrigel. The day of seeding was termed day 0 and spheroids were dosed at days 4, 7 and 11 with 250 µl of 0, 0.5, 1, 2, 4 and 10 mM aspirin and 0, 10, 20, 30, 1000 and 5000 µM metformin. When cells were dosed the wells were washed with PBS before the media was changed to remove all of the existing media.
Following the results of the initial experiments the drug concentrations and experimental setup were then modified. Cells were dosed at days 0, 4, 7 and 11 but the original media was not removed and spheroids were not washed with PBS as this was found to disrupt the matrigel. Instead, fresh media was added to the existing media. The concentrations were also changed so that spheroids were dosed with 0, 0.25, 0.5, 1, 2 and 4 mM aspirin and 0, 10, 20, 30, 100 and 1000 µM metformin to determine whether lower concentrations of the drugs would also have an antiproliferative effect. Spheroids were imaged at day 14.

4.3.2 Examining the effect of aspirin and metformin on spheroid growth

Cells were seeded into ADF plus supplements rather than dosing immediately and grown in spheroid media for 1 week, forming small spheroids with an average spheroid area of 0.03 $x10^5$ μm^2. They were then dosed with 0, 0.25, 0.5, 1, 2 and 4 mM aspirin and 0, 10, 20, 30, 100 and 1000 µM metformin for a further 1.5 weeks, dosing twice a week. Spheroids were imaged at day 18. At the end of the experiment spheroids were also stained with calcein AM and ethidium homodimer-1 as described in Chapter 2, Section 2.7.4 or stained for markers of cell proliferation, PCNA and cyclin D1, following the immunofluorescent protocol as described in Chapter 2, Section 2.7.5.

4.4 Results

4.4.1 Establishing 3D cell culture

Cells were seeded suspended in matrigel and cultured in Advanced DMEM/F12 (ADF) plus supplements B27, N2 and N-acetylcysteine to create 3D functional spheroids. It was observed that the media and supplements supported cell growth and that spheroids were formed from single cells. In 2D culture, cells grow in a monolayer with PC3 cells having a long and thin appearance. In 3D, the PC3 cells had a stellate morphology with spiky, branching protrusions around the perimeter (Figure 4.2). The spheroids did not contain a lumen as expected as they are considered to be a basal cell type due to the expression of keratin 5 and keratin 14 and lack of luminal markers PSA and AR (243).

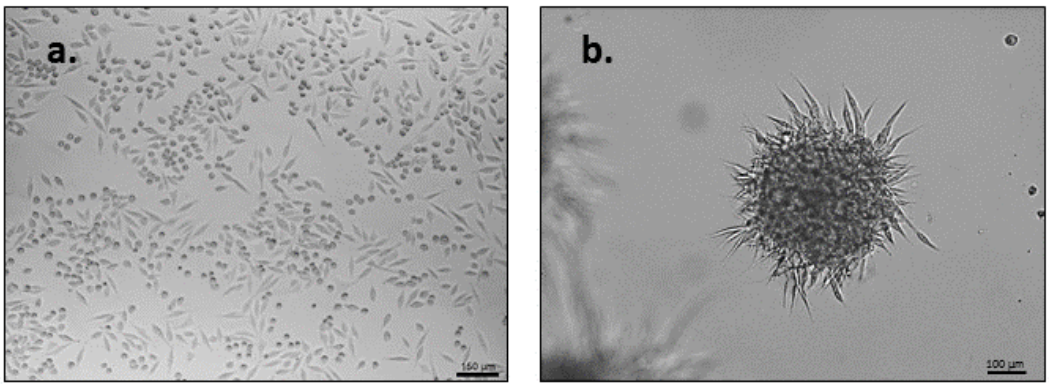


Figure 4.2: A comparison of PC3 cells grown in 2D and 3D cell culture.
(a) PC3 cells grown in 2D cell culture as a monolayer attached to plastic. **(b)** PC3 cells grown in 3D cell culture suspended in matrigel.

Non-tumorigenic PNT2 prostate epithelial cells and primary cells from a prostate with benign prostatic hyperplasia, H642/17, were also grown in 3D culture to compare the differences in spheroid morphology with the PC3 prostate cancer cell line. Unlike the PC3 cell line which displayed protrusions both the non-tumorigenic and the benign primary prostate cells had a rounded morphology and slower growth rates (Figure 4.3).

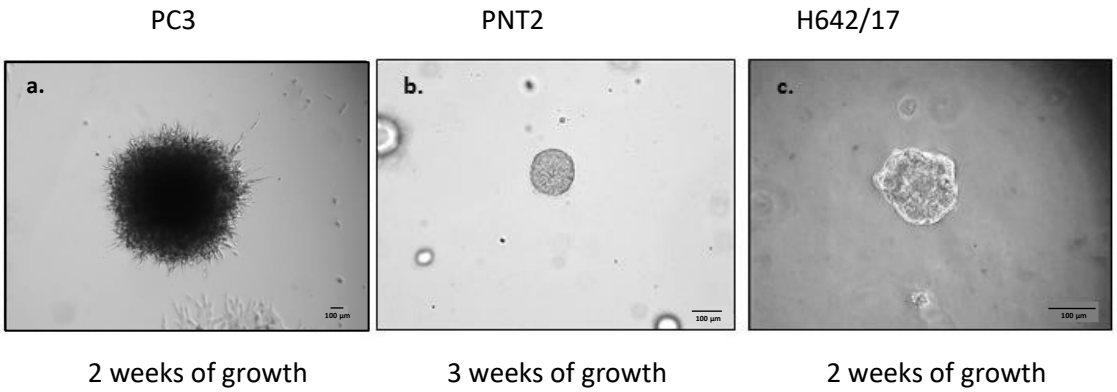


Figure 4.3: A comparison of cancerous and non-cancerous prostate cells grown in 3D culture.
(a) Cancerous PC3 cells **(b)** non-tumorigenic PNT2 cells and **(c)** primary prostate cells from a patient with benign prostatic hyperplasia, H642/17, displayed different morphologies when grown in 3D culture. PC3 and PNT2 cells were imaged at 5x magnification on a wide-field microscope while the H642/17 cells were imaged at 20x magnification on an Olympus CK2 microscope.

A seeding density trial was performed to assess how many cells to seed per well and at what time point the experiment should be terminated. It was necessary to ensure that spheroids were seeded at a low enough density so that they did not overlap and were imaged for as long as possible without becoming too large to fit within the imaging frame. The PC3 cell line was selected to examine the effect of the aspirin and metformin on proliferation in 3D culture as these were the only cells that showed an additive effect of the drugs in 2D cell culture (Chapter 3, Figures 3.4 and 3.5). PC3 cells were seeded at 40, 80, 160 and 240 cells per well and grown for three weeks. Spheroids were imaged once a week and the media was changed twice a week.

The PC3 spheroids grew quickly, displaying constant growth over the 3-week culturing period, and would have continued to grow if culturing was maintained (Figure 4.4). Two to three weeks was chosen as an appropriate time point to end future experiments as after this, some spheroids were too large to fit within the field of view and it would have been difficult to accurately record their size (Figure 4.5). After two weeks of growth spheroids had an average diameter of ~526 µM. At 80, 160 and 240 cells per well there was large overlap between the spheroids with too many cells seeded to individually assess spheroid size. Therefore, it was decided to seed 40 cells per well for future experiments.

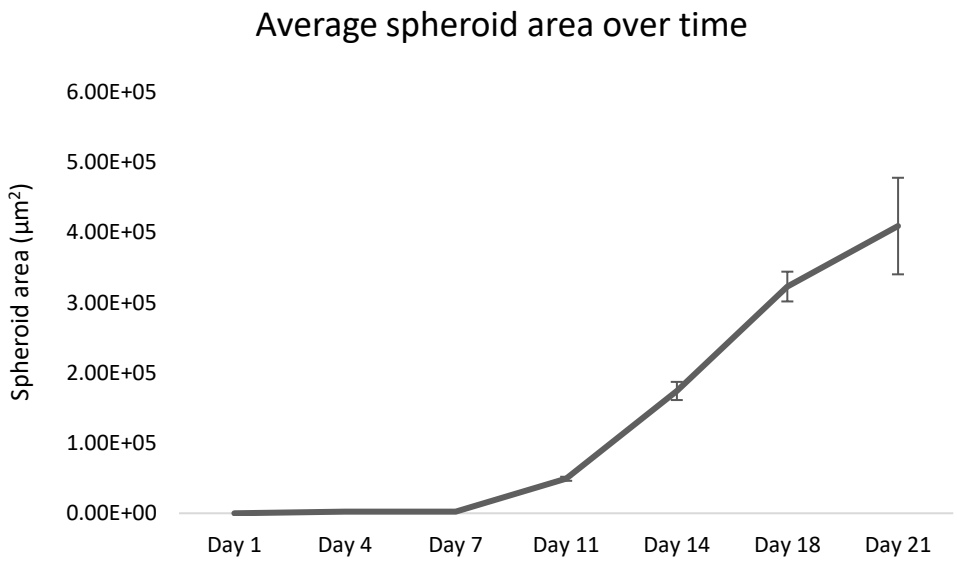


Figure 4.4: Average PC3 spheroid area over 21 days of culturing.
PC3 spheroids grew slowly until day 7 when spheroid area greatly increased with time. The spheroids would have continued to grow after day 21 if culturing was maintained. Data for one independent experiment performed in triplicate. Error bars represent standard error of mean.

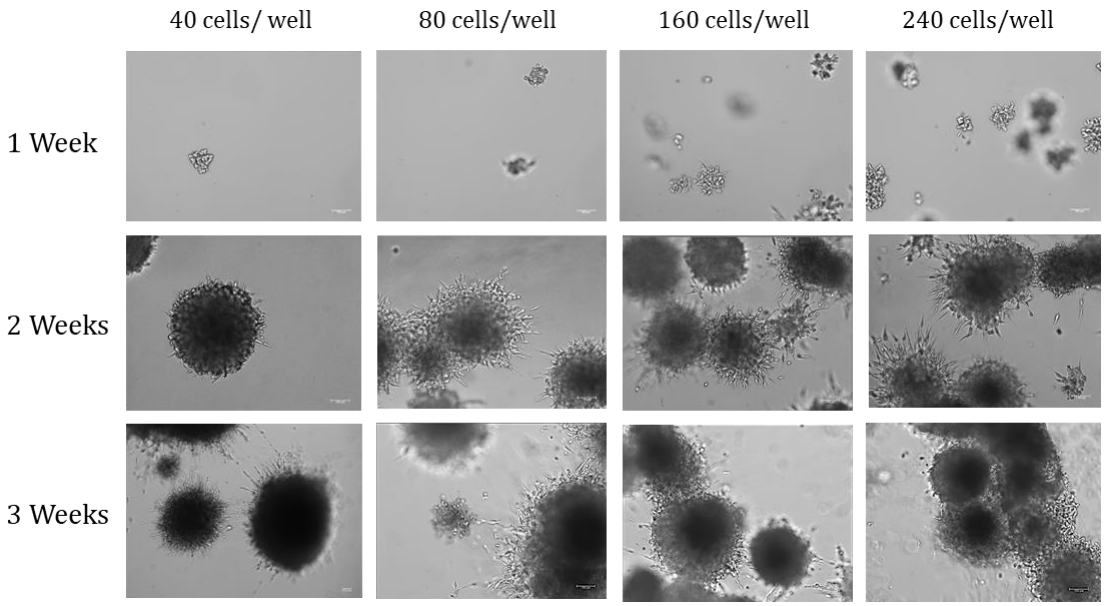


Figure 4.5: Seeding density trial for PC3 cells.
Single cells were seeded in matrigel at 40, 80, 160 and 240 cells per well and grown for 3 weeks. Images were taken using the Leica LAS X program on a wide-field microscope using the 5x objective.

4.4.2 Localization of markers of cell proliferation, PCNA and cyclin D1, within PC3 spheroids

Spheroids were stained for markers of cell proliferation, PCNA and cyclin D1, to determine the localization of proliferating cells within the spheroid and to provide a more detailed understanding of spheroid growth. When staining for markers of cell proliferation, PCNA and cyclin D1 were focused around the outer edges of the spheroid, with the centre remaining unstained (Figure 4.6). DAPI was observed throughout the spheroid. The spheroid protrusions were stained but did not appear as bright as the central region because they were composed of fewer cells. The surrounding matrigel did cause some minor background fluorescence. A negative control without the addition of the primary antibody confirmed that there was no nonspecific binding of the secondary antibody and that the labelling observed was due to the binding of the secondary antibody to the primary antibody (Figure 4.7**Figure**).

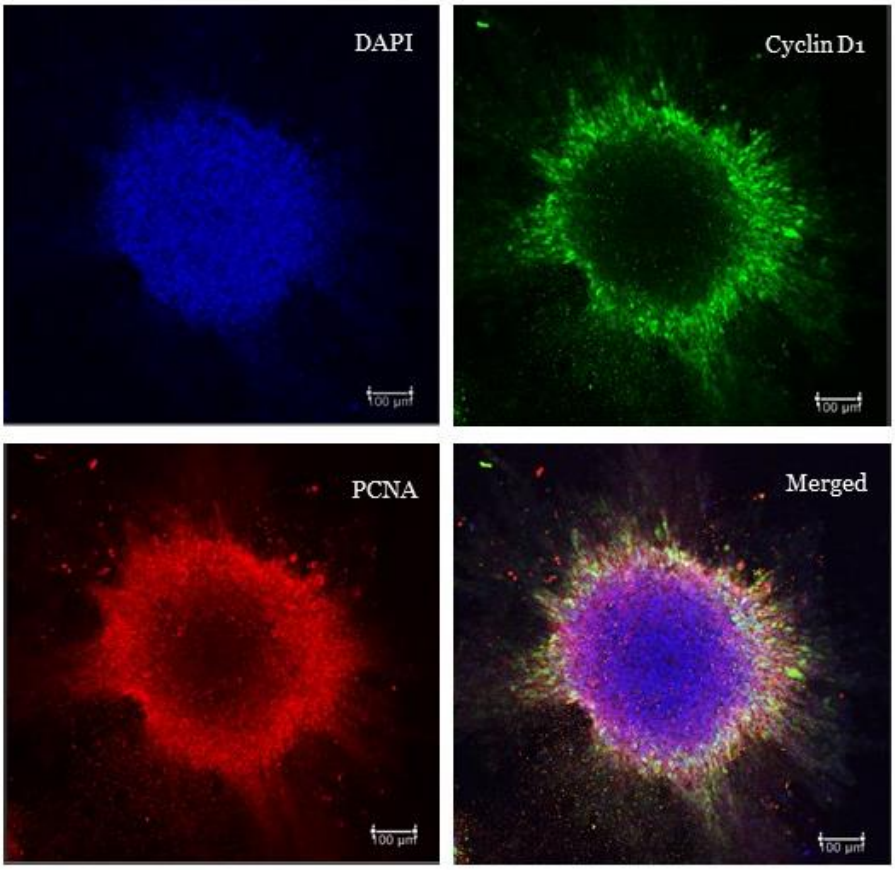


Figure 4.6: Immunofluorescence for markers of cell proliferation in a PC3 spheroid.
PC3 spheroids were grown for 18 days and stained with proliferation markers PCNA (red) and cyclin D1 (green) to determine their localization. DAPI (blue) was used to visualise cell nuclei. Spheroids were imaged on a confocal microscope using the HCX PL Fluotar 10x 0.3 Dry objective. Representative of three repeats.

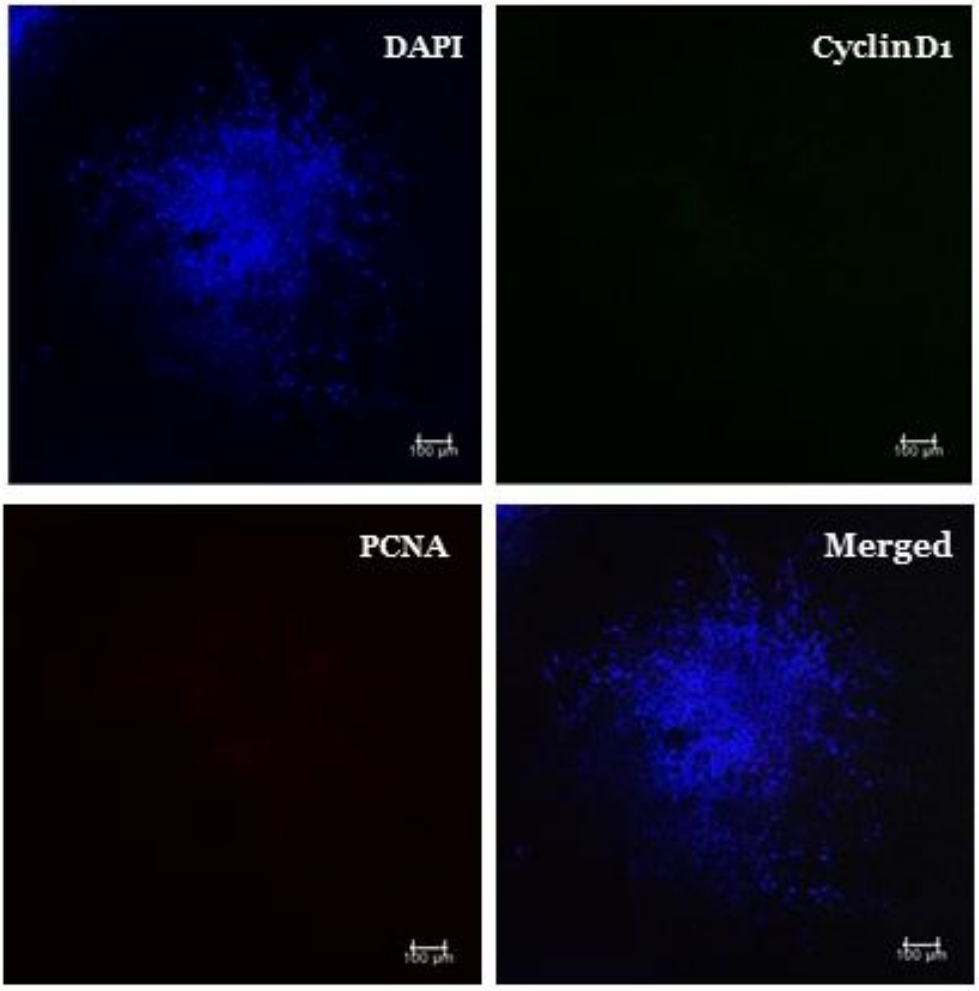


Figure 4.7: Negative control for immunofluorescence.
Spheroids were grown until day 18 and then underwent immunofluorescence for PCNA and cyclin D1 following the protocol described in in Chapter 2, Section 2.7.5 but without the addition of the primary antibody. Spheroids were imaged on a confocal microscope using the HCX PL Fluotar 10x 0.3 Dry objective. Representative of three repeats.

4.4.3 Localization of markers of cell proliferation throughout the spheroid growth period

To examine changes in spheroid proliferation over the peroid of growth, spheroids were fixed and stained with PCNA and cyclin D1 at days 4, 7, 11, 14, 18 and 21 (Figure 4.8). For spheroids grown from days 4-14 PCNA and cyclin D1 were observed throughout, indicating that all the cells were viable and proliferating. At days 18 and 21 PCNA and cyclin D1 were localized to the outer edges of the spheroid, forming a zone of proliferation. This was to be expected as studies have shown that the centre of large spheroids are often necrotic (244). It appears that PCNA stains further into the centre of the spheroid than cyclin D1.

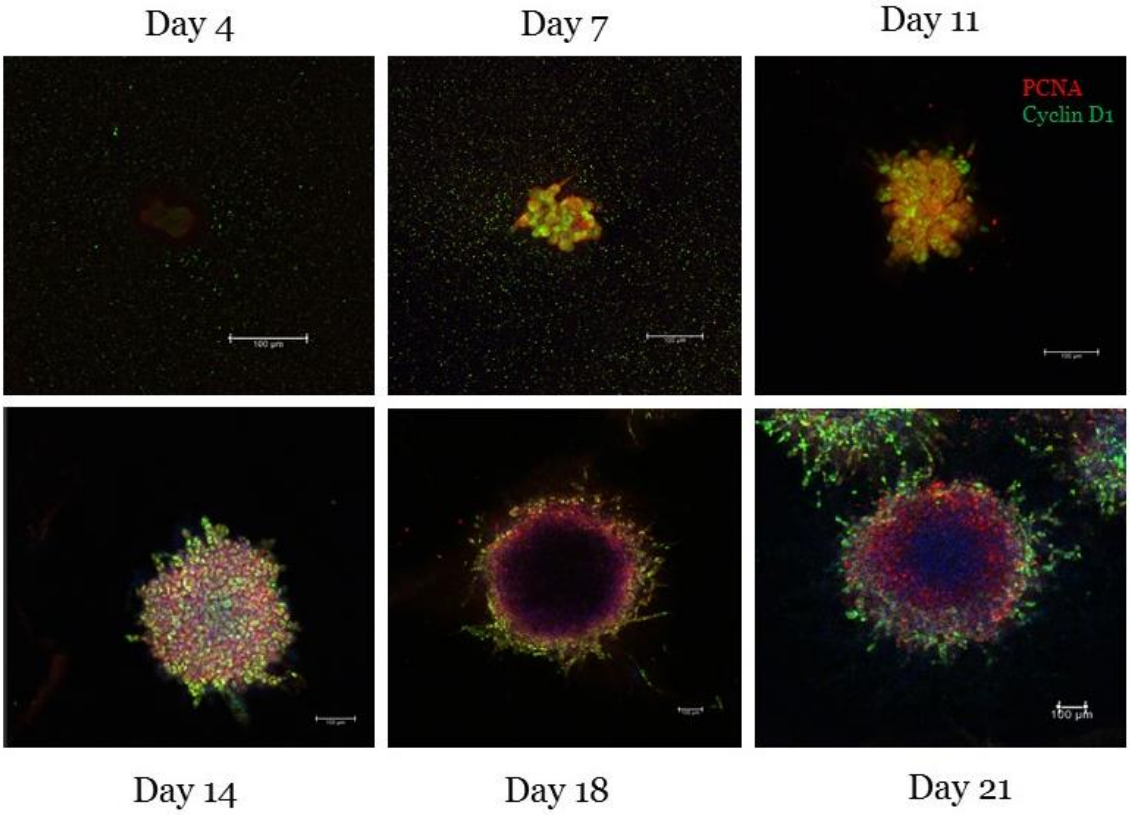


Figure 4.8: PC3 spheroid immunofluorescence for markers of cell proliferation during a three-week growth period.
Localization of PCNA (red) and cyclin D1 (green) was examined in PC3 spheroids at days 4, 7, 11, 14, 18 and 21. Representative of one independent experiment performed in triplicate. Spheroids were imaged on a confocal microscope using the HCX PL Fluotar 10x 0.3 Dry objective.

Together the data from the growth experiments suggest that spheroids up to day 21 contain actively proliferating cells and potentially a necrotic core.

4.4.4 Optimization of dosing PC3 spheroids with aspirin and metformin

Once the media requirements and time points for the PC3 3D cell culture were established, it was important to decide what concentrations of aspirin and metformin to use and establish a dosing regimen. Studies have shown that drugs have a different effect in 3D and 2D cell culture, so it was not known if the concentrations used in 2D for the combination studies would be optimum in 3D cell culture. Therefore, cells were seeded and dosed with the same doses used for the 2D culture experiments (Chapter 3, Section 3.3.4). Cells were dosed with 0, 0.5, 1, 2, 4, 10 mM aspirin or 10, 20, 30, 100 and 5000 µM metformin. Spheroids were analyzed using Matlab, which compressed the image stacks into 2D, located the spheroids and calculated spheroid area (Chapter 2, Section 2.7.3). Spheroids were highlighted in pink against a green background to easily identify each spheroid (Figure 4.9)

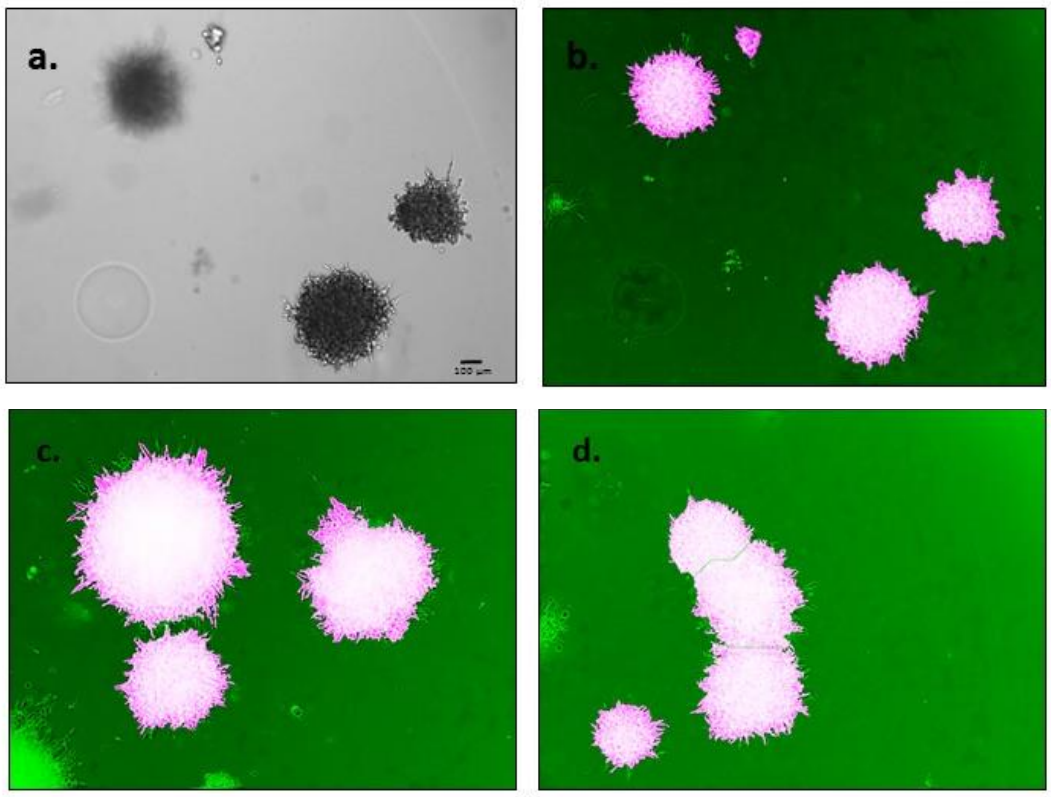
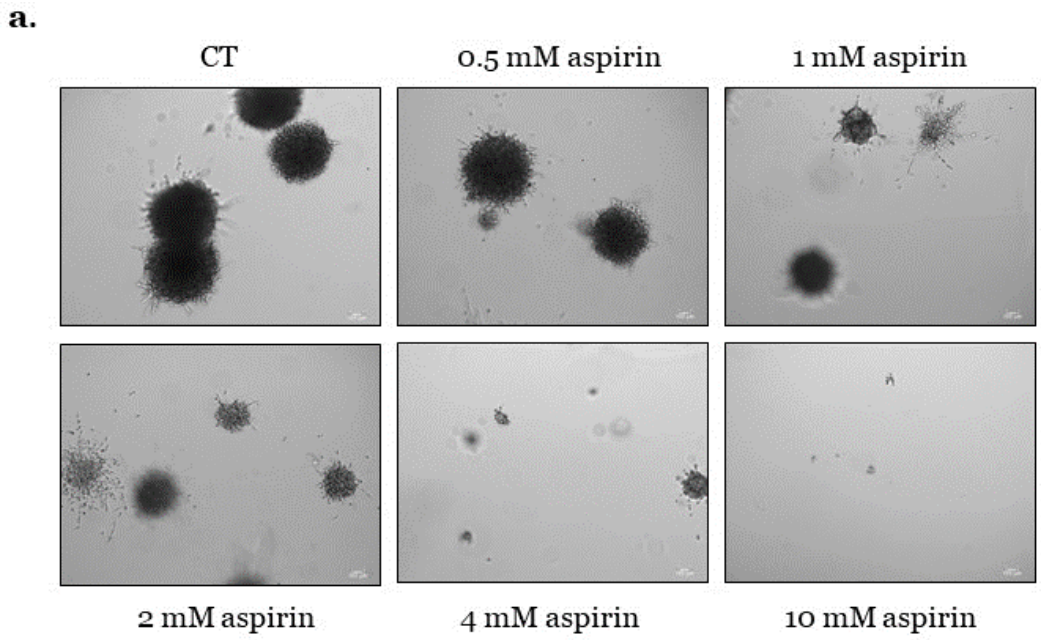


Figure 4.9: Matlab calculated spheroid area.
(a) 35 images of the spheroids were taken through the z plane to form a z-stack on a wide-field microscope **(b, c)** Matlab compressed the 35 images into a 2D image and calculated spheroid area; spheroids were highlighted in pink. **(d)** Spheroid area for spheroids which were overlapping were manually discarded so as not to skew the data.

The outer edges of the spheroids were not fully detected by the software as the spheroids had many protrusions, which can sometimes blend into the background and are difficult to distinguish. Spheroids that were partially out of frame were excluded. After running the images through Matlab the spheroids were checked manually, comparing the images with the calculated area. Spheroids which were only partially identified or were not separated from overlapping spheroids were manually excluded so as not to skew the data.

The day of seeding was termed day 0 and spheroids were dosed at days 4, 7 and 11 with 0, 0.5, 1, 2, 4 and 10 mM aspirin or 0, 10, 20, 30, 1000, 5000 µM metformin and then underwent imaging at day 14. It was observed that when cells were dosed with aspirin there was a dose-dependent decrease in median spheroid area with increase in aspirin concentration. Median spheroid area was 1.4 $x10^5$, 1.0 $x10^5$, 0.90 x 10^5, 0.25 $x10^5$, 0.11 $x10^5$ and 0.08 $x10^5$ μm^2 for the control, 0.5, 1, 2, 4 and 10 mM aspirin respectively (Figure 4.10). The decrease in spheroid area was significant from 1 mM aspirin and above. The greatest decrease was seen between 1 and 2 mM aspirin, with a drop in median spheroid area of 0.64 x 10^5 μm^2. Aspirin was toxic at 10 mM, with only one small spheroid observed.

For PC3 spheroids dosed with metformin a decrease in median spheroid area was observed at 20, 30, 100 and 5000 µM metformin (Figure 4.11). However, spheroid area was only significantly different from the control at 5000 µM metformin ($p<0.001$). At this concentration a large decrease was seen, with a drop of almost 60% in median area compared to that of the control.



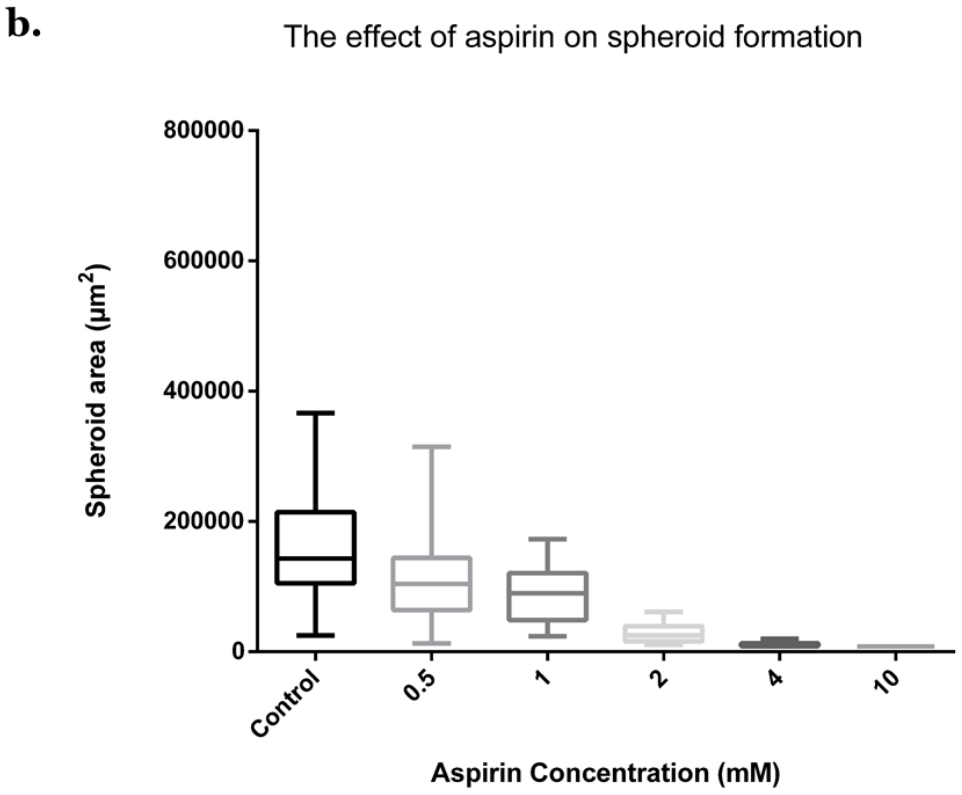


Figure 4.10: Optimising dosing with aspirin for 3D cell culture.
(a) PC3 spheroids dosed twice a week for two weeks with 0, 0.5, 1, 2, 4 and 10 mM aspirin and then imaged at day 14. **(b)** Box and whisker plot displays the median, interquartile range and the 5-95 percentile. Data from one independent experiment performed in triplicate.

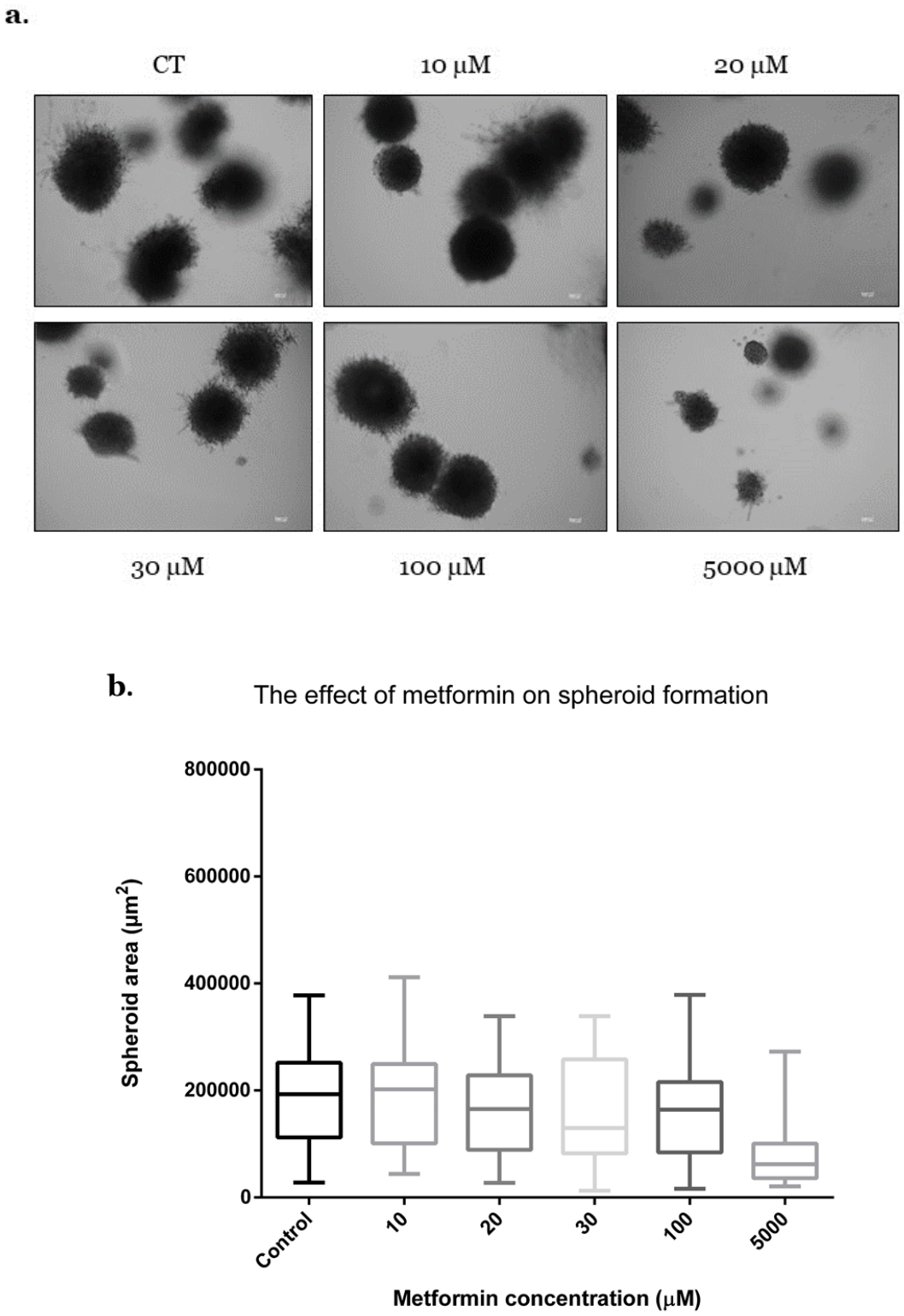


Figure 4.11: Optimising dosing with metformin for 3D cell culture.
(a) PC3 spheroids were dosed for 2 weeks with 0, 10, 20, 30, 100 and 5000 μM metformin and then imaged at day 14. **(b)** Box and whisker plot displays the median, interquartile range, and the 5-95 percentile. Data from one independent experiment performed in triplicate.

The previous experiments were performed to assess the effect aspirin and metformin on spheroid formation and to optimize dosing conditions. The experiments provided strong preliminary data on the effect of the drugs on proliferation in 3D, indicating that drugs do inhibit spheroid growth. After one experiment it was decided to modify the model to reduce the highest concentrations of both drugs, using more clinically achievable concentrations. In addition, it was decided to seed the cells directly into media containing the drugs instead of dosing after 4 days to examine the ability of a single cell to form a spheroid. It was thought that this would better model preventing the formation of a tumour within a person who is already taking aspirin or metformin. Instead of removing spent media during dosing, the fresh media was added to the existing media to minimize the disruptiveness to the matrigel.

4.4.5 Aspirin dose-dependently inhibits PC3 spheroid formation

With the new culture conditions PC3 cells were dosed at days 0, 4, 7 and 11 with 0, 0.25, 0.5, 1, 2 and 4 mM aspirin or 0, 10, 20, 30, 100 and 1000 µM metformin. Again, a dose-dependent decrease in median spheroid area with increase in aspirin concentration was observed (Figure 4.12). The decrease in spheroid area was dose-dependent, decreasing to a minimum median area of 0.11 $x10^5$ μm^2 for spheroids dosed with 4 mM aspirin, which was the same as seen in the previous experiment (Figure 4.10). A significant effect was observed between 0.5 mM and 4 mM aspirin (p= 0.12, <0.001, <0.001, <0.001, and <0.001 for 0.25, 0.5, 1, 2 and 4 mM aspirin respectively compared to the control). Median spheroid area was 1.75 $x10^5$, 1.37 $x10^5$, 1.03 $x10^5$, 0.61 $x10^5$, 0.26 $x10^5$ and 0.11 $x10^5$ for the control, 0.25, 0.5, 1, 2 and 4 mM aspirin respectively. Median spheroid areas were similar to the previous experiment (Figure 4.10), despite the changes in the method. The largest different in median spheroid area between the two experiments was when the cells were dosed with 1 mM aspirin for which in the previous experiment it was 0.9 x 10^5 whereas in this new experimental setup median spheroid area was 0.61 $x10^5$ indicating that lower concentrations have a greater effect on spheroid formation. Interestingly, for both experiments aspirin was more effective at lower concentrations in 3D culture than in 2D, causing a significant reduction in proliferation at 0.5 mM which was not observed in the 2D experiments (Chapter 3, Figure 3.5).

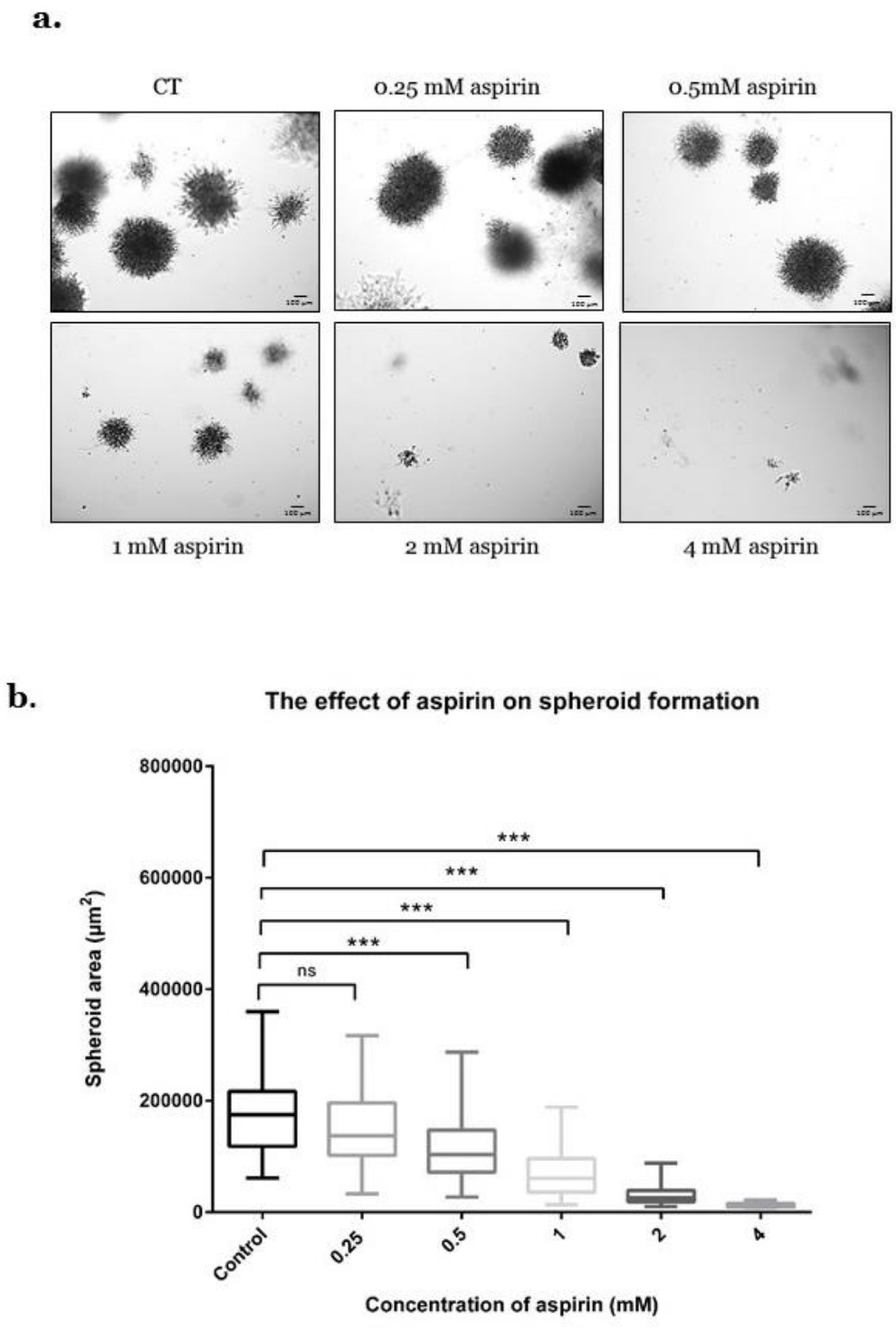
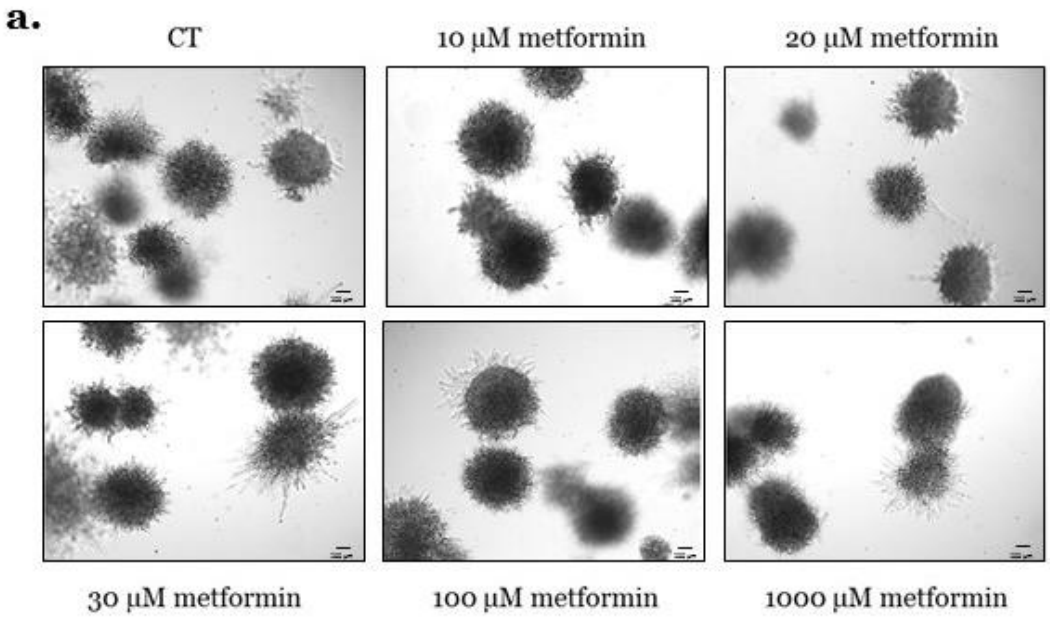


Figure 4.12: The effect of aspirin on PC3 spheroid formation.
(a) PC3 spheroids dosed for two weeks with 0, 0.25, 0.5, 1, 2 and 4 mM aspirin. **(b)** Change in spheroid area for spheroids dosed with 0.25, 0.5, 1, 2 and 4mM aspirin at day 14. Box and whisker plot displays the median, interquartile range and the 5-95 percentile. Data were analysed using a Kruskal-Wallis one-way ANOVA and Dunn's post hoc test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data from three independent experiments performed in quadruplet.

4.4.6 Metformin has no significant effect on spheroid formation at clinically relevant concentrations

This experiment tested whether clinically achievable doses of metformin affect spheroid formation. The maximum concentration of metformin was reduced to 1000 µM metformin from 5000 µM in the previous experiment for reasons described in Chapter 4, Section 4.4.4. It was seen that there was no significant effect on spheroid area for spheroids dosed with 10-100 µM metformin (Figure 4.13). However, at 1000 µM metformin there was a significant decrease in median spheroid area compared to the control ($p= 0.15$, >0.99, 0.85, >0.99 and 0.046 for 10, 20, 30, 100 and 1000 µM). This suggests that concentrations above those that are therapeutically relevant, 40 µM, are required to influence spheroid formation and supports the 2D cell culture data where 30 µM metformin did not affect cell proliferation (Chapter 3, Figure 3.5) but 1000 µM caused a decrease in cyclin D1 expression (Chapter 3, Figure 3.11).



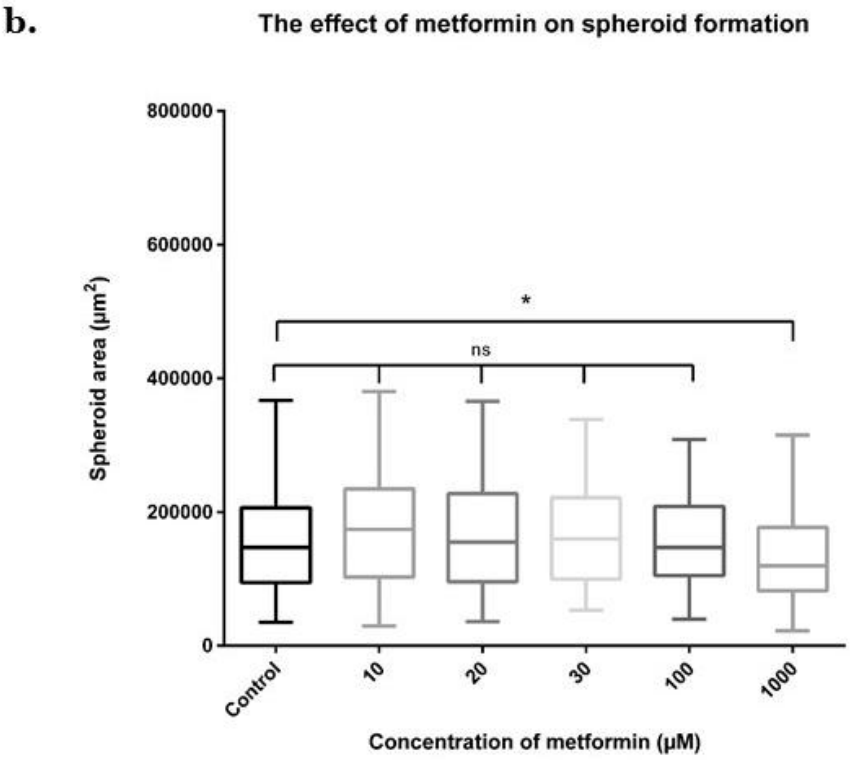


Figure 4.13: The effect of metformin on PC3 spheroid formation.
(a) PC3 spheroids after two weeks of being dosed with 0, 10, 20, 30, 100 and 1000 µM metformin**. (b)** Change in spheroid area for spheroids dosed with 10, 20, 30, 100 and 1000 µM metformin at day 14. Box and whisker plot displays the median, interquartile range and the 5-95 percentile. Data were analysed using a Kruskal-Wallis one-way ANOVA and Dunn's post hoc test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data from three independent experiments performed in quadruplet.

4.4.7 Aspirin dose-dependently inhibits PC3 spheroid growth

The next experiment aimed to study the effect of aspirin on PC3 spheroid growth. Cells were dosed after one week of growth, once they had formed small spheroids with an average spheroid area of 0.03 x 10^5 µm^2. They were then dosed with 0, 0.25, 0.5, 1, 2 and 4 mM aspirin and 0, 10, 20, 30, 100 and 1000 µM metformin for a further 1.5 weeks, dosing twice a week. Spheroids were imaged at day 18.

Similar to the spheroid formation experiment, a dose-dependent decrease in spheroid area was observed when cells were dosed with aspirin (Figure 4.14). Median area was 2.8 x10^5, 3.0 x10^5, 2.4 x10^5, 2.2 x10^5, 1.8 x10^5 and 0.5 x10^5 µm^2 for the control, 0.25, 0.5, 1, 2 and 4 mM aspirin respectively, with a significant decrease from 1 mM aspirin onwards (p = >0.99, 0.39, 0.03, <0.001, and <0.001 for 0.25, 0.5, 1, 2 and 4 mM compared to the control). Spheroid area was not as affected as in the previous experiment, when the cells were seeded directly into the media containing the drugs (Figure 4.12), where a significant effect was seen from 0.5 mM aspirin.

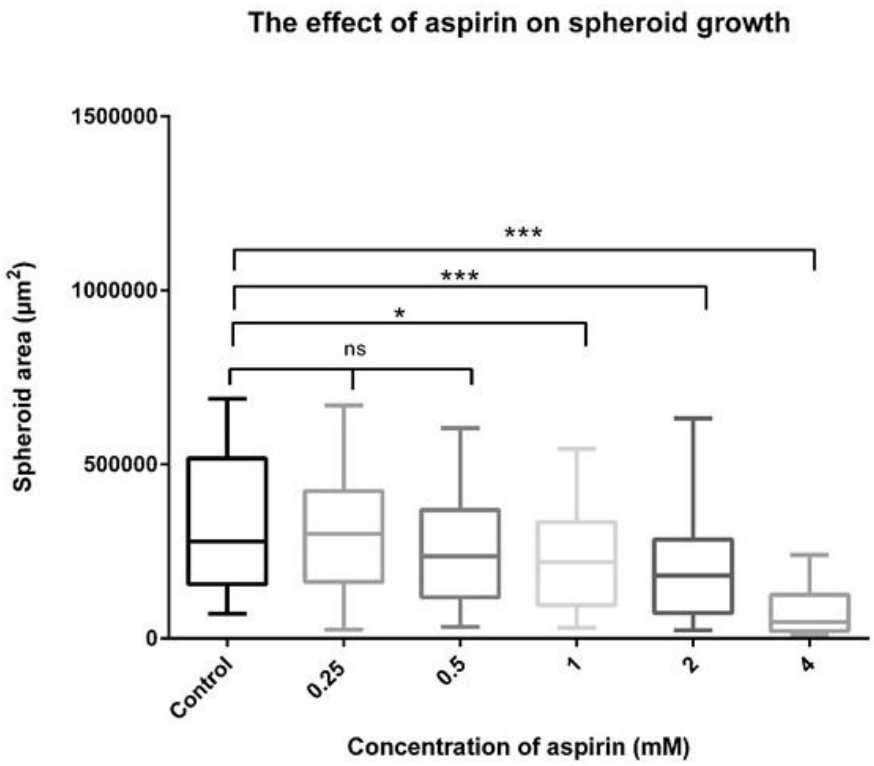


Figure 4.14: The effect of aspirin on PC3 spheroid growth.
Change in spheroid area for spheroids dosed with 0.25, 0.5, 1, 2 and 4 mM aspirin at day 18. Spheroids were dosed for 11 days. Box and whisker plot displays the median, interquartile range and the 5-95 percentile. Data were analysed using a Kruskal-Wallis one-way ANOVA and Dunn's post hoc test for multiple comparisons. * p <0.05, ** p <0.01, *** p < 0.001. Data represents three independent experiments performed in quadruplet.

4.4.8 Metformin has no significant effect on spheroid growth at clinically relevant concentrations

In a similar experiment, when cells were dosed with metformin there was a decrease in spheroid area, which was significant at 100 µM (p= 0.01) and 1000 µM (p= 0.002) (Figure 4.15). Spheroid growth was more affected by metformin than spheroid formation for which a significant effect was only seen at 1000 µM. Median spheroid area was 2.78 x10^5, 2.05 x10^5, 2.05 x10^5, 1.75 x10^5, 1.56 x10^5 and 1.29 x10^5 µm^2 for the control, 10, 20, 30, 100 and 1000 µM metformin respectively. This was a decrease in median area of 44% and 64% for 100 and 1000 µM metformin compared with the control. Again, this data supported the results from the 2D cell culture experiments where metformin alone had no effect on cell proliferation at clinically relevant concentrations, (Chapter 3, Figure 3.5).

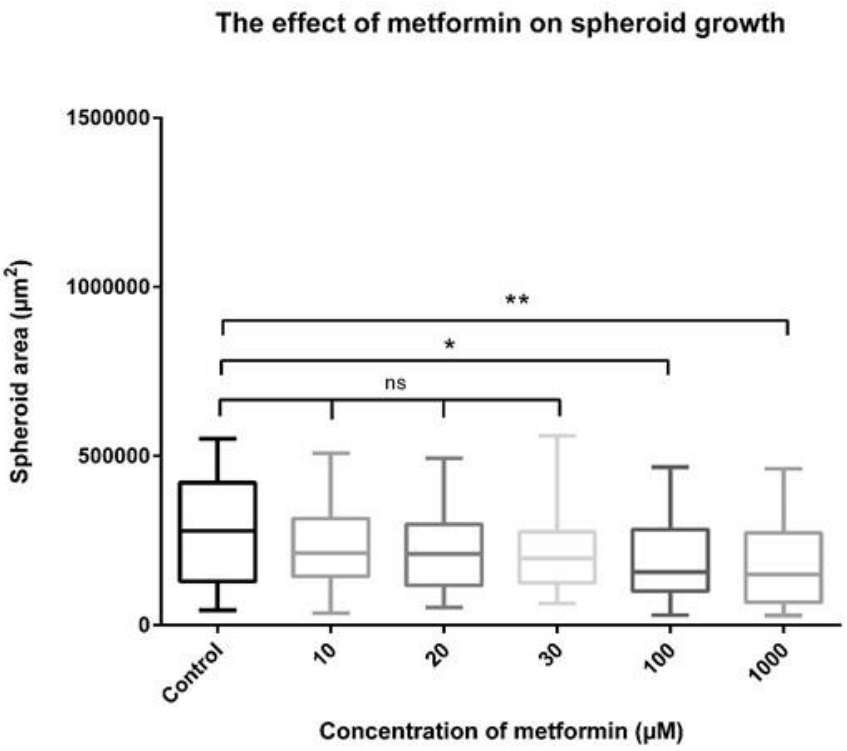


Figure 4.15: The effect of metformin on PC3 spheroid growth.
Change in spheroid area for spheroids dosed with 10, 20, 30, 100 and 1000 µM metformin at day 18. Spheroids were dosed for 11 days. Box and whisker plot displays the median, interquartile range and 5-95 percentile**.** Data were analysed using a Kruskal-Wallis one-way ANOVA and Dunn's post hoc test for multiple comparisons. * p <0.05, ** p <0.01, *** p < 0.001. Data represents three independent experiments performed in quadruplet.

4.4.9 Metformin enhances the efficacy of aspirin to inhibit spheroid growth

To investigate whether the drugs had an additive effect on spheroid growth as observed in the 2D culture experiments, Chapter 3, Figures 3.4 and 3.5, spheroids were dosed with a combination of aspirin (0.25 or 1 mM) and metformin (30 µM). The concentrations 0.25 and 1 mM aspirin were chosen as they include a concentration that no effect spheroid growth on its own (0.25 mM) and a concentration that does have an effect on its own but does not drastically reduce spheroid growth (1mM) as seen in Chapter 4, Section 4.4.7. 30 µM metformin was chosen as this was the highest clinically relevant concentration of metformin and in the 2D culture experiments this concentration had no effect alone but enhanced the effect of aspirin when used in combination.

For both combinations a decrease in spheroid area was observed with the combination of 1 mM aspirin and 30 µM metformin having the greatest effect (Figure 4.16). Median area was 2.77 x 10^5, 3.01 x 10^5, 2.33 x 10^5, 2.44 x 10^5, 1.88 x 10^5 and 1.67 x 10^5 μm^2 for the control, 0.25 mM aspirin, 1 mM aspirin, 30 µM metformin, the combination of 0.25 mM aspirin and 30 µM metformin and the combination of 1 mM aspirin and 30 µM metformin respectively. Compared to the control, there was a significant difference in median spheroid area for the combination of 0.25 mM aspirin and 30 µM metformin, with a decrease of 32%. This was considerably greater than the decrease in median area when spheroids were dosed with 0.25 aspirin and 30 µM which was 0% and 13% compared to the control respectively. Despite this, the decreased failed to reach significance ($p>0.05$). The combination of 1 mM aspirin with 30 µM metformin caused a decrease in median spheroid area of 40% compared to the control which was highly significant ($p<0.001$). While there was a large reduction in median spheroid area compared to dosing with 1 mM aspirin alone (0.66 x10^5 μm^2) it did not reach significance and indicates that the decrease observed is due to aspirin.

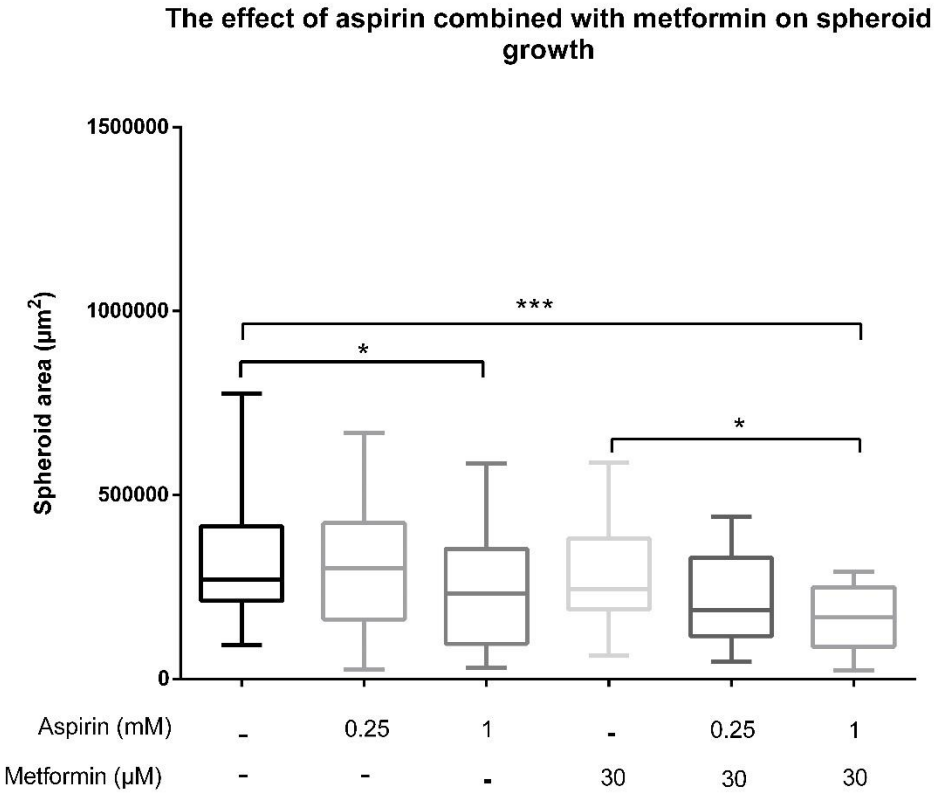


Figure 4.16: The effect of aspirin combined with metformin on PC3 spheroid growth.
Change in spheroid area after dosing with either 0.25 or 1 mM aspirin alone and in combination with 30 µM metformin at day 18. . Spheroids were dosed for 11 days. Box and whisker plot displays the median, interquartile range and the 5-95 percentile**.** Data were analysed using a Kruskal-Wallis one-way ANOVA and Dunn's post hoc test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data represents three independent experiments performed in quadruplet.

4.4.10 Aspirin and metformin do not induce cell death in 3D cell culture

After examining changes in spheroid area, the spheroids were stained with calcein AM and ethidium homodimer-1 to examine changes in cell viability. It was seen in the previous experiments that aspirin inhibited spheroid formation and growth, reducing spheroid area in both experiments (Figure 4.12 and Figure 4.14). This experiment was used to determine if the drugs were inhibiting cell proliferation and the spheroids were growth arrested or whether the cells were undergoing cell death. Staining with calcein AM and ethidium homodimer identified that PC3 spheroids contained a necrotic core (Figure 4.17) with the center staining red, indicating that the cells membranes were disrupted and ethidium homodimer 1 could enter. This supported the immunofluorescence data which showed that cells in the center of the spheroid did not express PCNA or cyclin D1 and were not proliferating. The outer zone was mostly composed of live cells which were stained green.

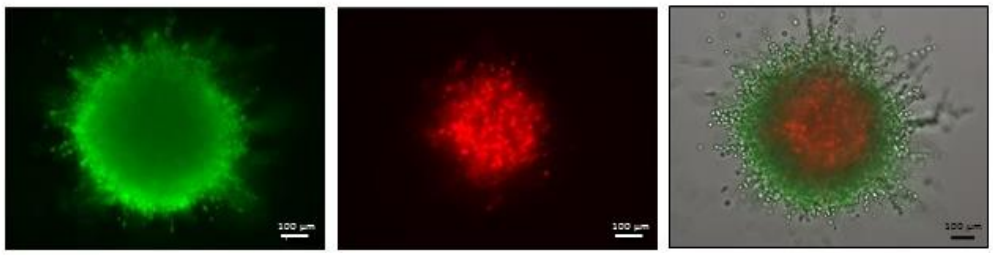


Figure 4.17: PC3 spheroids stained with calcein AM and ethidium homodimer-1.
Calcein AM stains live cells green, indicating intracellular esterase activity, whilst ethidium homodimer-1 stains dead cells red, indicating loss of plasma membrane integrity. Representative of experiments repeated three times.

When the cells were dosed with aspirin and metformin the spheroids maintained the pattern of live and dead cells seen in the untreated samples (Figure 4.18). Spheroids dosed with the highest concentration of the drugs, which caused the greatest decrease in spheroid area during the spheroid formation and growth experiments, were still composed of live cells with almost no cell death. This indicates that the drugs do not induce cell death in PC3 3D cell culture and suggests that the changes observed in the spheroid formation and growth experiments were due to aspirin and metformins inhibition of proliferation.

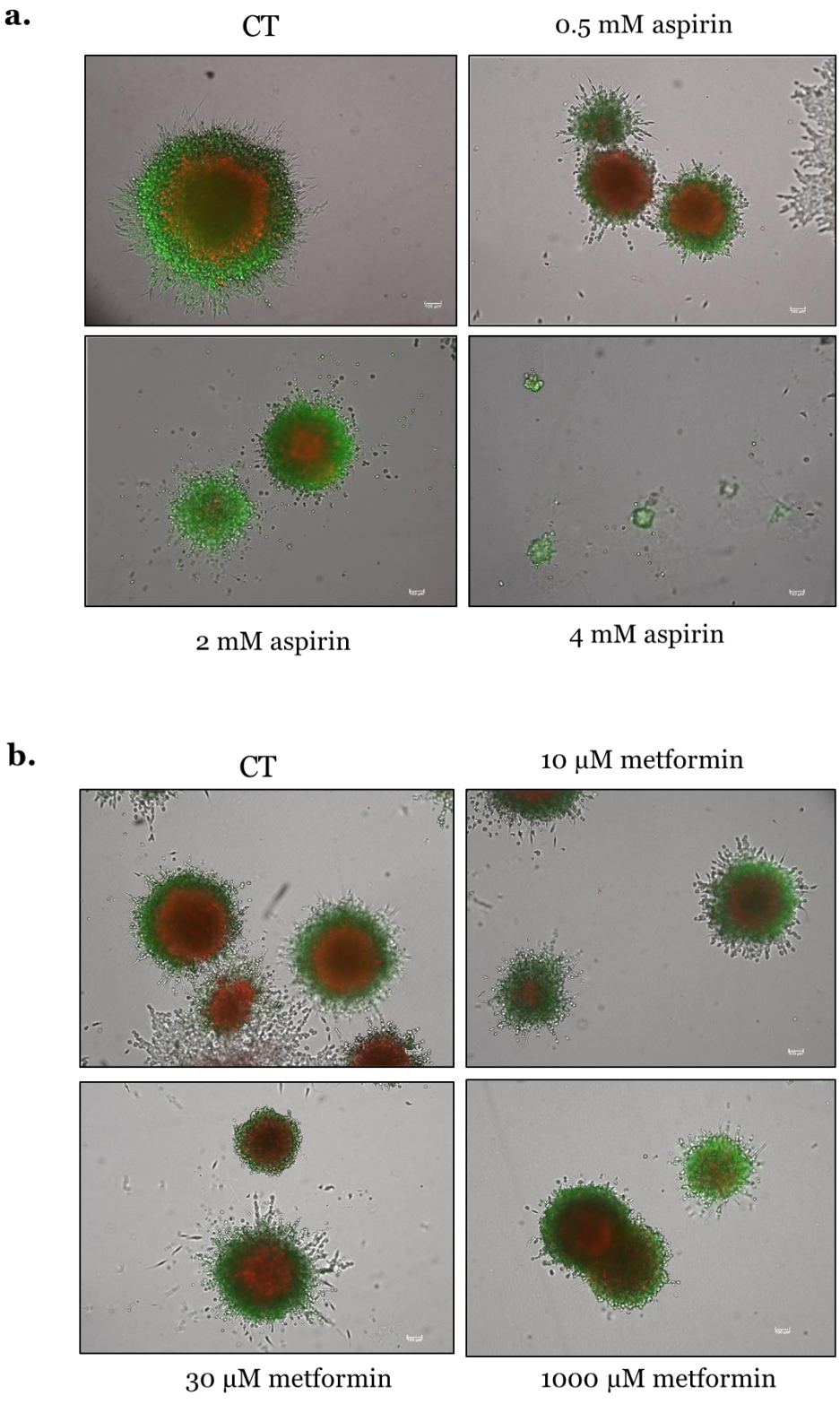


Figure 4.18: Calcein AM and ethidium homodimer-1 staining in PC3 spheroids dosed with aspirin or metformin.
PC3 spheroids were dosed with **(a)** aspirin or (**b)** metformin and stained with calcein AM (green) and ethidium homodimer-1 (red). Representative of experiment repeated three times.

4.5 Discussion

In this chapter, the effect of aspirin and metformin on cell proliferation in 3D cell culture was examined. Culturing the cells as spheroids identified the differences between 2D and 3D cell culture in terms of growth and response to treatment. It has been reported that these differences are associated with alterations in gene expression and result in the spheroids possessing a more similar genetic profile to that seen with tumour masses *in vivo* (241). Growing the cells as a spheroid provides drug diffusion gradients with less accessibility of the drug to the cells in the middle, making the model more representative of the effect on a tissue. *Breslin and O'Driscoll* have also shown that the expression of proteins such as transporters associated with drug resistance and proteins involved in cell survival are increased in 3D cultures compared to 2D conferring higher innate resistance to treatments (245).

In terms of spheroid structure, *Edmonson et al* have stated that spheroids can adopt four different shapes; round, mass, grape-like and stellate (241). The spheroid structure depends on the cell line but also the culture conditions and the extracellular matrix surrounding the cells. Growing the PC3, PNT2 and primary prostate cells as spheroids demonstrated key differences between cell type and morphology. The more advanced and invasive PC3 cells possessed a stellate morphology and formed protrusions into the surrounding matrigel. This morphology has also been reported by *Björk et al* (246). The non-tumorigenic PNT2 and primary cells formed much smaller, rounded spheroids. It is encouraging that in our model the PC3 and non-tumorigenic spheroids morphologically appear to demonstrate the mesenchymal and epithelial properties of the cells. Localization of markers of cell prolifertion, PCNA and cyclin D1, during the PC3 spheriod growth period was examined using immunofluorescence. PCNA and cyclin D1 were dispersed throughout the spheroid from day 1-14 but were localized to the outer layer of cells at days 18 and 21. Spheroid with a radii of 200 µm and larger have been reported to have a zone of proliferation which is restricted to the outer layer of cells with quiescent cells on the inside (247). Large spheroids will also have a necrotic centre. Immunofluorescence confirmed that cells on the periphery were actively growing with high expression of both proteins. The decrease in oxygen availability towards the centere of the spheroid is a rate limiting factor in spheroid growth (248) and so may contribute to the decrease in cell proliferation observed towards the spheroid centre. An important limitation of this experiment was that it was not established whether the antibodies had penetrated the spheroid centre. While DAPI did, the use of other antibodies such as those for hypoxia (CA-9 or HIF-1) would be benefical to confirm that the method works.

In assessing spheroid formation, it was observed that aspirin markedly affected spheroid size, reducing the median area in a dose-dependent manner that was significant from 0.5 mM, a therapeutically achievable concentration. This was an encouraging result, implying that aspirin does inhibit the ability of single cells to form spheroids. The benefit of administering aspirin pre-diagnostically is often debated with some studies such as the Physicians Health Study finding that administering aspirin before cancer diagnosis is associated with a lower risk of lethal prostate cancer (117) whereas other epidemiological studies have shown no pre-diagnostic effect (249) (250). The data from this experiment supports the suggestion that it is beneficial to administer aspirin before cancer diagnosis.

Examining the effect of aspirin or metformin on PC3 spheroid growth by dosing with once the spheroid had formed gave an indication of the effect of the drugs on proliferation in an existing tumour as it was also of interest to see what effect aspirin and metformin may have when taken after cancer diagnosis. Spheroids dosed with aspirin showed a dose-dependent decrease in spheroid area, which was significant from 1 mM onwards. While the reduction in spheroid area was apparent it was less of an effect than seen in the spheroid formation experiment. This may be because spheroids were only dosed once the spheroid had formed (after 1 week of growth) so it was harder for the drugs to penetrate the cells in the centre of the spheroid, allowing some cells to maintain their normal proliferation rate. Studies have shown that spheroids possess drug diffusion gradients which increase with the size of the spheroid and affect response to treatment (251). Despite this, in both models aspirin had a potent effect, greatly reducing spheroid area even at low concentrations, and support the use of aspirin both pre and post-diagnostically.

There are many papers which provide strong evidence of metformin's anti-cancer action both through *in vitro* work and population studies (252) (253). However, in the spheroid formation and spheroid growth experiments a change in spheroid area at clinically relevant concentrations of metformin, 30 µM or lower, was not observed. This was consistent with the 2D cell culture data where metformin also had no effect on cell proliferation at these concentrations.

Spheroids dosed with the combination of aspirin and metformin experienced a greater decrease in median spheroid area than when dosed with each drug alone indicating that metformin increases inhibition of spheroid growth by aspirin. This may mean that when metformin is used in combination with aspirin, lower doses of aspirin will be required. It is also encouraging that this model supports the PC3 2D culture data where an additive effect of the drugs was seen through ^{3}H- thymidine incorporation and cell counting.

When examining cell death it was seen that the spheroids potentially contain a necrotic core and an outer layer of live, proliferating cells. As previously mentioned spheroids display a diffusion gradient for drugs, but this is also true for oxygen, waste and nutrients which causes different depths of the spheroid to be in different nutritional states and the centre to become necrotic (254) (255). When the spheroids were dosed with either aspirin or metformin there was no visual change in cell death indicating that the reduction in spheroid area was due to inhibition of cell proliferation rather than increased cell death, consistent with the PC3 data from the 2D cell culture experiments (Chapter 3, Figures 3.5 and 3.19). The spheroids dosed with higher concentrations of aspirin, such as 4 mM, were much smaller in size and presumably because of their size did not contain a necrotic core. As mentioned previously spheroids with a radii of 200 µm or greater will have a zone of proliferation and then a quiescent or necrotic centre. In the cell death experiments PC3 spheroids were grown for 18 days and had an average spheroid radii of 297 µm. While this study examined cell death rather than apoptosis, in future experiments it would also be of interest to see if the cells in the centre are apoptotic with use of a caspase inhibitor such as Q-VD-OPh.

In this chapter it was seen that aspirin had a large effect on spheroid formation and growth, reducing median spheroid area at low concentrations. Conversely, metformin only caused a reduction in spheroid formation and growth at concentrations which were higher than those considered clinically relevant. Therefore, the next question was whether the drugs exert their anti-cancer effects through other phenotypes.

Chapter 5

The effect of aspirin and metformin on cell migration

5.1 Introduction

5.1.1 Cell migration and invasion in prostate cancer

Nearly all prostate cancer associated mortality occurs due to the metastasis of the cancer, with the most common secondary sites being the lymph nodes, bones, lungs and liver (256). In men with advanced prostate cancer, around 80 percent will have cancer that has spread to the bones (257). The bone microenvironment is favourable for prostate cancer metastasis due to constant cell turnover (258) and the expression of chemokines (i.e. CXCL12) which interact with chemokine receptors on the prostate cancer cells (i.e. CXRCR4) to mediate metastasis (259). Bone structure is normally maintained by a careful balance of bone matrix production by osteoblasts and bone resorption by osteoclasts. While many signalling molecules are involved in this process, the most important are receptor activator of nuclear factor-$_{K}$β ligand (RANKL), expressed by osteoblasts, and its receptor RANK, expressed by osteoclasts. Prostate cancer bone metastases destroy bone by releasing factors such as parathyroid hormone-related peptide to drive osteoblast production of RANKL, leading to increased bone resorption. Once in the bones the cancer can develop or spread to new sites. A study by *Gundem et al* reported that the metastasis of cancer from these secondary sites is a common feature of prostate cancer, introducing new mutations and creating future sub clones (260). This makes the cancer even more difficult to control as it increases the number of locations to where the cancer must be contained and the number of mutations that are present.

The process by which tumours metastasize involves multiple steps, with the cells migrating to the blood vessels, undergoing intravasation, surviving in the circulatory or lymphatic system, extravasation and colonizing a new site (261). This requires a change in the molecular phenotype of the tumour cells with the production of migration inducing proteins such as N-cadherin and the down regulation of epithelial proteins such as E-cadherin during EMT (Figure 5.1). Transcription factors are also extremely significant in the process of EMT, acting as molecular switches to regulate expression of genes, which are key to cell migration as well as the expression of each other. In particular, Slug, Snail, Twist-1 and Zeb-1 are often highlighted as major players, promoting migration by down regulating the abundance of E-cadherin and upregulating levels of N-cadherin (262). These factors are activated early in EMT and play fundamental roles in cancer, fibrosis and development (263). Intracellularly, E-cadherin binds to α-catenin, β-catenin and p120-catenin linking adherence junctions to the actin cytoskeleton.

For cell movement to occur, E-cadherin is downregulated by transcription factors such as Slug, Snail, Twist and ZEB which leads to translocation of membrane bound β-catenin to the nucleus where it controls the expression of numerous genes such as c-myc (264). This subsequently causes an upregulation of mesenchymal markers such as N-cadherin and vimentin and expression of N-cadherin mediates Rho-induced stress fibres to causes the rearrangement of the cytoskeleton. The formation of lamellipodia due to Rac1 and Cdc42 activation mediate cell movement. Matrix metalloproteinases (MMPs) and integrins also play an important role in cell migration. MMPs cause extracellular matrix remodelling during development, inflammation and wound healing by degrading the basement membrane and modifying expression of cell adhesion molecules (265). Integrins are transmembrane receptors which bind cells to the extracellular matrix and mediate signals between the two. They also cause extracellular matrix remodelling through regulation of localization and activity of proteases (266). Both MMPs and integrins have been shown to be upregulated in cancer (267) (268).

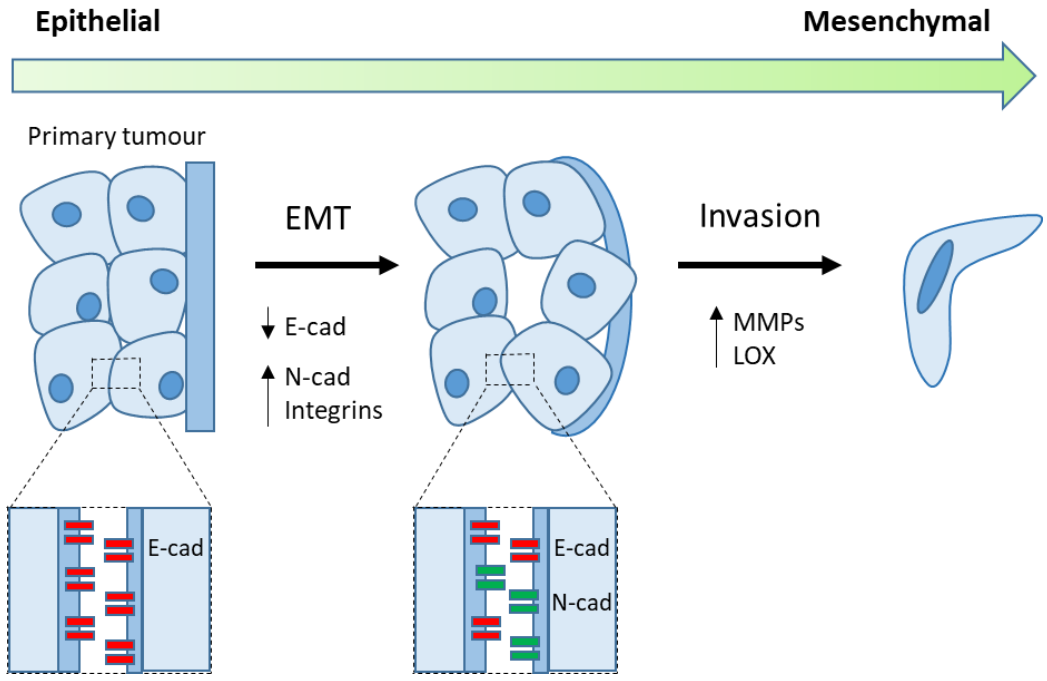


Figure 5.1: The epithelial to mesenchymal transition.
Epithelial cells have high levels of E-cadherin, anchoring them to neighbouring cells. Cancer cells often become more mesenchymal with a down regulation of E-cadherin and an upregulation of proteins such as N-cadherin, integrins and matrix metalloproteinases (MMPs). This allows them to migrate away from the primary tumour, invade and grow in surrounding areas.

While surgery, radiotherapy, hormone therapy and chemotherapy work well with localized prostate cancer, advanced prostate cancer is much more difficult to treat as it is impossible to remove all the cancer with surgery or to control its spread with radio and chemotherapies. Furthermore, as prostate cancer develops the cells often become androgen independent which causes hormone deprivation therapy to become ineffective and confers a growth advantage to the cells. Despite the obvious importance of metastasis in prostate cancer development, only a few studies to date have examined the impact of aspirin or metformin on prostate cancer cell migration. Furthermore, no studies have examined the effect of combining both drugs on cell migration in this cancer. There is a need for treatments, which slow down the progression of prostate cancer, providing time for clinicians to diagnose the disease at an earlier stage, for treatments to take effect and to delay the need for more aggressive therapies.

5.2 Hypotheses and aims

In this chapter, the effects of aspirin and metformin on prostate cancer cell migration are described.

Hypothesis: Based on the results from Chapter 3 and 4 which show that metformin increases the efficacy of aspirin in reducing prostate cancer cell proliferation, it is hypothesised that metformin will also increase the efficacy of aspirin in reducing the migratory capacity of prostate cancer cells and promote a more epithelial phenotype.

Aims and objectives:

1. To determine the effect of aspirin and metformin alone and in combination on cell migration.
2. To examine the effect of aspirin and metformin alone and in combination on gene and protein expression of markers of EMT.

5.3 Materials and methods

5.3.1 Seeding and dosing cells for the wound healing assay, western blotting and qPCR

For the wound healing assay, western blotting and qPCR cells were seeded in T25 flasks as described in Chapter 2, Section 2.3.1. Aspirin and metformin were reconstituted as described in Chapter 2, Section 2.2 and cells were dosed for 96 hours with 0, 10, 20, 30 and 1000 μM metformin and 0, 0.25, 0.5, 1, 2 and 4 mM aspirin with the media being collected and replaced every 24 hours. At the end of 96 hours the wound healing assay was performed as described in Chapter 2, Section 2.5, western blotting as described in Chapter 2, Section 2.4 and qPCR as described in Chapter 2, Section 2.6. Western blotting examined markers of epithelial to mesenchymal transition (EMT), including E-cadherin and N-cadherin while qPCR examined mRNA levels of E-cadherin, N-cadherin, slug and MMP9. Statistical analysis was performed as described in Chapter 2, Section 2.8.

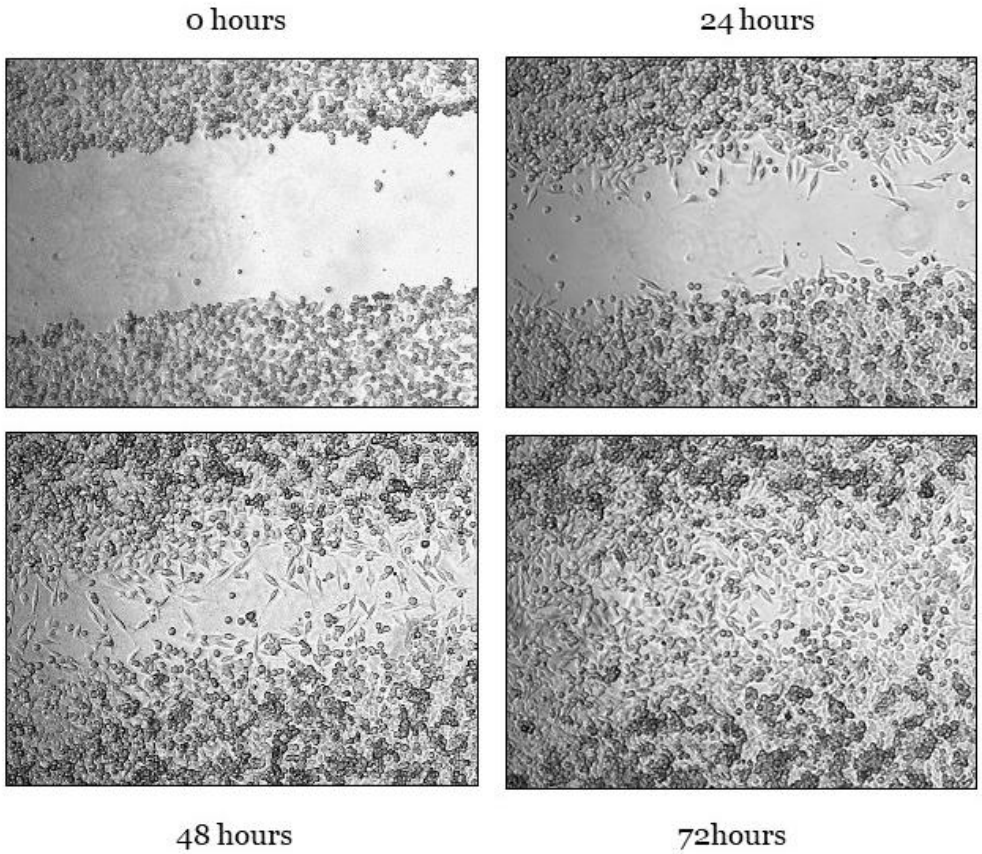
5.4 Results

5.4.1 Characterisation of PC3 and DU145 cell movement

As it was seen in Chapter 4 that the results from the 3D proliferation assays supported the 2D proliferation data, the experiments to examine the effects of the drugs on cell migration were performed in 2D. First, to investigate the cells ability to migrate, a wound healing assay was performed. This allowed real time analysis of cell movement, taking images every 10 minutes over 72 hours. The PC3 and DU145 cell lines were chosen as they are both migratory whereas the LNCaP and PNT2 cells are not. The DU145 and PC3 cells were pretreated with the proliferation inhibitor mitomycin C to prevent cell proliferation, as both cell lines proliferate at a different rate, to ensure that only changes in cell motility was measured. The PC3 cells moved individually with some cells being more motile than others and moving back and forwards between both sides of the gap (Figure 5.2 a). The cells moved at a steady rate, and after 72 hours the gap was fully closed. Unlike the PC3 cells, the DU145 cells moved together as a sheet indicating that they require close contact with other cells for movement (Figure 5.2 b).

a.

PC3



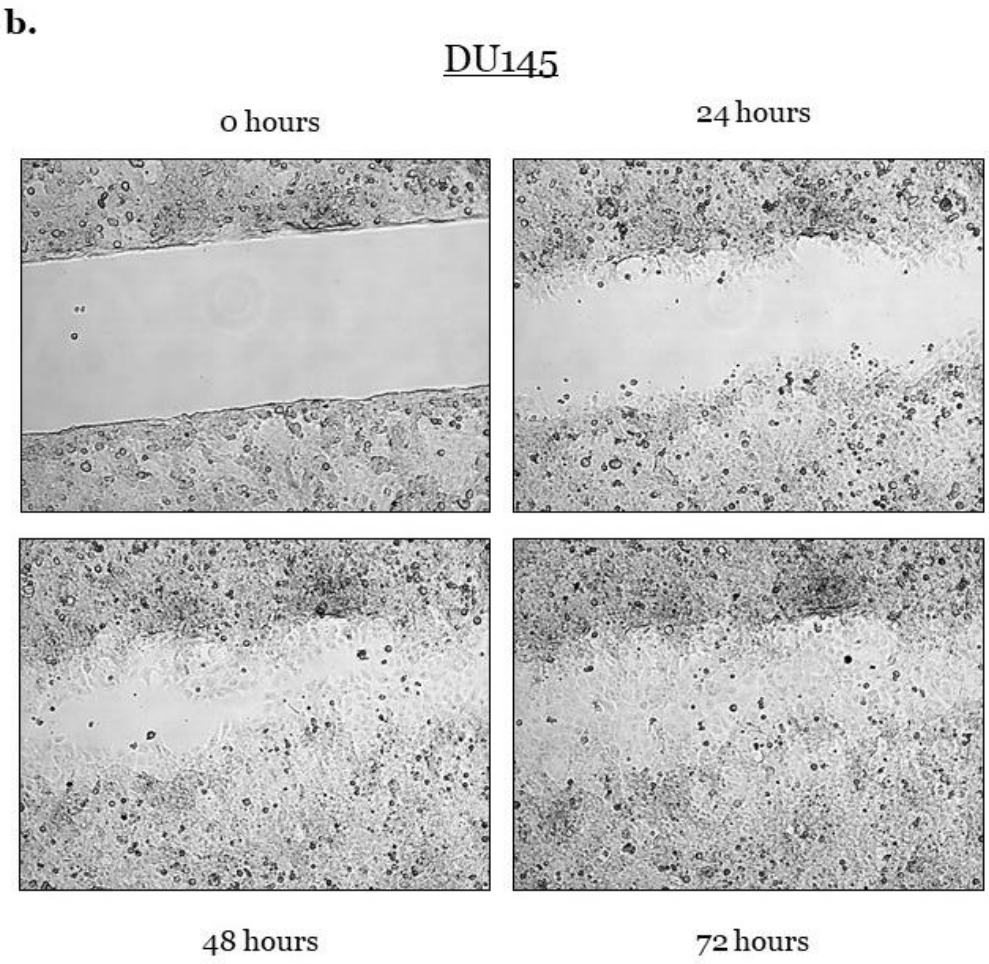


Figure 5.2: Migration of PC3 and DU145 cells.
(a) PC3 and **(b)** DU145 cells were seeded with a proliferation inhibitor, mitomycin C (20 µg/ml), and imaged every 10 minutes to examine cell migration over 72 hours.

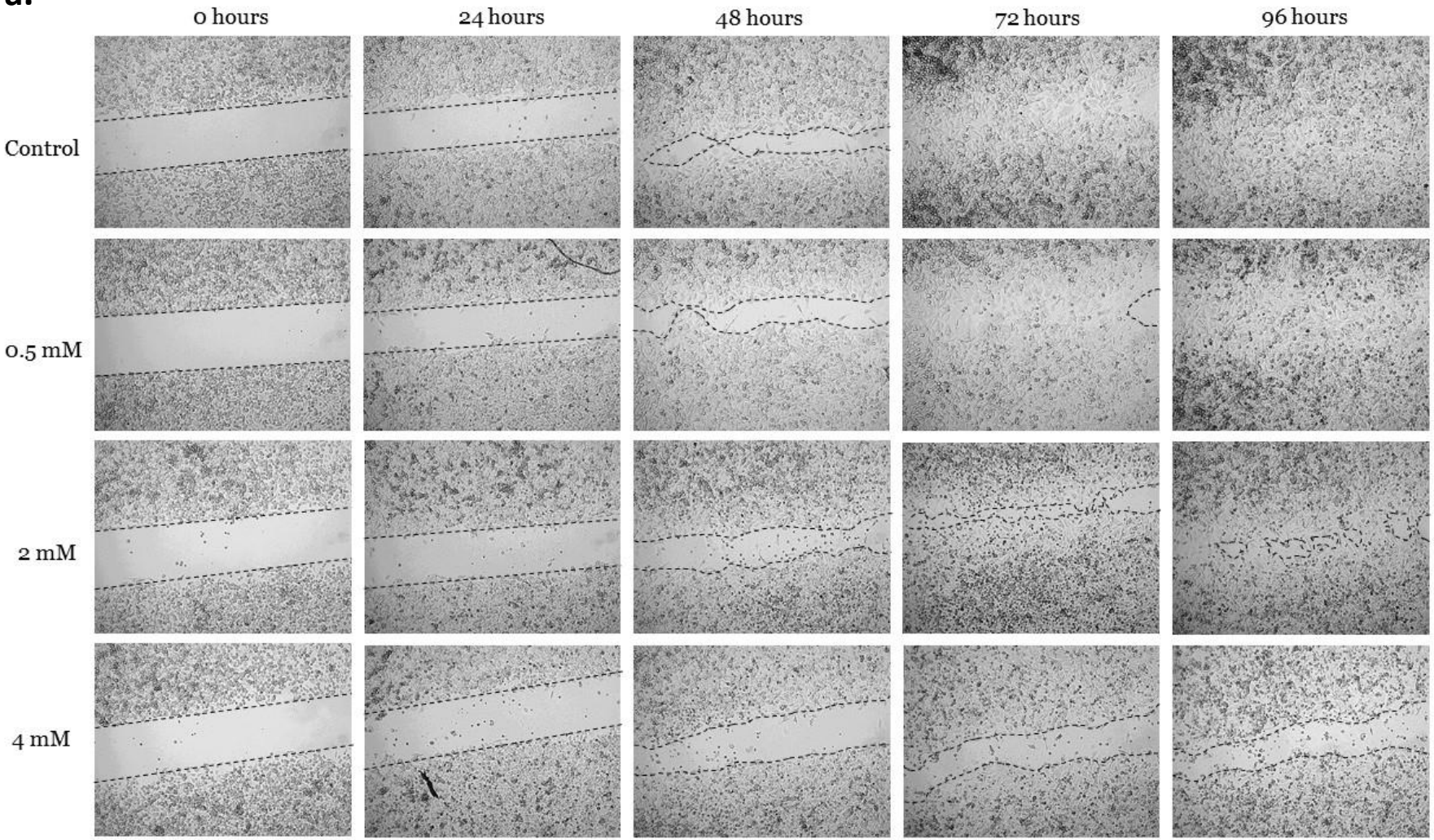
For a full video of PC3 cell migration see https://streamable.com/na5lm. For a full video of DU145 cell migration see https://streamable.com/kaebi.

5.4.2 Aspirin dose dependently inhibits cell migration

After it was determined whether the cells moved individually or through collective cell migration, the effect of the drugs on cell movement was examined. For this, the PC3 cell line was chosen as it is the most advanced of the three prostate cancer cell lines and is the most motile. In the previous experiment is was seen that PC3s cells utilised single cell migration and were more metastatic that the DU145s. Additionally, this cell line took on a spikey morphology when grown in 3D cell culture, indicative of a highly metastatic cell line. PC3 cells were pre-treated with aspirin, metformin or a combination of the two drugs every 24 hours for a total of 96 hours and then were assessed in the wound healing assay.

Cells pre-treated with aspirin (0.5, 2 and 4 mM) migrated more slowly than the control, in a dose-dependent manner (Figure 5.3). While the gap completely closed after 72 hours for the control cells, it took 96 hours when cells were treated with 0.5 mM aspirin and the cells were unable to close the gap after the 96 hours when dosed with either 2 or 4 mM aspirin. The largest difference in gap area was observed at 60 hours of imaging, with a difference of 14% ($p=0.045$), 16% ($p=0.04$) and 19% ($p=0.04$) in gap area for cells dosed with 0.5, 2 and 4 mM aspirin compared to the control respectively. Dosing the cells with the highest concentration of aspirin, 4 mM, had the greatest effect and caused a significant difference in gap area at 36, 72, 84 and 96 hours of imaging ($p=0.048$, 0.01, 0.046, 0.049 respectively).

a.


0 hours
24 hours
48 hours
72 hours
96 hours
Control
0.5 mM
2 mM
4 mM

b.

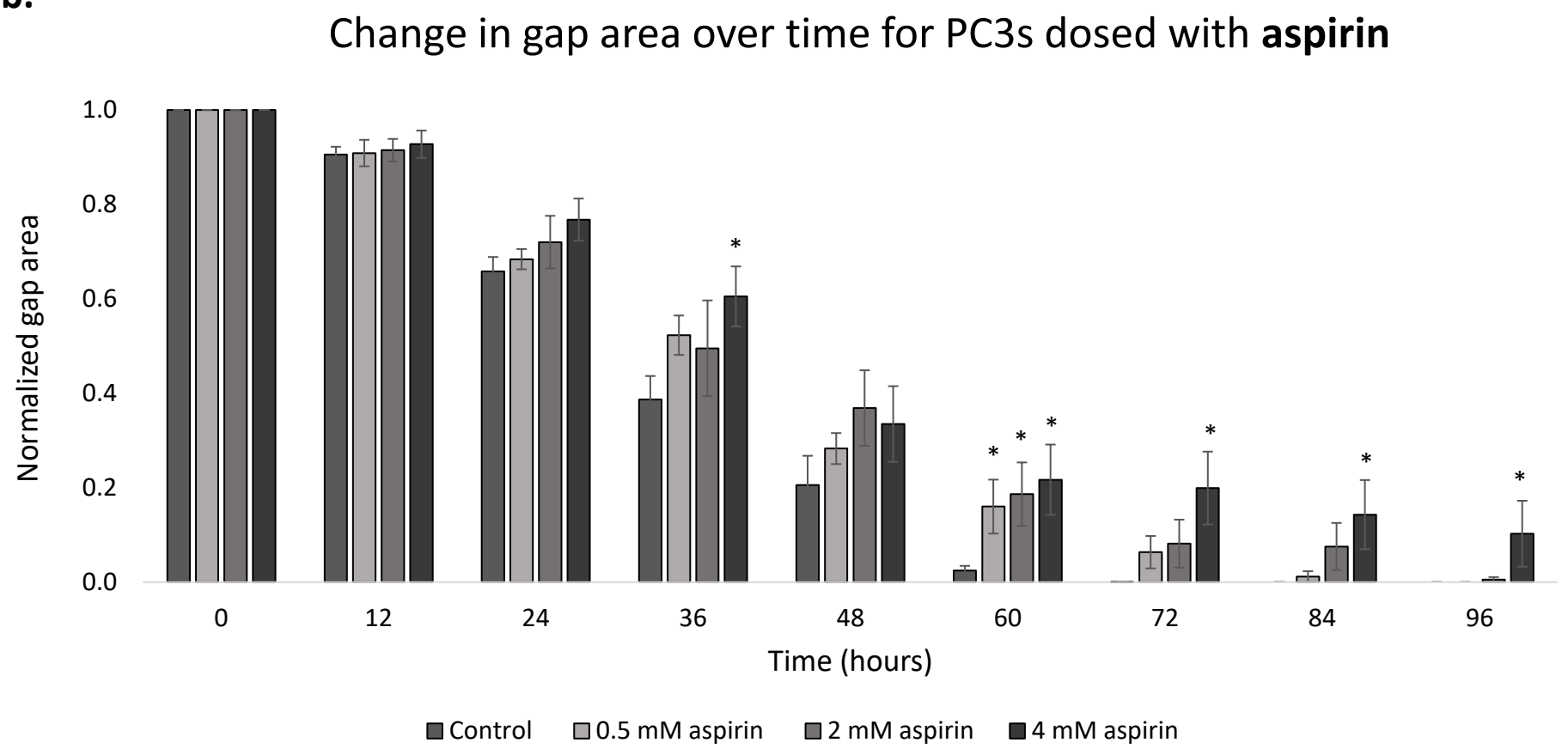
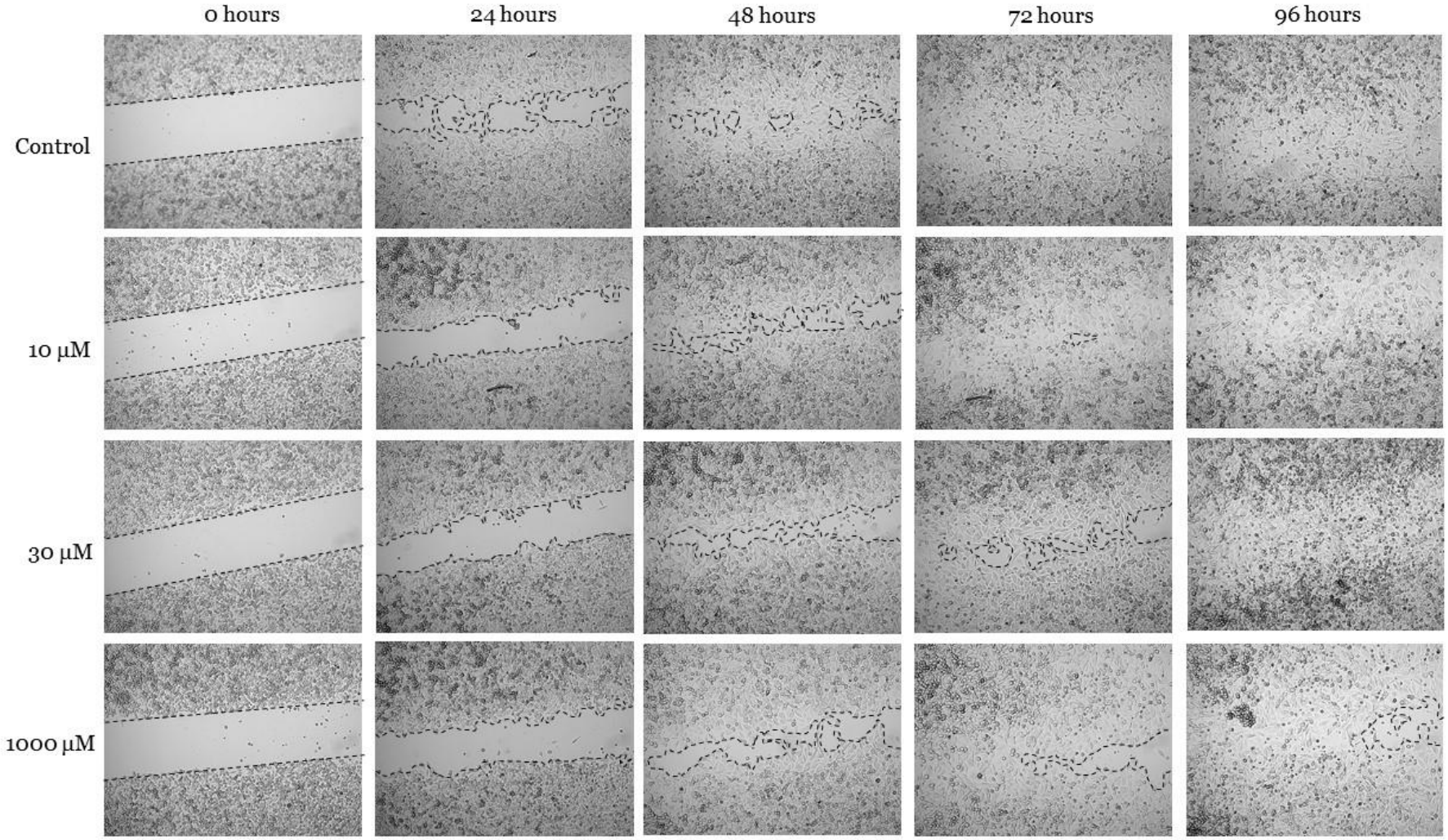


Figure 5.3: Wound healing assay for PC3 cells pre-treated with aspirin.
(a) PC3 cells were pre-dosed with aspirin for 96 hours and then seeded in an Ibidi insert to perform a wound healing assay. Mitomycin C, 20 µg/ml, was used as a proliferation inhibitor. The gap was imaged every 12 hours to examine cell migration over 96 hours and gap area was calculated using ImageJ. **(b)** Change in the area of the gap over time. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA each time point and Dunnett's post hoc test. Where there is an asterisk it is the condition compared to the control of that timepoint. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data represents three independent experiments performed in triplicate.

5.4.3 Metformin dose-dependently inhibits cell migration

As with aspirin, when the PC3 cells were pre-treated for 96 hours with metformin (10, 30 and 1000 µM) cell migration was reduced dose-dependently (Figure 5.4). Compared to the 72 hours it took for the control cells to close the gap, it took those dosed with 10 µM metformin 84 hours while cells dosed with 30 or 1000 µM metformin did not close the gap within the 96 hours of imaging. This was an important result, indicating that whilst clinically relevant concentrations of metformin (30 µM) has no effect on cell growth or survival (Chapters 3 and 4) it does dramatically inhibit cell migration, a fundamental aspect of prostate cancer development. The difference in gap area for 30 µM metformin compared to the control was significant at 36 hours ($p = 0.008$) and 60 hours ($p= 0.001$). The difference in gap area for cells treated with 1000 µM metformin was more obvious and was significant from 24 hours onwards ($p= 0.02, 0.008, 0.04, 0.002, 0.008, 0.002$ and 0.049 for 24-96 hours compared to the control). Similar to aspirin, the biggest difference in gap area for pre-dosed cells was at 60 hours, with a difference of 9%, 19% and 20% in gap area for 10, 30 and 1000 µM metformin compared to the control respectively.

a.


0 hours
24 hours
48 hours
72 hours
96 hours
Control
10 µM
30 µM
1000 µM

b.

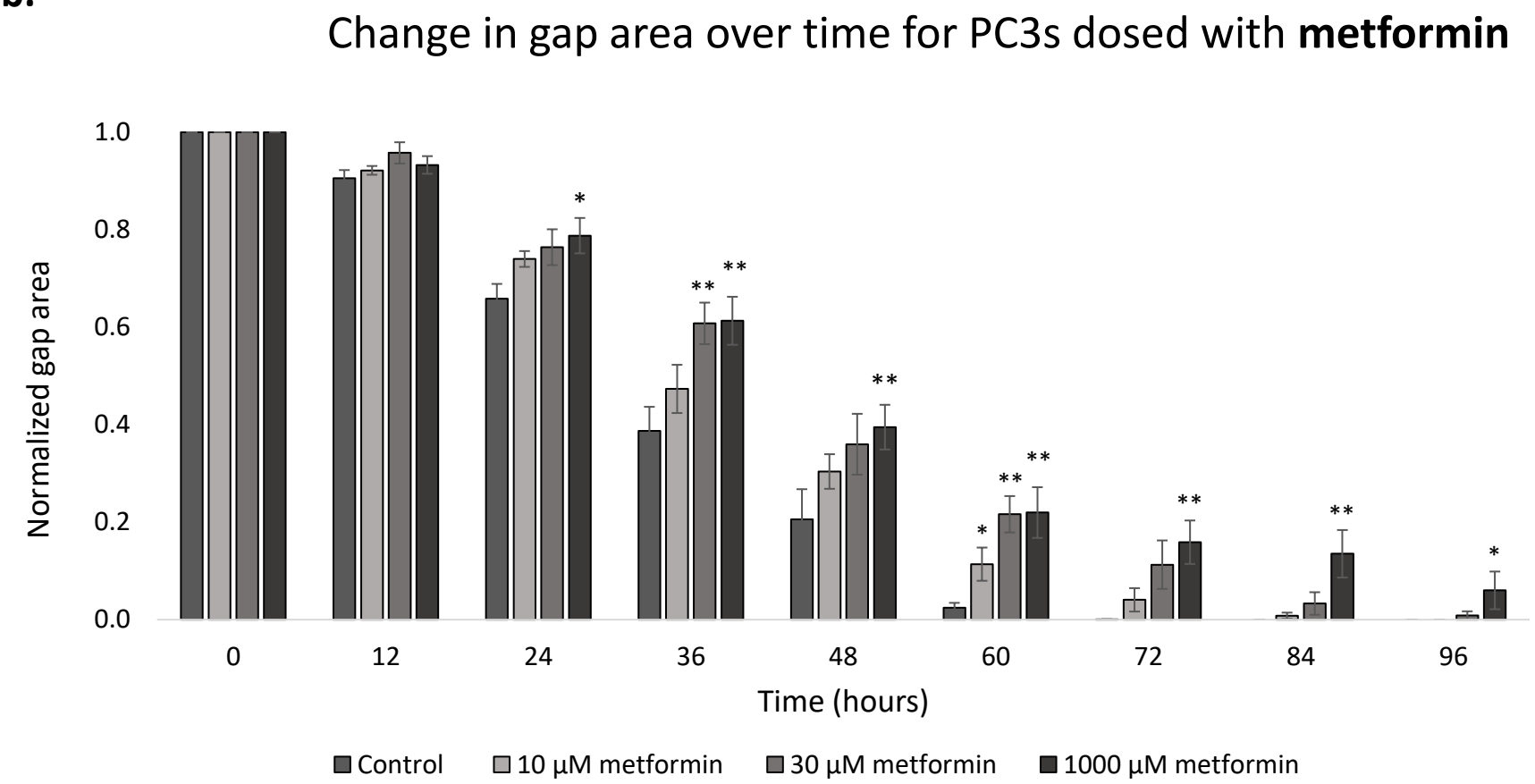
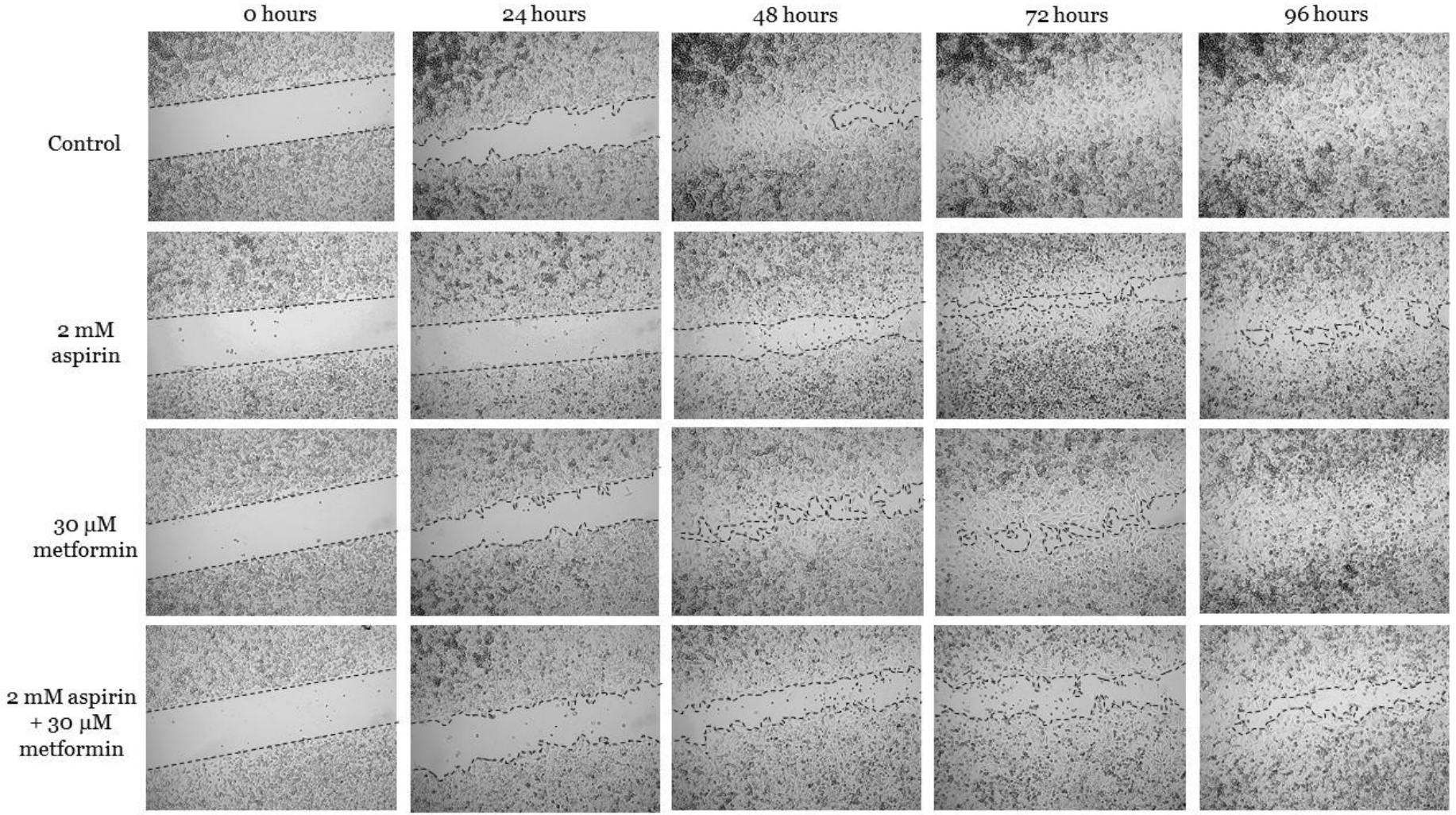


Figure 5.4: Wound healing assay for PC3 cells pre-dosed with metformin.
(a) PC3 cells were pre-dosed with metformin for 96 hours and then seeded in an Ibidi insert to perform a wound healing assay. Mitomycin C, 20 µg/ml, was used as a proliferation inhibitor. The gap was imaged every 12 hours to examine cell migration over 96 hours and gap area was calculated using ImageJ. **(b)** Change in the area of the gap over time. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA at each time point and Dunnett's post hoc test. Where there is an asterisk it is the condition compared to the control of that timepoint. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data represents three independent experiments performed in triplicate.

5.4.4 The combination of aspirin and metformin causes the greatest reduction in cell migration

When the PC3 cells were pre-treated with a combination of both drugs, 2 mM aspirin and 30 µM metformin, cell migration was reduced more than by dosing with each drug alone. At the end of 96 hours of imaging, cells dosed with the combination of both drugs did not close the gap (Figure 5.5). There was a significant difference in gap area for the combination compared to the control from 24 hours of imaging onwards ($p= 0.04, 0.006, 0.04, 0.002, 0.045, 0.049$ and 0.03 for 24 to 96 hrs respectively). Whilst the combination was not significantly different from dosing with aspirin or metformin alone it was observed that metformin inhibited cell migration more quickly than aspirin and that the combination of both drugs was more effective, as seen by a significant difference in gap area compared to the control at 24 hours. This may indicate that using aspirin and metformin together could have the greatest potential to inhibit cell migration.

a.


0 hours
24 hours
48 hours
72 hours
96 hours
Control
2 mM aspirin
30 μM metformin
2 mM aspirin + 30 μM metformin

b.

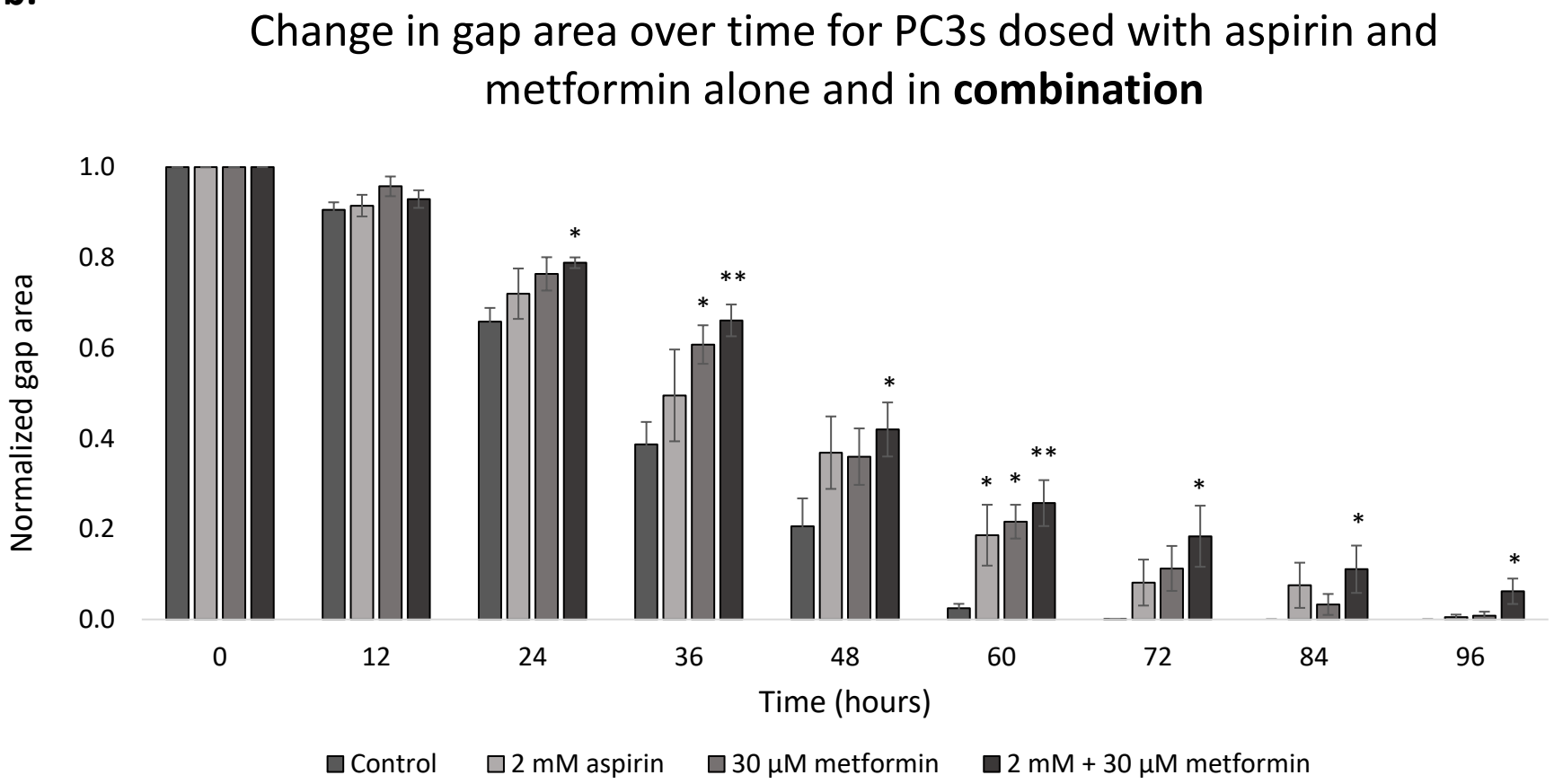


Figure 5.5: Wound healing assay for PC3 cells pre-dosed with aspirin and metformin alone or in combination together.
(a) PC3 cells were pre-dosed with either aspirin, metformin or a combination of both drugs for 96 hours and then seeded in an Ibidi insert to perform a wound healing assay. Mitomycin C, 20 µg/ml, was used as a proliferation inhibitor. The gap was imaged every 12 hours to examine cell migration over 96 hours and gap area was calculated using ImageJ. **(b)** Change in the area of the gap over time. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA at each time point and Tukey's post hoc test for multiple comparisons. Where there is an asterisk it is the condition compared to the control of that timepoint. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data represents three independent experiments performed in triplicate.

5.4.5 Alterations in markers of cell migration following aspirin and metformin treatment

As the wound healing experiment identified that both aspirin and metformin reduce the migratory capacity of the cells, western blotting was performed to examine protein expression of markers of cell migration and invasion. A panel of four prostate cell lines was used to determine the differential abundance of epithelial-cadherin (E-cadherin) and neural-cadherin (N-cadherin) (Figure 5.6). These proteins are some of the most commonly used markers of cell migration and invasion (269) and as both drugs affected the individual cell migration of PC3 cells in the previous experiment it was assumed that they may be mediated via the expression of molecules involved in cell to cell junctions. As expected, the most mesenchymal cell line, PC3, had the lowest expression of E-cadherin and was the only cell line to express N-cadherin. The cancerous DU145 and LNCaP cell lines as well as the non-tumorigenic PNT2 cell line all expressed E-cadherin but not N-cadherin. The LNCaP and PNT2 cells expressed two bands for E-cadherin, which are reported to be the immature (130 kda) and the mature forms of the E-cadherin protein (120 kda). The two bands are only observed in the more epithelial cell lines when there is a high level of the protein, however, it is not always seen (270) (271). The lower band is used to determine E-cadherin expression as determined from previous experiments of the IMEG research group.

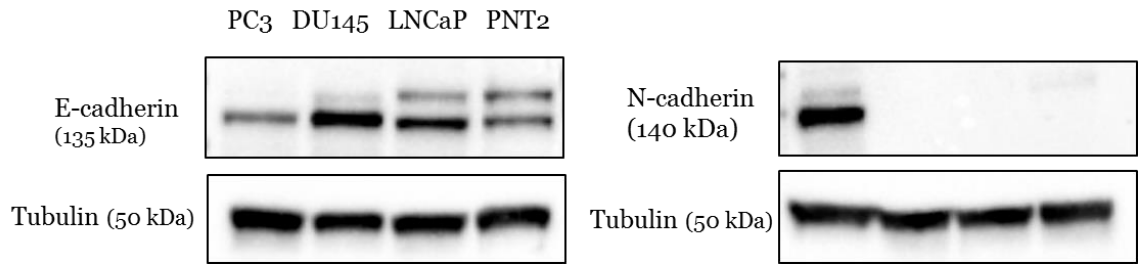


Figure 5.6: E-cadherin and N-cadherin expression in prostate cancer and epithelial cell lines.
Expression of E-cadherin (135 kDa) and N-cadherin (140 kDa) in PC3, DU145, LNCaP and PNT2 cell lines. The housekeeping gene tubulin was used as a loading control.

5.4.5.1 Aspirin and metformin increase E-cadherin expression in the PC3 cell line

Dosing the PC3 cell line with aspirin (0.25-4 mM) caused an increase in the abundance of E-cadherin in a dose-dependent manner (Figure 5.7). There was a significant increase from 1 mM aspirin onwards, p= 0.03, 0.04 and 0.04 for 1 to 4 mM respectively. For N-cadherin, the changes in protein levels varied and a significant change was not observed (p >0.05).

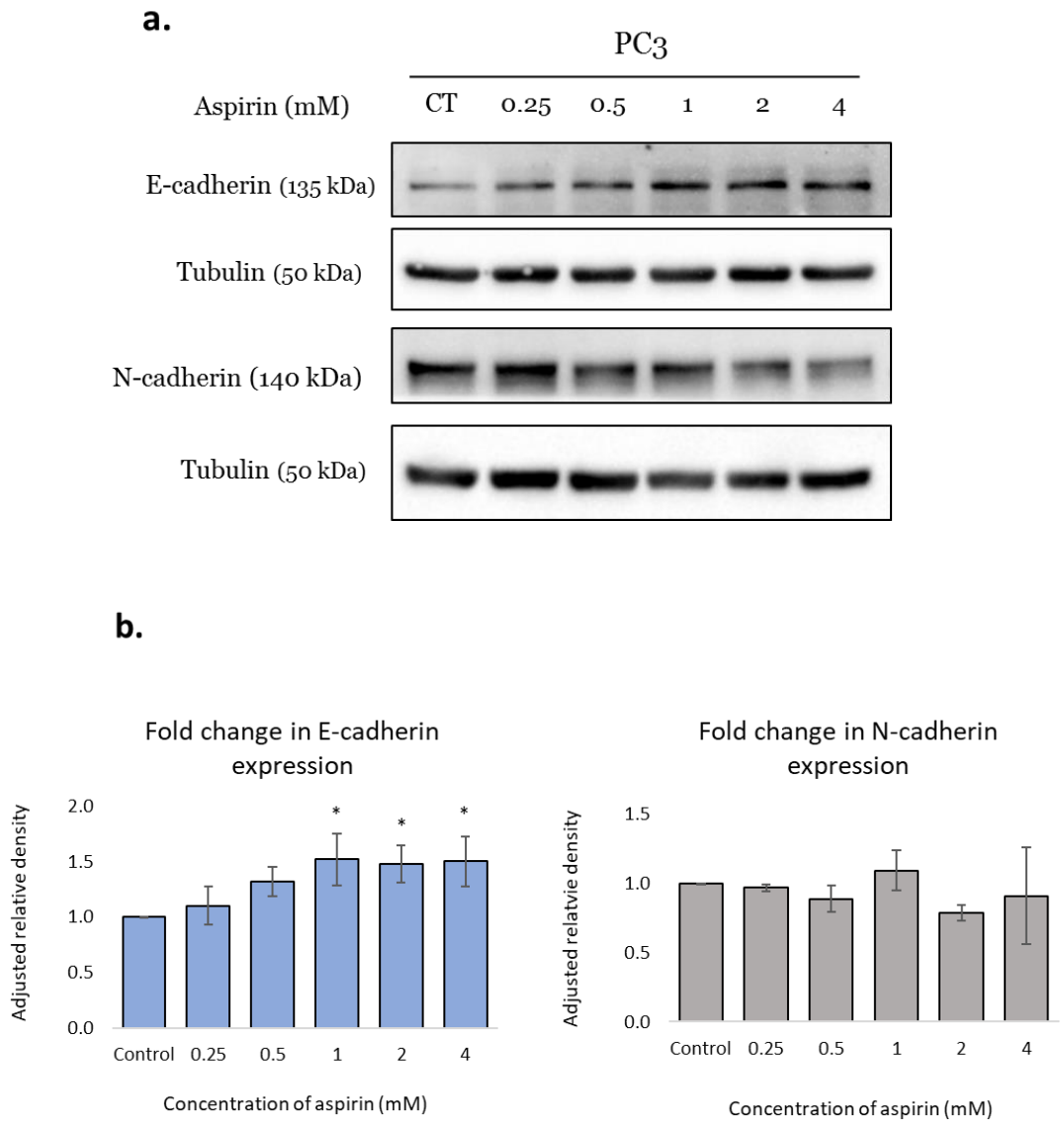


Figure 5.7: E-cadherin and N-cadherin levels in PC3 cells dosed with aspirin.
(a) Abundance of markers of cell migration, E-cadherin and N-cadherin, in PC3 cells dosed with aspirin every 24 hours for a total of 96 hours. **(b)** Densitometry was performed to quantify protein levels. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA and Dunnett's post hoc test. * p <0.05, ** p <0.01, *** p <0.001. Data represents three independents.

When the PC3 cell line was dosed with metformin (10-1000 µM) there was a dose-dependent increase in E-cadherin and decrease in N-cadherin (Figure 5.8). The increase in E-cadherin was significant from 30 µM metformin (p= 0.03, 0.01 and 0.01 for 30, 100 and 1000 µM respectively) while the decrease in N-cadherin occurred at a lower dose, with a significant decrease from 20 µM metformin onwards (p= 0.03, 0.03, 0.03 and 0.01 for 20 to 1000 µM metformin respectively) indicating that metformin promotes a more epithelial-like phenotype.

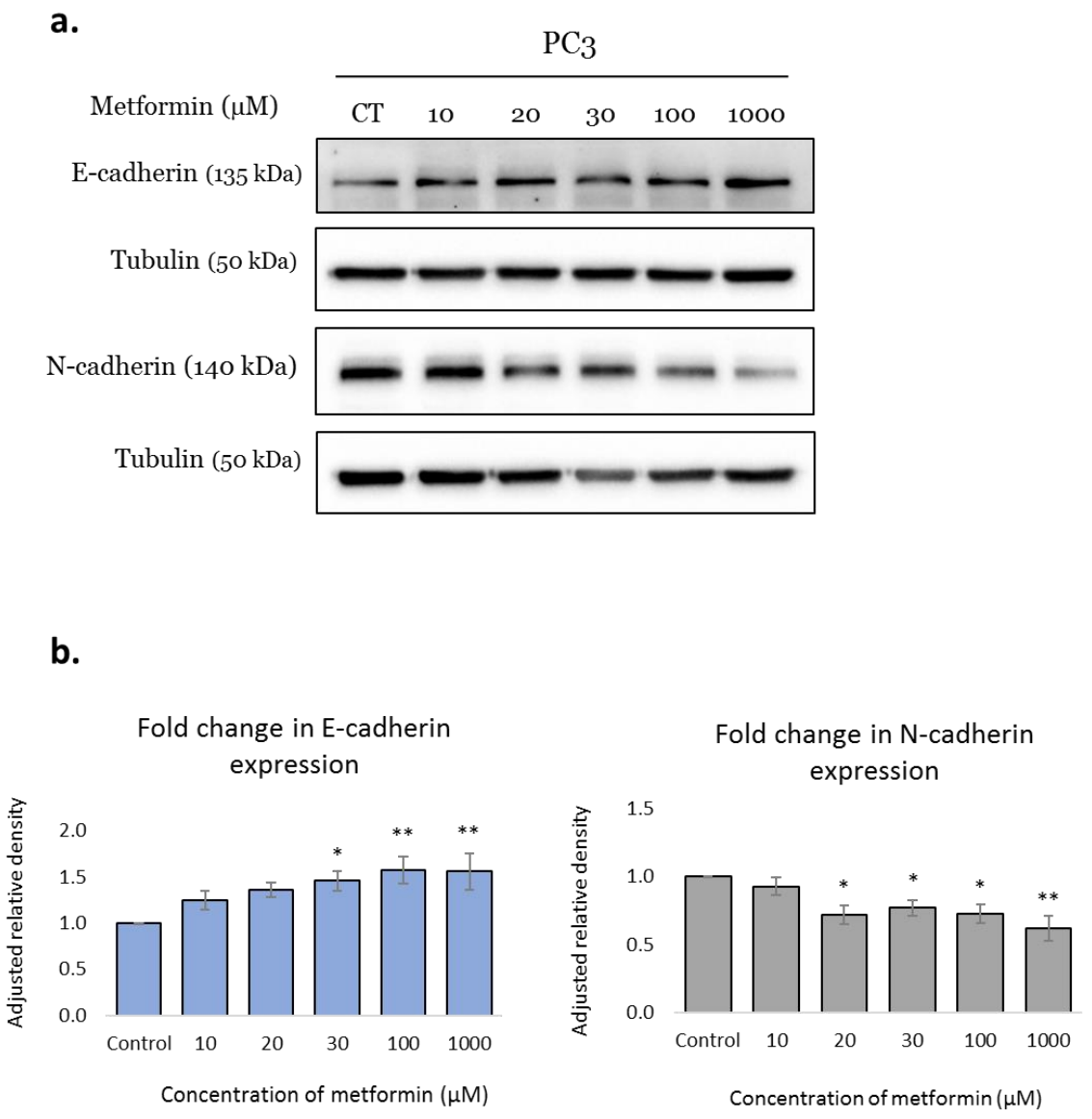


Figure 5.8: E-cadherin and N-cadherin levels in PC3 cells dosed with metformin.
a) Protein levels of markers of cell migration, E-cadherin and N-cadherin, in PC3 cells dosed with metformin every 24 hours for a total of 96 hours. **(b)** Densitometry was performed to quantify protein levels. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA and Dunnett's post hoc test. * p <0.05, ** p <0.01, *** p <0.001. Data represents three independent experiments.

Dosing the PC3 cell line with a combination of aspirin (2 mM) and metformin (30 µM) caused the greatest increase in E-cadherin expression (p=0.008) and decrease in N-cadherin expression (p= 0.04) (Figure 5.9). However, as seen with the wound healing experiment, this was not significantly different to dosing with the drugs alone.

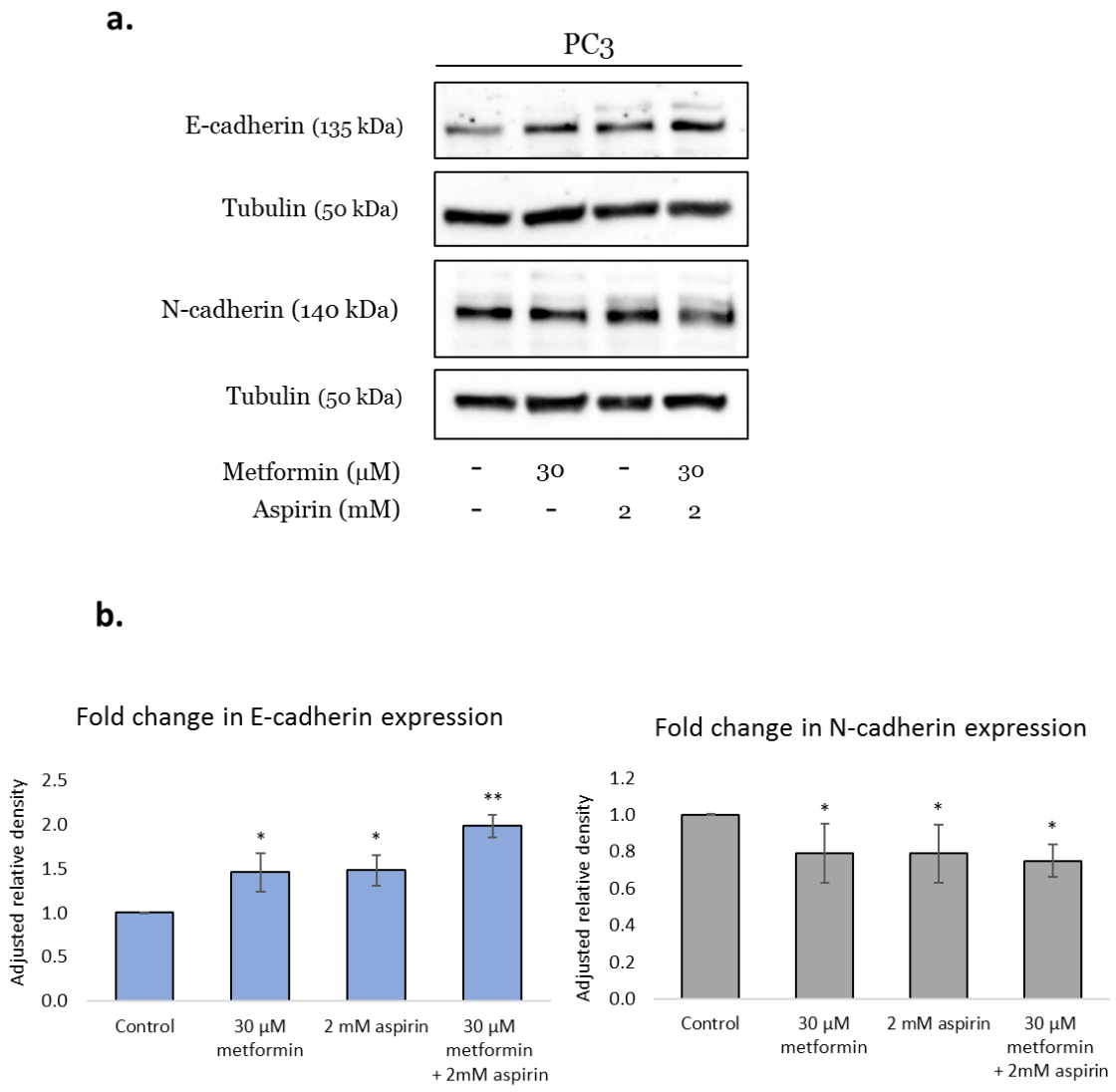


Figure 5.9: E-cadherin and N-cadherin abundance in PC3 cells dosed with a combination of aspirin and metformin.
(a) Levels of markers of cell migration, E-cadherin and N-cadherin, in PC3 cells dosed with either aspirin or metformin alone and in combination every 24 hours for a total of 96 hours. **(b)** Densitometry was performed to quantify protein levels. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA and Tukey's test for multiple comparisons. * p <0.05, ** p <0.01, *** p <0.001. Data represents three independent experiments.

5.4.5.2 Aspirin and metformin increase E-cadherin mRNA levels and decrease N-cadherin mRNA levels in the PC3 cell line.

qPCR was performed to examine mRNA levels of E-cadherin and N-cadherin when PC3 cells were dosed with aspirin, metformin or a combination of the two drugs. This, along with the western blotting data determined whether the drugs alter expression of these proteins pre or post translationally. Melt curves were graphed for each gene of interest to confirm amplification of a specific product (Figure 5.10).

For E-cadherin, a dose-dependent increase in mRNA levels was observed when the cells were dosed with aspirin, reaching a maximum fold change of 2.85 for 4 mM aspirin (p=0.01) (Figure 5.11 a). While there also appeared to be a dose-dependent increase in E-cadherin mRNA levels when cells were dosed with metformin this was only significant at 1000 µM, reaching a maximum fold change of 2.38 (p =0.04) (Figure 5.11 b). Conversely, a dose-dependent decrease in N-cadherin was observed when cells were dosed with aspirin (Figure 5.11 d), decreasing average expression by over 50% at 4 mM aspirin (p=0.01). A large decrease in N-cadherin of around 66% average mRNA levels was observed when PC3 cells were dosed with metformin which did not appear to be concentration dependent (p=0.01, 0.01 and <0.01 for 10, 30 and 1000 µM respectively) (Figure 5.11 e). As seen with western blotting, the combination of aspirin (2 mM) and metformin (30 µM) caused the largest increase in E-cadherin but was not significantly different to dosing with each drug alone (Figure 5.11 c). The combination was also no different to dosing with each drug alone for N-cadherin (Figure 5.11 f). Therefore, the qPCR data indicated that 2 mM aspirin altered mRNA levels of both E-cadherin and N-cadherin which consequently affected their downstream protein expression (Figure 5.7). The qPCR for 30µM metformin, indicated that metformin decreases N-cadherin mRNA but not E-cadherin and so the increase observed in western blotting suggests post-translational regulation (Figure 5.8).

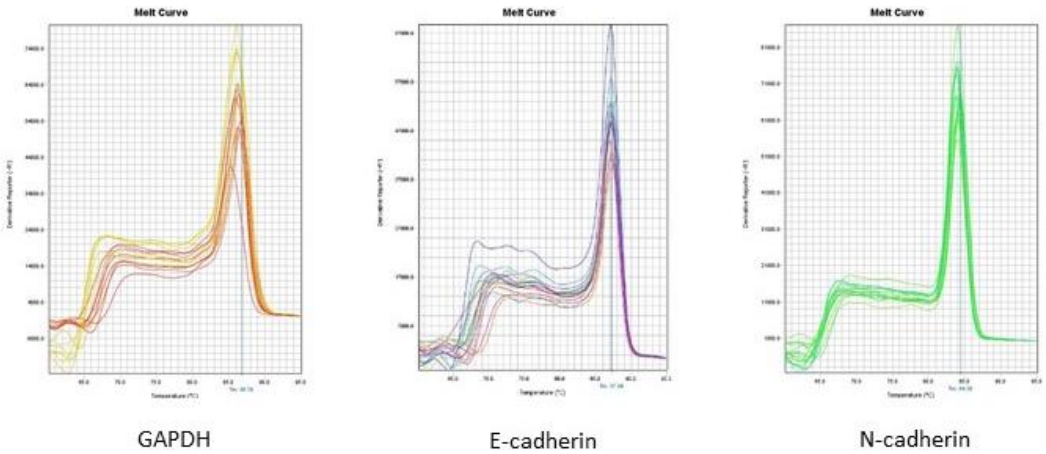


Figure 5.10: qPCR melt curves for GAPDH, E-cadherin and N-cadherin.
Melt curves show a single peak for each gene of interest and confirm amplification of a specific product.

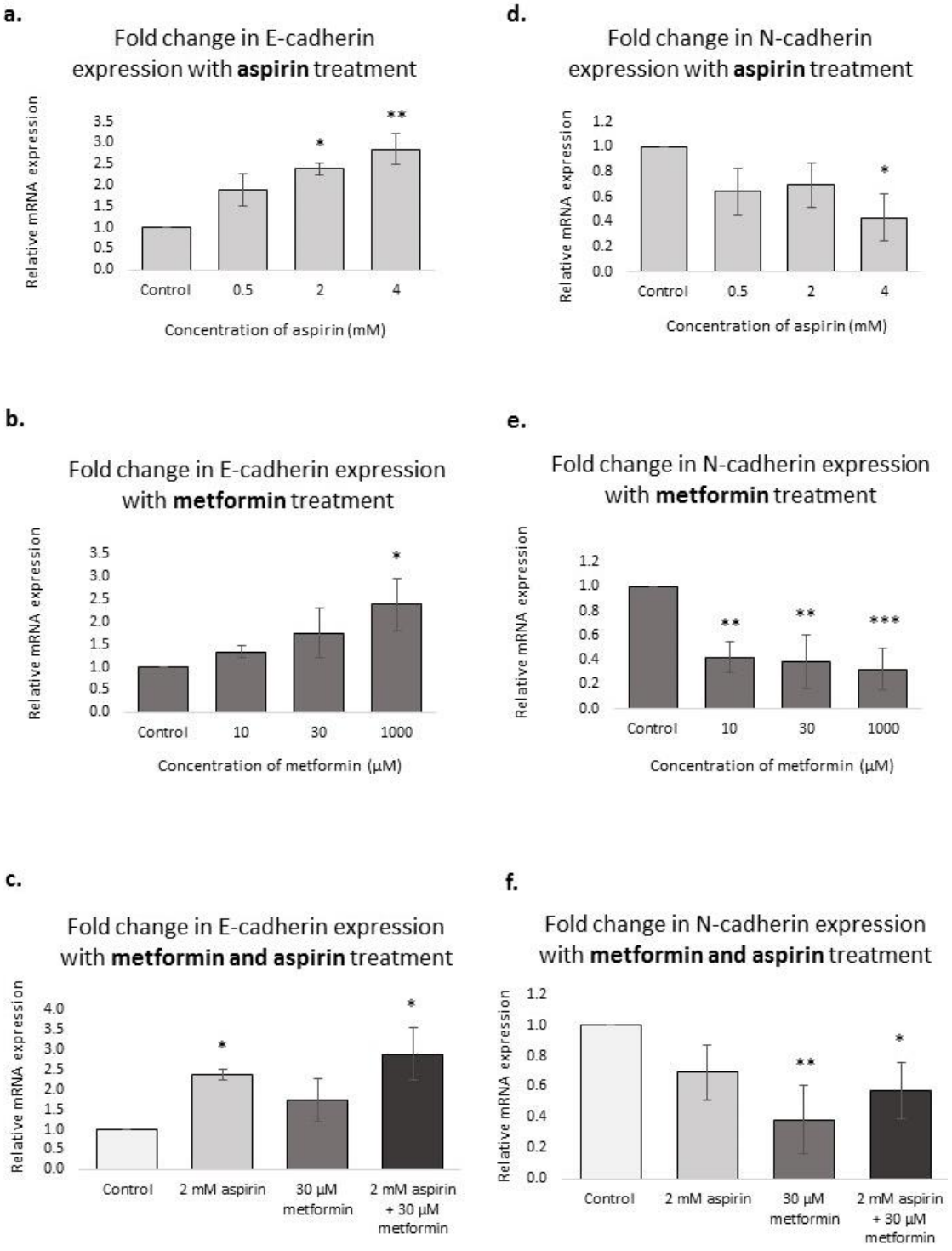


Figure 5.11: E-cadherin and N-cadherin mRNA levels in PC3 cells determined by qPCR.
PC3 cells were dosed every 24 hours for a total of 96 hours with **(a, d)** aspirin **(b, e)** metformin or **(c, f)** aspirin and metformin in combination together. E-cadherin **(a, b, c)** and N-cadherin **(d, e, f)** were normalized to the housekeeping gene GAPDH. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA and either Dunnett's **(a, b, d, e)** or Tukey's **(c, f)** post hoc tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data represents three independent experiments performed in triplicate.

5.4.5.3 Aspirin and metformin decrease MMP-9 and Slug mRNA levels in the PC3 cell line

As aspirin and metformin caused both pre and post translational modifications to N-cadherin and E-cadherin, qPCR for matrix metalloproteinase-9 (MMP-9) and the transcription factor SNAI2 (Slug) was also performed to examine the effect of the drugs on other markers of EMT. Melt curves were graphed for each gene of interest (Figure 5.12).

When PC3 cells were dosed with either aspirin or metformin a dose-dependent decrease in both MMP-9 and Slug mRNA expression was observed also indicating that the cells become more epithelial and less migratory (Figure 5.13). For MMP-9 the reduction in gene expression was significant even at the lowest concentrations of the drugs, reducing average expression by 35% at 0.5 mM aspirin ($p= 0.005$) and 57% at 10 µM metformin ($p<0.001$). The reduction in expression increased with increasing drug concentration, reducing MMP-9 by 70% at 4 mM aspirin ($p<0.001$) and 80% at 1000 µM metformin ($p<0.001$). Similarly, Slug expression decreased at all concentrations tested, with a reduction of 32% at 0.5 mM aspirin ($p= 0.003$) and 55% at 10 µM metformin ($p= 0.004$). Slug expression reached a maximum decrease in average expression of 46% at 4 mM aspirin ($p<0.001$). As seen with N-cadherin, a concentration dependent decrease in Slug was not observed when the cells were dosed with metformin. However, it is important to note that metformin was highly effective at suppressing MMP-9 and Slug even at very low concentrations (10µM) which is important for treatment. Dosing with a combination of aspirin (2 mM) and metformin (30 µM) caused a large decrease in MMP-9 and Slug mRNA levels but this was no different to dosing with each drug alone, supporting qPCR for E-cadherin and N-cadherin and the western blotting data.

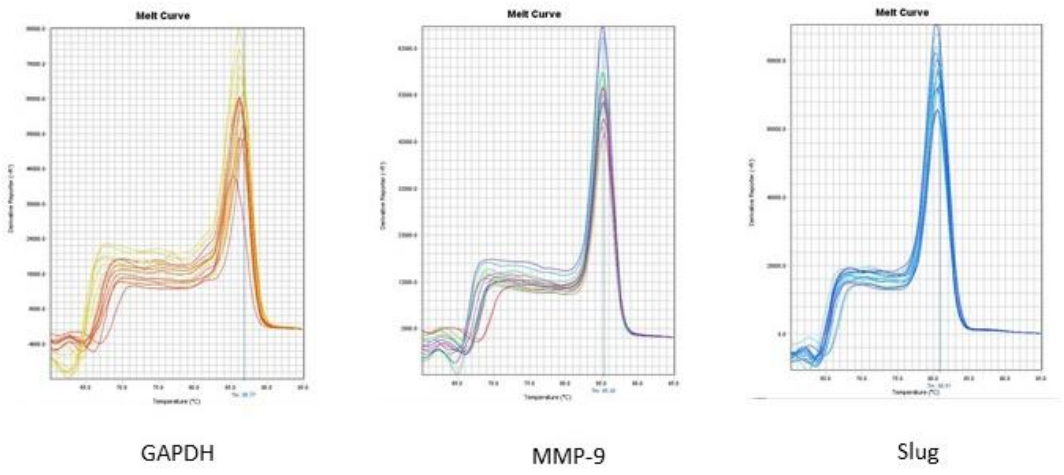


Figure 5.12: qPCR melt curves for GAPDH, MMP-9 and Slug.
Melt curves show a single peak for each gene of interest and confirm amplification of a specific product.

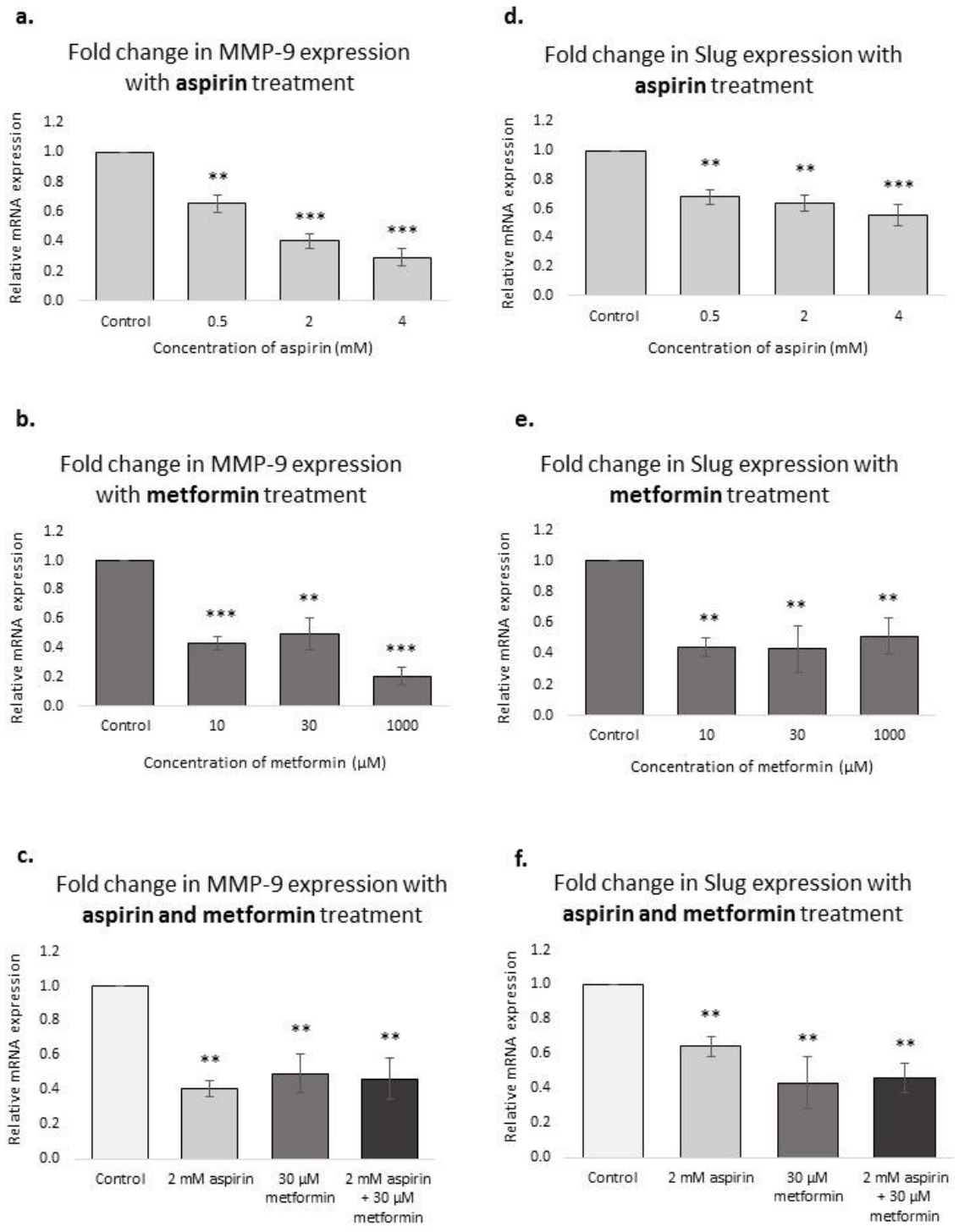


Figure 5.13: MMP-9 and Slug mRNA levels in PC3 cells determined by qPCR.
PC3 cells were dosed every 24 hours for a total of 96 hours with **(a, d)** aspirin **(b, e)** metformin or **(c, f)** aspirin and metformin in combination together. MMP-9 **(a, b, c)** and Slug **(d, e, f)** were normalized to the housekeeping gene GAPDH. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA and either Dunnett's **(a, b, d, e)** or Tukey's **(c, f)** post hoc tests. * p <0.05, ** p <0.01, *** p <0.001. Data represents three independent experiments performed in triplicate.

5.4.5.4 Aspirin and metformin increase E-cadherin expression in the LNCaP cell line

E-cadherin and N-cadherin protein abundance was also examined in the LNCaP cell line. This cell line represents an earlier stage of prostate cancer than the PC3 and DU145 cells, a stage which is still dependent on androgens, and provides insight into how the drugs may affect cancer progression. These cells migrate extremely slowly and so have more of an epithelial profile than the PC3 cells with no N-cadherin and high levels of E-cadherin. The LNCaP cells were dosed with 0-4 mM aspirin as were the other cell lines, however, western blotting could not be performed for cells dosed with 4 mM aspirin as there was not enough lysate due to the toxic effect of aspirin at this concentration as explained in Chapter 3, Section 3.4.4. For cells dosed with either 0-2 mM aspirin or 0-1000 µM metformin there was a non-significant increase in E-cadherin (Figure 5.14). N-cadherin was not examined as the LNCaP cell line normally has very low levels of this protein and so an anticipated decrease in this protein would not have been seen.

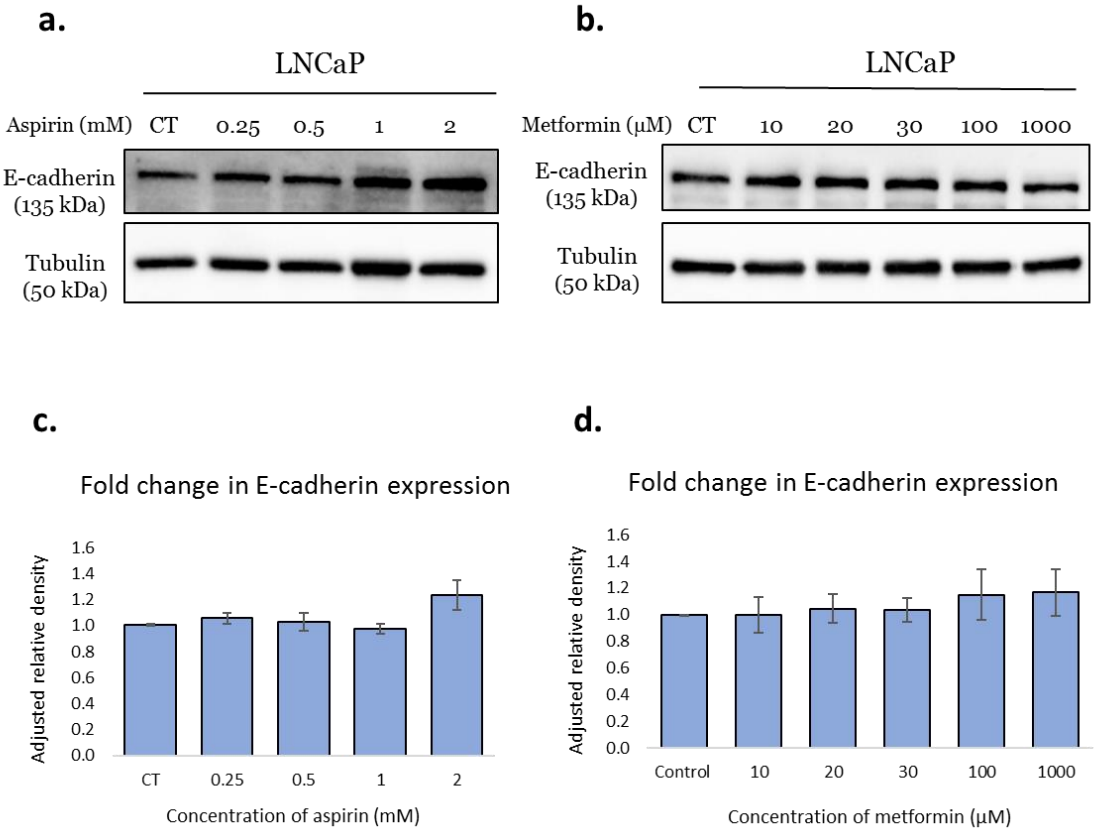


Figure 5.14: E-cadherin levels in LNCaP cells dosed with aspirin or metformin.
Abundance of E-cadherin in LNCaP cells dosed with aspirin **(a)** or metformin **(b)** every 24 hours for a total of 96 hours. Densitometry was performed to quantify protein levels **(c, d)**. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA and Dunnett's post hoc test.

When the LNCaP cell line was dosed with the combination of aspirin (2 mM) and metformin (30 µM) E-cadherin was potentially increased although this was not significant (Figure 5.15).

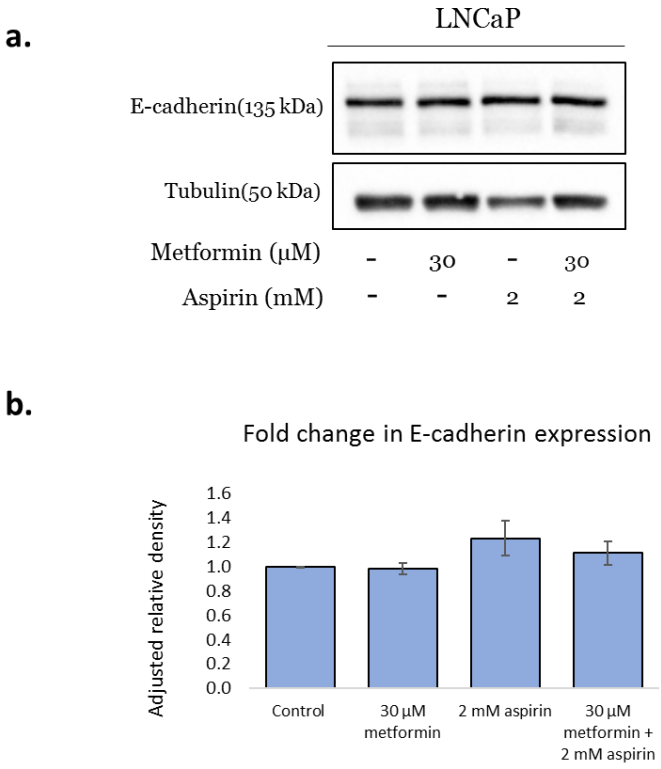


Figure 5.15: E-cadherin levels in LNCaP cells dosed with a combination of aspirin and metformin.
(a) Abundance of E-cadherin in LNCaP cells dosed with either aspirin or metformin alone and in combination every 24 hours for a total of 96 hours. **(b)** Densitometry was performed to quantify protein levels. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA and Tukey's test for multiple comparisons.

5.4.5.5 Aspirin and metformin have no effect on E-cadherin expression in the PNT2 cell line

The PNT2 cells are a non-tumorigenic epithelial prostate cell line and can be used as a control. As with the LNCaP cells they have high levels of E-cadherin and do not express N-cadherin. E-cadherin abundance did not change when the PNT2 cells were dosed with either aspirin or metformin (Figure 5.16).

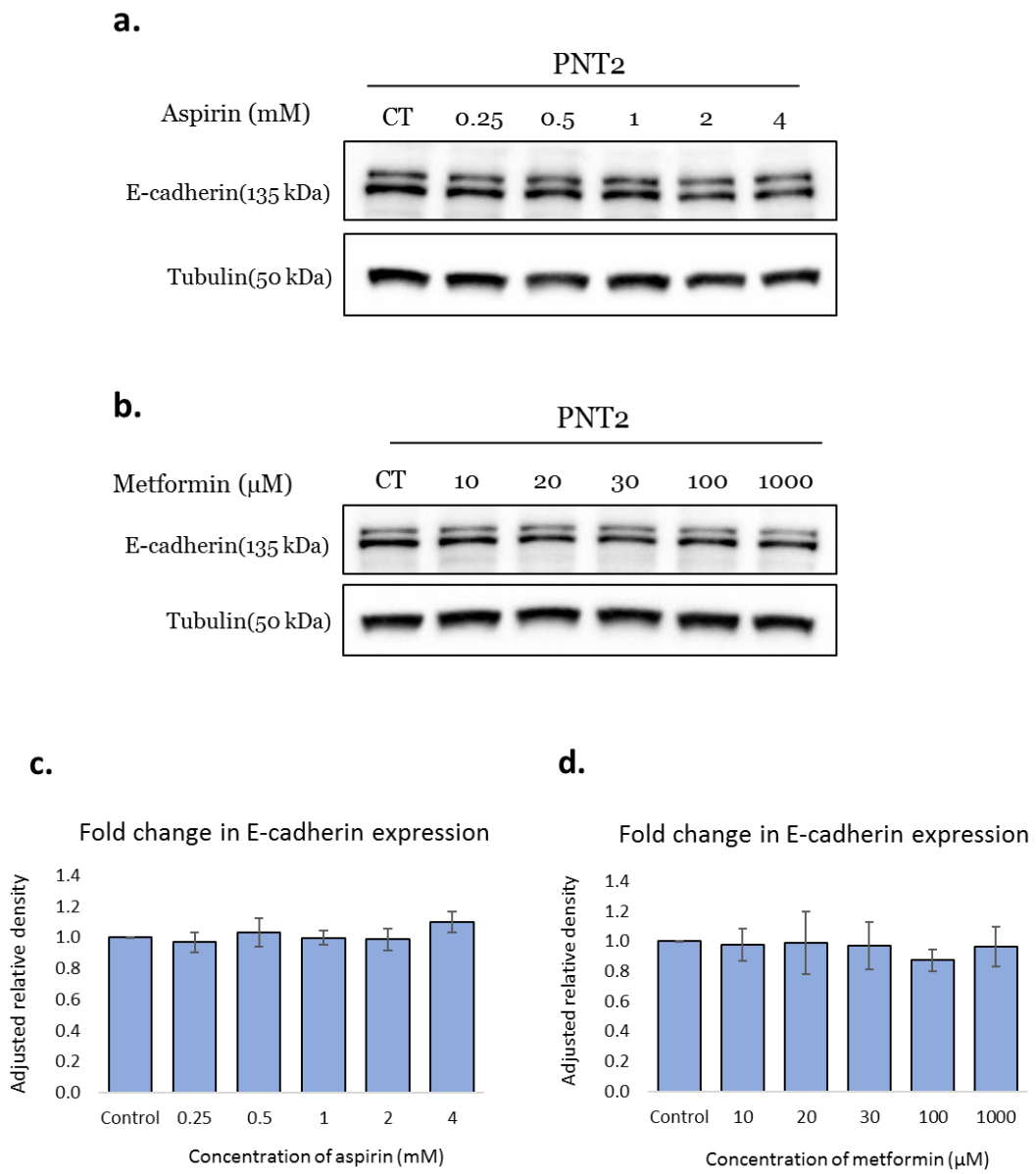


Figure 5.16: E-cadherin levels in PNT2 cells dosed with aspirin or metformin.
Abundance of E-cadherin in PNT2 cells dosed with aspirin **(a)** or metformin **(b)** every 24 hours for a total of 96 hours. Densitometry was performed to quantify protein levels **(c, d)**. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA and Dunnett's post hoc test

Importantly the combination of aspirin and metformin also had no significant effect on E-cadherin levels (Figure 5.17) indicating that the combination of the drugs does not alter the epithelial phenotype of this cell line.

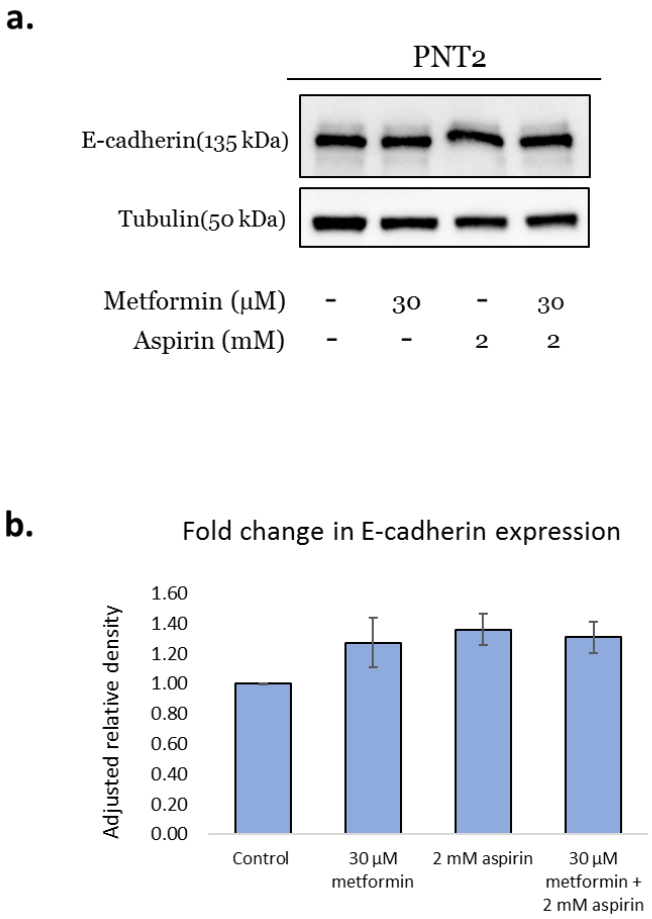


Figure 5.17: E-cadherin abundance in PNT2 cells dosed with a combination of aspirin and metformin. **(a)** Levels of E-cadherin in PNT2 cells dosed with either aspirin or metformin alone and in combination every 24 hours for a total of 96 hours. **(b)** Densitometry was performed to quantify protein levels. Error bars represent standard error of mean. Data was analysed using a one-way ANOVA and Tukey's test for multiple comparisons.

5.5 Discussion

5.5.1 Aspirin and metformin and prostate cell motility

In the wound healing assay, the PC3 cells were highly motile, with individual cells moving back and forth between each side of the gap. In contrast, the DU145 cells remained in close contact, moving as a sheet. During cancer migration, both single cells and clusters of cells can detach from primary tumours, enter the blood stream and migrate to new areas to colonize (264). This movement is regulated by adhesion molecules which are involved in localized cell migration such as E-cadherin, N-cadherin, integrins and MMPs but also makes use of leaky tumour vasculature to squeeze in between epithelial cells and enter the blood system (272). It is thought that tumour cells also release factors such as damage-associated molecular pattern (DAMP) molecules to increase vascular permeability (273). To exit the circulatory system, integrins present on circulating tumour cells bind to intercellular adhesion molecule 1 or vascular cell adhesion molecule 1 present on the vascular cell surface to mediate extravasation (272). While moving as a sheet may not permit the rapid movement of individual cells it still provides several advantages, allowing the cells to maintain tissue organization and cohesiveness (274). It also propagates mechanical signalling through cell-cell junctions, distributing tasks to each cell and protects metastatic clusters from immune assault (274). Collective cell migration is more commonly seen in epithelial cancers and these cells often have higher survivability in response to chemotherapeutics due to tumour interaction with ECM molecules, making treatment more difficult(275). It has been reported that an upregulation of integrins causes a pro-survival effect due to intracellular signalling through the focal adhesion kinase (FAK), PINCH/AKT signal transducer, STAT3 and glycogen synthase kinase 3 (GSK3) (276). However, both types of cell migration tend to be less responsive to chemotherapy as migrating cells temporarily stop proliferating which allows them to avoid treatments which targets the cell cycle (277). While the migration of individual cells can be problematic due to the rapid formation of new colonies, migration as a sheet can allow tumours to take over their surrounding areas quickly and are the more aggressive form in circulation, worsening prognosis. Therefore, it is necessary to identify drugs which can prevent both types of movement and keep the cancer localized. In this study, only the effect of aspirin and metformin on PC3 single cell migration was examined. PC3s are more motile than DU145s and are derived from the bone rather than the brain which is a common secondary site in prostate cancer. Furthermore, in Chapter 3 an additive effect of the aspirin and metformin was observed on cell proliferation in the PC3 cell line but not the DU145s and so it was of interest to see if the drugs also have an additive effect on cell migration.

In the wound healing assay, both aspirin and metformin reduced cell migration of PC3 cells in a dose-dependent manner, preventing closure of the gap after the 96 hours of imaging at the higher concentrations of each drug. In Chapters 3 and 4 therapeutic concentrations of metformin, ≤ 30 μM, had no effect on cell proliferation. In contrast, the effect of metformin on cell migration was much more apparent, indicating that it may play more of an anti-metastatic role in cancer treatment. Indeed, epidemiological findings suggest that both aspirin and metformin reduce the risk of distant metastases in several cancers. In a meta-analysis consisting of 5 randomised controlled trials daily aspirin (75 mg) was shown to prevent distant metastasis in colorectal cancer (108). Another study determined that aspirin reduces the risk of bone metastasis in men with prostate cancer after a median follow up of 70 months (278). Similarly, metformin has also been shown to improve distant metastasis free survival as observed in a study which examined men who took a median dose of 500 mg metformin twice daily for a median duration of 58.2 months (279). Interestingly, in a study which examined metformin use in diabetic patients for the treatment of pancreatic cancer there was a statistically significant increase in survival after 5 years for metformin users compared to non-metformin users, but only for patients who received metformin when the disease was nonmetastatic. This may imply that metformin is more beneficial at the earlier stages of cancer progression when the cancer is localized and prevents cancer metastasis (280).

However, while both aspirin and metformin have both been suggested to inhibit cell migration little work has been done examining the effect of combining them. In the wound healing assay, the combination of aspirin and metformin had the greatest effect on cell motility. Interestingly it has previously been reported that the combination of aspirin and high concentration metformin (3 or 5 mM) significantly reduced cell migration of pancreatic cancer cells (187). It is also encouraging that in this thesis the combined effect of aspirin and metformin on cell motility was observed at much lower metformin concentrations than in the previous study which are more clinically relevant.

5.5.2 Aspirin and metformin and markers of EMT

E-cadherin forms tight junctions with neighbouring cells, anchoring the cells to each other. Its levels are frequently reduced in the more advanced stages of cancer which allows the cells to migrate away from each other and form secondary sites of cancer (281). Conversely, N-cadherin is often upregulated in cancer, promoting cell migration and invasion by inducing cellular polarity and organising the actin cytoskeleton to form actin bundles for migration (282). In western blotting and qPCR both drugs caused an increase in PC3 E-cadherin expression and a decrease in N-cadherin expression. Interestingly, when the cells were dosed with 30 μM metformin an increase in E-cadherin was observed by western blotting but not qPCR, indicating that the protein is post-translationally regulated. In breast cancer cells metformin has been shown to alter the transcriptome and proteome of cells, rewiring signalling pathways through many transcriptional, translational and post-translational modifications (283). E-cadherin is also known to undergo post-translational modification in response to stimuli such as ER stress and apoptosis (284). Despite this, no study to date has shown that metformin post-translationally alters E-cadherin.

PC3 cells treated with aspirin also showed a dose-dependent reduction in both MMP-9 and Slug expression. Treatment with metformin greatly reduced MMP-9 and Slug but did not appear to be concentration dependent. Slug is a well-known repressor of E-cadherin and so as Slug expression decreases E-cadherin expression increases. While this was seen when cells were dosed with aspirin it was not observed with metformin where the increase in E-cadherin continued with increase in concentration of metformin despite Slug levels remaining the same. While Slug is a key transcription factor involved in EMT it is not the only E-cadherin repressor and the data suggests that metformin inhibits other repressors of E-cadherin independently of Slug. Metformin is known to modulate several EMT transcription factors including Snail (285), ZEB (286) and Twist (287), however the mechanisms by which requires further investigation. While metformin is known to decrease MMP-9 expression in other cancers (288) to the best of my knowledge this is the first time a decrease in MMP-9 has been observed in prostate cells with metformin treatment. Aspirin has previously been shown to reduce MMP-9 in prostate cancer and in separate studies both aspirin and metformin have been suggested to decrease MMP-9 and Slug expression through a reduction in NF_KB activation (146) (289) (290). Targeting MMP has been shown to reduce invasion of prostate cancer cells and potentially induce apoptosis (291). Similarly Slug upregulation leads to metastatic prostate cancer (292) and so is a potential target for treatment.

In this thesis, dosing with either aspirin or metformin demonstrated that the drugs alone inhibit cell migration. However, the data for the combination was mixed. While the wound healing assay showed that the combination of aspirin and metformin caused the greatest reduction in cell motility, the changes observed with the combinations were often no different to dosing with the drugs alone. It may be that the effect is very minimal so was only seen in the wound healing assay as the cells were exposed to the drugs for 96 hours longer than the cells for western blotting and qPCR. Indeed, in the wound healing assay the effect of the combination becomes more apparent over time. In addition, testing lower concentrations of the drugs, which are not effective when the drug is used on its own should be considered in the future. It is thought that the drugs both target similar pathways to reduce cell migration and so using concentrations which already cause a large decrease in markers of cell movement could make it more difficult to see an enhanced effect when the drugs are used in combination.

Chapter 6

General Discussion

6.1 Biological and clinical implications of main findings

Repositioning established medicines is a major opportunity in the field of cancer research. Not only does it save time and money in drug development, but the side effects of the drugs are well documented, reducing the possibility for patient harm. Aspirin and metformin are two therapeutics which have been in use for many years, aspirin is a nonsteroidal anti-inflammatory drug while metformin is the first line treatment for type II diabetes. More recently *in vitro*, *in vivo* and epidemiological studies have shown that both drugs possess anti-cancer effects, slowing down cancer development and reducing cancer associated mortality. However, while many studies have been conducted examining the benefit of aspirin treatment for colon cancer, reports of its actions in prostate cancer are limited. Current literature supports the use of either aspirin or metformin in prostate cancer treatment, however most *in vitro* studies use doses which are supra pharmacological and there is a lack of information regarding the effect of combining the drugs for prostate cancer treatment.

This project used lab-based techniques to examine the effect of aspirin and metformin on non-tumorigenic (PNT2) and cancerous (PC3, DU145 and LNCaP) prostate cell lines, specifically looking at changes in cell proliferation, apoptosis and cell migration using doses which are clinically relevant. The genomic diversity seen in cancer due to sustained cell proliferation results in a heterogenous molecular profile and makes personalized therapies difficult to implement. In prostate cancer tumour heterogeneity is a problem for treatment (293) (294), with a study reporting that in a cohort of 304 patients extensive heterogeneity was seen for Gleason scored samples in 89% of the patients and DNA ploidy for 40% of the patients, posing a major challenge to biomarker research and making personalized therapy less effective (295). In addition, increased cell survival through deregulation of apoptotic pathways propagates the incorporation of new mutations into the tumour and reduces treatment success. Cell migration also plays a key role in cancer progression, enabling the cancer to spread to other sites in the body, such as the bones, lungs and liver which leads to a poor prognosis. The 5-year survival rate for localized and locally advanced prostate cancer are almost 100% and 95% respectively whereas for metastatic prostate cancer it is around 30% (296). This sharp decrease demonstrates the importance of identifying drugs which can prevent cancer migration and keep the cancer localized to the prostate.

The results from this thesis provide further evidence of the use of aspirin and metformin as anti-cancer agents and discuss the value of combining the drugs to decrease cell proliferation and cell migration.

It was seen in Chapter 3 that aspirin induces apoptosis in only the LNCaP cell line and at high concentrations (2 mM) and it was suggested that it could be in part through stabilization of the tumour suppressor p53 and activation of its downstream targets. Localized prostate tumours usually express wildtype p53 while mutations and/or allelic losses in the p53 gene are more often associated with metastatic prostate cancer and tumour recurrence (297). p53 mutations which lead to a non-functional version of the protein have been reported in 3-20% of prostate cancer cases at the time of diagnosis (298). Therefore, the ability of aspirin to activate p53 could be beneficial in most patients (>80%) potentially reducing the number of cases which progress to metastatic disease. Interestingly, aspirin had differential effects in the LNCaP cell line which were dependent on dose, causing inhibition of cell proliferation at 0.5 mM and cell death at 2 mM. It was encouraging that low dose aspirin (0.5 mM) caused a reduction in cell proliferation, suggesting that lower doses will still have anti-cancer activity reducing the risk of side effects associated with high dose aspirin. Daily aspirin is normally given at 75 mg- 325 mg with the upper end representing a regular strength aspirin tablet (299). Aspirin to treat short-term pain can be taken at a maximum of 3600 mg every 24 hours for a few days (300) but no longer than 3 days due to potential side effects. While translating *in vivo* doses to *in vitro* culture is often controversial, in this study concentrations of 0.25-4 mM aspirin were used following reports that human plasma aspirin levels are around 1 mM (301) and 2.5 mM (289) when therapeutic doses of aspirin are given.

Metformin is the first line agent for treatment of type two diabetes and is normally given at a maximum dose of 2,000 mg per day (302) although some studies state that this can go up to 2,500 mg (303). In a study which examined systemic plasma concentrations after intake of 2,500 mg metformin, plasma concentration in the portal vein was between 40 and 70 µM and after hepatic uptake this concentration was reduced to 10-40 µM (182). In another controlled clinical study, maximum metformin plasma levels did not exceed 30 µmol/L (304). In this thesis, metformin had no effect on cell proliferation or apoptosis at concentrations up to 30 µM in any of the cell lines. While this is controversial to the multitude of studies which support the proposition that metformin has anti-proliferative effects, many of the studies are conducted at supra-pharmacological metformin concentrations. Indeed, in this study 100 and 1000 µM

metformin did cause a reduction in spheroid area when examining spheroid growth implying that concentrations higher than those found in the plasma do have an anti-proliferative effect.

In terms of examining the combined effect of the drugs on cell proliferation, an additive effect was observed in the PC3, potentially due to the lack of the PTEN tumour suppressor in this cell line. Lack of PTEN leads to an overactive PI3K pathway, a pathway which is targeted by both drugs (Chapter 3). Using the drugs in combination may reduce the dose necessary to have an effect and reduce adverse patient reactions, although this is clearly dependent on the genotype of the tumour cells.

Examining the effect of the drugs on PC3 cell migration (Chapter 5) provided evidence that both drugs individually prevent cell migration as previously reported in other studies (147) (305). This is an important feature for cancer treatment and suggests that it may be beneficial to administer the drugs for localized prostate cancer to prevent metastasis. Treatment with aspirin or metformin alone caused the cells to adopt a more epithelial phenotype as seen by an increase in E-cadherin, and a reduction in N-cadherin, MMP-9 and Slug with western blotting and qPCR. This data supports previous studies, where both aspirin and metformin decreased MMP-9 and Slug expression and reversed EMT through inhibition of the canonical NF_KB pathway in prostate cancer cells (146) (306). The results from the combination of both drugs were variable for cell migration and show an enhanced but not additive effect. Both drugs target similar pathways including the NF_KB, PI3K/AKT, STAT3 and MAPK pathways which have been suggested to contribute to their anti-migratory effects. Aspirin specifically inhibits IKK-β activation whereas metformin reduces NF_KB activation through activation of AMPK (307) or independently of AMPK (306), with inhibitors of AMPK and PI3K signalling abrogating its effects. These studies primarily examine the use of metformin in cardiovascular disease prevention as prolonged activation of the NF_KB pathway is known to play an important role in eliciting signals which trigger chronic inflammation and disease development (308). The connection of NF_KB to prostate cancer cell growth, survival and cell movement (309) makes drugs that have the capacity to target NF_KB activation, such as aspirin and metformin, well suited for cancer treatment. Importantly this study establishes that the anti-metastatic activity of the drugs can be achieved at therapeutically relevant doses in prostate cancer, particularly when used in combination.

There is much interest in the potential use of aspirin and metformin alongside conventional therapies. Both aspirin and metformin have been proposed to have synergistic effects with

standard cancer treatments such as chemotherapy and radiotherapy, with studies suggesting patient outcome is improved when they are used together (191) (310) (195) (196). NF_KB activation appears to be increased during radiotherapy (311) and chemotherapy (312) and so the mechanisms by which aspirin and metformin target NF_KB signalling could reduce resistance to chemo and radiotherapy, improving patient outcome. In terms of surgery, inhibition of cell migration observed with both drugs could help keep the cancer localized prior to surgery, providing more time for men to decide what treatment option they want to proceed with.

In this thesis, the combination of aspirin and metformin demonstrated that the drugs have potential efficacy. However, it is important to also consider the effects of the drugs on different hallmarks of cancer and how targeting multiple hallmarks at the same time may be most beneficial therapeutically. While aspirin was more effective at inhibiting prostate cancer cell proliferation (Chapter 3 and 4) metformin was more effective at inhibiting cell migration and did so at an earlier time point than aspirin (Chapter 5). Therefore, when the drugs are used in combination, aspirin may act to reduce tumour cell growth while metformin prevents tumour metastasis, targeting different hallmarks to provide a practical benefit. Indeed in a recent nature review, it was suggested that combining drugs based on their effect on independent cancer hallmarks simultaneously targets multiple phenotypic behaviours of cancer cells, improving effectiveness of treatment (313). In addition, tumours are known to possess intra-tumour heterogeneity which results from genetic and non-genetic determinants and leads therapeutic resistance (314). Therefore, depending on the genetic composition of the tumour some patients may respond better to drugs which target cell proliferation pathways while others respond better to those which target cell migration pathways. The pleiotropic nature of both aspirin and metformin allows the drugs to target multiple signalling pathways so that heterogeneous tumours may still respond to treatment.

In all, it was concluded that aspirin and metformin have differential effects depending on the cancer subtype (Figure 6.1). In the wild type p53, androgen dependent cells, such as the LNCaPs, aspirin alone was seen to influence cell proliferation. In the mutant p53, androgen independent cells, such as the PC3s, although aspirin alone could inhibit cell proliferation it was the combination of aspirin and metformin that had the greatest effect on cell proliferation and cell migration. This indicates that in the more advanced stages of prostate cancer the combination of aspirin and metformin (targeting two distinct hallmarks of cancer) would be most effective for treatment.

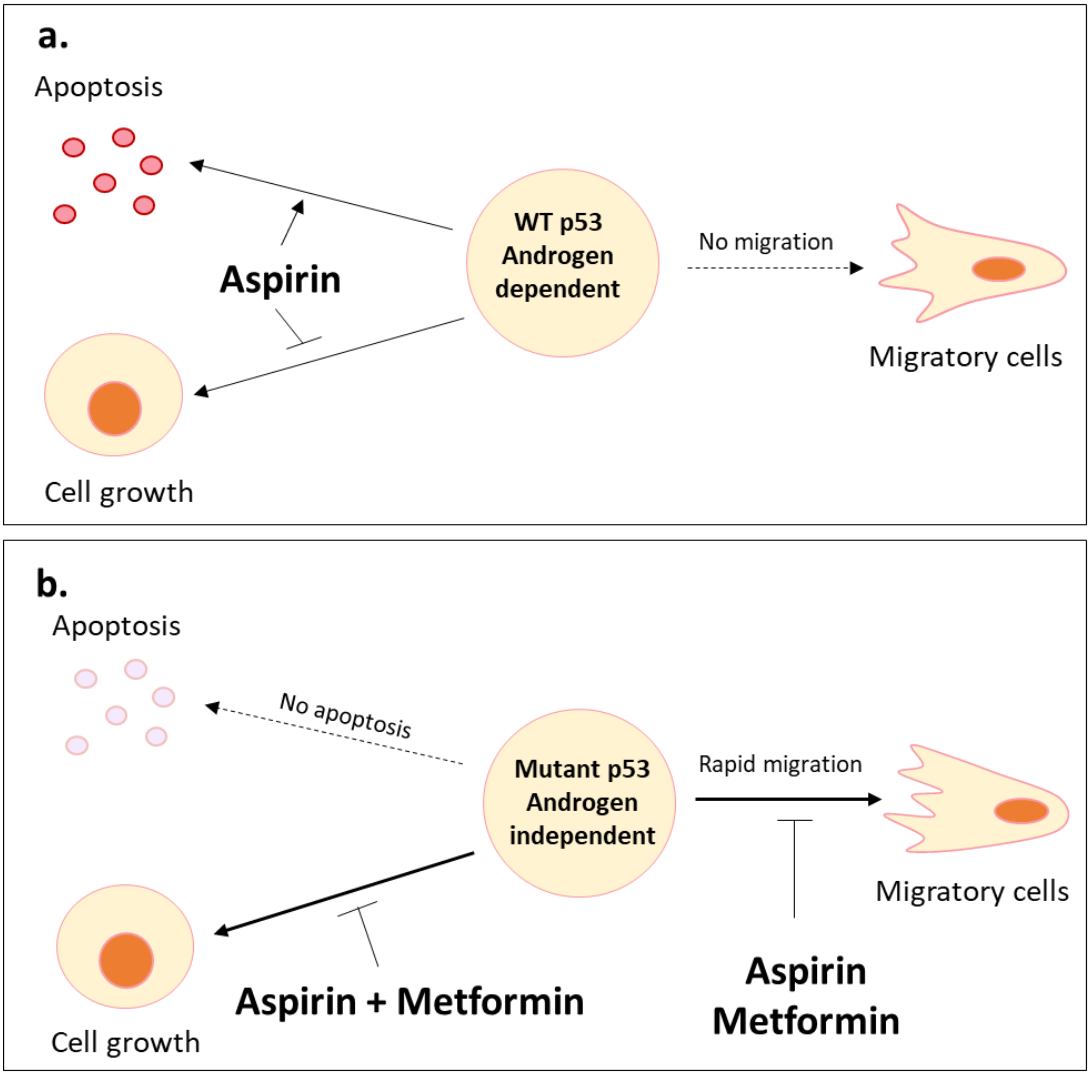


Figure 6.1: Aspirin and metformin have differential effects depending on cancer subtype.
(a) In wild type p53, androgen dependent cells such as LNCaPs, aspirin alone induces apoptosis and inhibits cell proliferation. **(b)** In mutant p53, androgen independent cells such as PC3s the combination of aspirin and metformin inhibits cell proliferation and rapid cell migration more effectively that when the drugs are given individually. In this cancer subtype the drugs do not induce apoptosis.

6.2 Limitations of the research

The four prostate cell lines used in this thesis attempt to encompass various stages of prostate cancer including non-tumorigenic, androgen dependent and androgen independent cancer. However, all three of the cancerous cell lines are from the more advanced, metastatic stages of the disease. Therefore, a limitation of the study is that the effect of the drugs on localized prostate cancer was not examined. Towards the end of the project I obtained P4E6 cells, an early stage prostate cancer cell line, from a collaboration (Maitland Lab, University of York) and in future work it would be useful to examine the effects of the drugs on this early stage prostate cancer cell line. It would also be interesting to grow the cells in 3D to see if there are differences in the spheroids morphologies. The PC3, DU145, LNCaP and PNT2 cells are all established commercial cell lines and contain some of the more commonly observed mutations in cancer such as loss of important tumour suppressors such as PTEN or p53. The fact that their mutation status is well known and documented is valuable as it allows a greater analysis of signalling pathways to aid in understanding of how the drugs exert their anti-cancer effects. However, while cell lines are useful to study specific signalling pathways access to primary cultures would have been beneficial, providing a heterogeneous population of cells which maintain many of the key markers and functions seen in cells *in vivo*.

Translating *in vivo* doses to *in vitro* culture is difficult and often controversial and so another limitation of this work is the debate over what drug concentrations are considered therapeutically relevant. In this study, 2 mM aspirin and 30 µM metformin were selected as the maximum doses for each drug. While literature supports these concentrations there are doubts whether 2 mM aspirin is achievable in the prostate. Therefore, more studies that examine the concentration of aspirin which reaches the prostate are needed before it can confidently be stated that 2 mM aspirin is therapeutically relevant. However, an important consideration for these studies is that people take aspirin and metformin daily for years. Metformin has been shown to accumulate in the mitochondria and many epidemiological studies have identified that aspirin and metformin have a long-term benefit which increases with duration of treatment (315). The studies in this thesis examined the short-term effects of the drugs and so a lower concentration over a longer period of time may have a similar effect to a higher concentration over a shorter period of time. Future studies which examine the effect of low dose, long term treatment may also be informative and clinically useful. Indeed, cells have been maintained in

low dose aspirin for extended periods of time, for example >50 weeks, to determine whether the cells respond differently to long term dosing and if the cells become more resistant to the drugs over time.

A final limitation was that while the proteins and genes examined in this study are useful to determine the effect of the drugs on hallmarks of cancer, such as proliferation and migration, they do not confirm the effect of the drugs on specific signalling pathways. In future work it would be beneficial to examine the expression of proteins further downstream such as the E2F family or ribosomal S6 kinase. FACs would also to determine what stage of the cell cycle the drugs inhibit and if this is altered through treatment with the combination of aspirin and metformin. Knockout studies would also be useful to determine the function of specific genes in response to treatment, of particular interest would be the NFκB protein complex and members of the PI3K pathway.

6.3 Future research

Multiple signalling pathways have been implicated in the anti-migratory effects of aspirin and metformin including the TGF- beta, AMPK, mTOR and STAT3 pathways as well as miRNAs. In a study by *Yue et al* it was noted that both aspirin and metformin modulate the AMPK, mTOR, STAT3 and NFκB pathways and could potentially affect cell migration via these mechanisms (17). Interestingly, PC3 cells do not express STAT3 (316) and so the ability of aspirin and metformin to inhibit cell migration in this cell line is not through the STAT3 pathway. In addition, the number of miRNAs which have been directly or indirectly linked with EMT is extensive and constantly increasing (317). Recently metformin has been shown to inhibit TGF-β induced EMT and modulate the expression of miRNAs, specifically miR30a, to reduce prostate cancer cell migration (318). Similarly, aspirin has been shown to reprogram the mesenchymal to epithelial transition (MET) and delay cell migration through the TGF-β/SMAD4 pathway in breast cancer (319). With many studies highlighting the pleiotropic nature of both drugs it would be interesting to examine the effect of combining aspirin and metformin on key signalling molecules involved in the PI3K/ATK, NFκB and TGFβ pathways. In this study, different effects were seen in cancer subtypes, with an additive effect of the drugs observed in the PC3 cell line suggesting that in androgen independent, p53 negative, PTEN negative cells both drugs inhibit the PI3K pathway to reduce cell proliferation (Figure 6.2 a). Conversely in androgen dependent, p53 positive cells, PTEN negative cells, such as the LNCaPs, aspirin could inhibit cell proliferation and induce apoptosis through inhibition of the PI3K pathway, inhibition of androgen receptor signalling and activation of p53 (Figure 6.2 b). In migratory cells, inhibition of NFκB by both aspirin and metformin as well as inhibition of other pathways previously mentioned in this section by either of the drugs could act to reverse EMT, preventing cancer metastasis (Figure 6.2 c). It would be interesting to examine the mechanisms by which aspirin and metformin exert their anti-cancer effects and understand which cancers may benefit most from treatment.

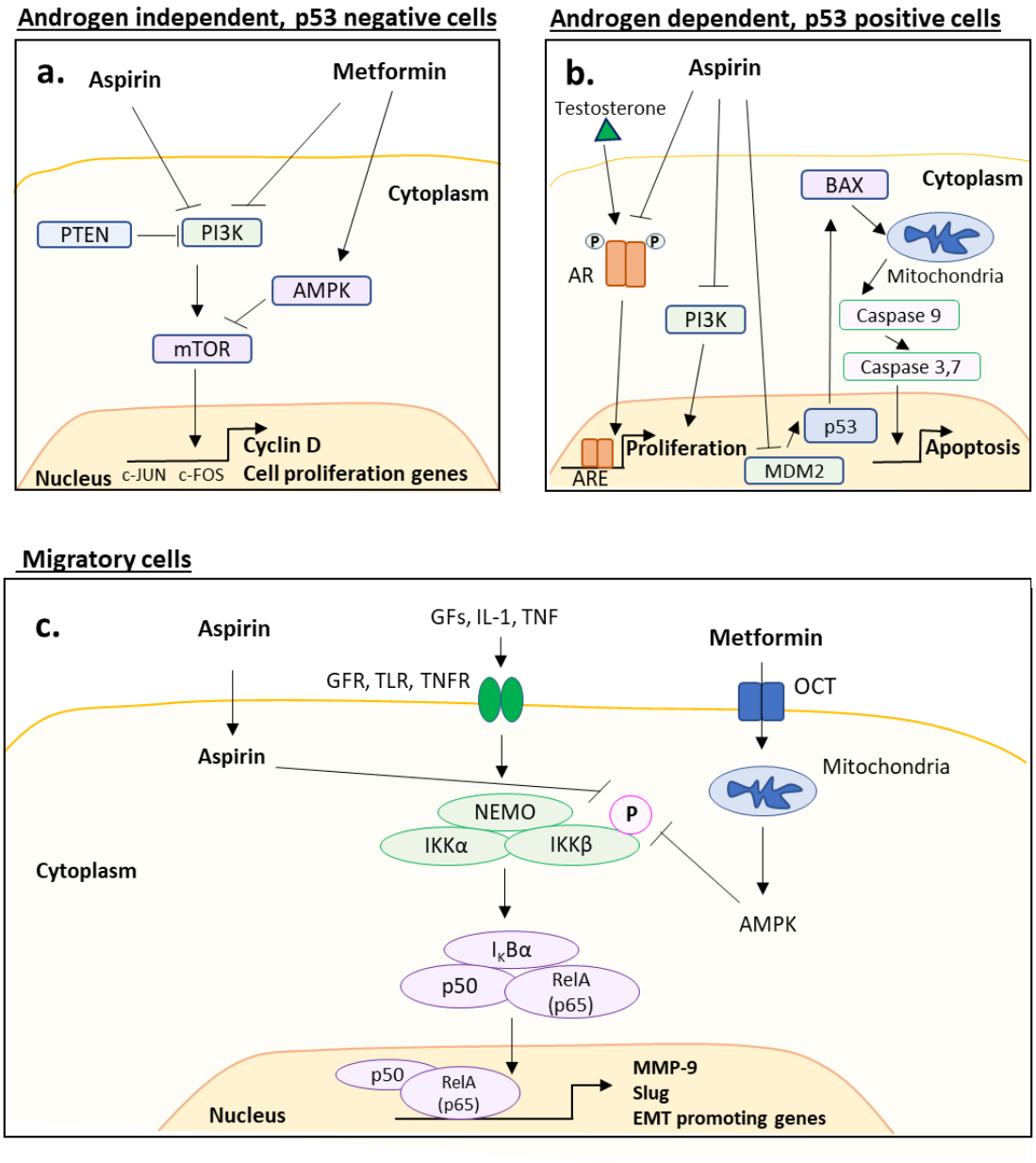


Figure 6.2: Proposed mechanisms by which aspirin and metformin may reduce cell proliferation and migration in cancer cells.
(a) In androgen independent, p53 negative cancer cells aspirin and metformin exert additive effects by targeting the PI3K pathway to reduce cell proliferation. This effect is enhanced in cells which are negative for the tumour suppressor PTEN which normally prevents overactive PI3K signalling. **(b)** In androgen responsive, p53 positive cancer cells aspirin inhibits cell proliferation by downregulating the androgen receptor mRNA and protein expression. This prevents the androgen receptor from downregulating cell cycle inhibitors such as p16 and p21 and enhancing the activity of CDKs. Aspirin also induces apoptosis by preventing MDM2 from binding to p53 and stabilization of p53. The PI3K pathway is also active in LNCaP cells and so aspirin may also reduce cell proliferation through inhibition of this pathway. **(c)** Aspirin and metformin target the $NF_{\kappa}B$ pathway to prevent cell migration. Aspirin inhibits IKK-β activation while metformin activates AMPK to reduce $NF_{\kappa}B$ activation.

Examining the effect of the drugs on cell proliferation in 3D cell culture identified that the spheroids appeared to contain a necrotic, hypoxic core. Hypoxia has been shown to promote cancer progression, leading to excessive dysfunctional vascularisation and the acquisition of mesenchymal traits which allow cancer to migrate to secondary sites (320). Metformin has been shown to reduce hypoxia in solid tumours (196), reducing cancer progression and dissemination and allowing therapeutics such a radiotherapy which requires oxygen to have a greater effect. In radiation therapy, oxygen assists in the chemical reactions that cause DNA damage when cells absorb energy from the ionizing radiation (321). Cells that have a normal level of oxygen are around two to three times more sensitive to radiotherapy than hypoxic cells such as those in the centre of a tumour (315)(314). Currently there is an ongoing clinical trial, ASPIRE, which examines whether aspirin improves radiotherapy in rectal cancer (323). The ADD-Aspirin trial is also recruiting participants to determine whether aspirin use after treatment for an early stage cancer prevents recurrence and reduces cancer associated mortality. It examines aspirin use for breast, colon/rectum, gastro and prostate cancers and in prostate cancer examines men who have undergone radiotherapy or a radical prostatectomy (123). It will be interesting to see the outcomes of both trials to help determine whether aspirin prevents cancer recurrence and improves outcome after primary treatment. Similarly, metformin has been shown to reduce the oxygen consumption rate and hypoxia in cells through inhibition of mitochondrial complex I (324). A study by *Spratt et al*, undertaken in men who underwent external-beam radiation therapy for localized prostate cancer identified a strong association between metformin use and clinical benefit in prostate cancer patients. There was improvement in all outcomes for patients who were taking metformin, including; PSA-recurrence free survival, distant metastases free survival, overall survival, prostate cancer specific mortality and the development of castration-resistant prostate cancer (325). It would be interesting to see if combining aspirin and metformin further reduces hypoxia and thus increases sensitivity to conventional therapy.

In addition, while our model was simplified to include only the extracellular matrix (matrigel) and cancer cells, other 3D models include non-cancerous cells found within the tumour microenvironment such as fibroblasts and immune cells (326). These cells secrete factors such as cytokines and growth factors which are important for cancer development and growth. It may be that the drugs are more effective at targeting stromal cells than epithelial and therefore, it would be interesting to include stromal and immune cells in our 3D model to see if the drug induced changes in tumour microenvironment affect prostate spheroid growth.

Both aspirin and metformin significantly affected cell migration, reducing the migratory capacity of the cells in the wound healing assay and causing the cells to adopt a more epithelial phenotype as seen with markers of EMT. However, it would have been interesting to conduct studies examining the effect of the drugs on cell invasion as well as migration. While the markers of EMT examined are characteristic of both cell migration and invasion conducting a transwell migration and invasion assay would have provided information about the cells ability to move through a physical barrier towards a chemoattractant such as growth media, further establishing the drugs effect on cancer metastatic processes (327). In addition, while E-cadherin and N-cadherin are two of the most reliable indicators of EMT the use of more markers such as integrins or the twist and ZEB families would have been valuable to better understand the drug induced changes to adhesion molecules and EMT transcription factors. As stated in Chapter 5, cells migrate either individually or as a cluster, with collective migration being the most commonly observed method in epithelial cancers. In this thesis, the effect of aspirin and metformin on individual cell migration was examined using the PC3 cell line but it was noted that the DU145 cells migrate by keeping in close contact with each other. In future experiments it would be interesting to also conduct migration experiments with this cell line to examine whether the drugs have a differential effect on collective cell migration. Examining the signalling pathways targeted by aspirin and metformin will help to determine whether the drugs also alter expression of proteins involved in EMT in these cells and will be effective in preventing both types of cell migration.

One of the major considerations in prescribing aspirin and metformin is deciding who will most benefit. Metformin is taken up by organic cation transporters, most commonly OCT1, and extruded by multidrug and toxic compound extrusion transporters 1 and 2 (MATE 1 and 2) which are highly expressed in the liver (328) but are also expressed by cells of the prostate (329) (330). However, studies have shown that in men with prostate cancer, having polymorphisms in OCT1 or MATE1 or high levels of MATE2 can cause a reduction in responsiveness to metformin treatment (331) (330). Furthermore, other genetic factors also affect response to metformin treatment, such as polymorphisms in Serine-threonine kinase 11 (STK11) also known as LKB1, an important protein involved in metformins downstream PI3K/AKT signalling (332). It would be interesting to examine the expression of OCT1, MATE2 and STK11 in our cell lines to determine their association with response. Similarly, genetic polymorphisms have also been associated with aspirin resistance, mostly in the COX-1/2, P2Y receptors (P2Y), and glucose-6-phosphatase isomerase (GPI) genes (333). P2Y is a platelet membrane receptor and plays a key role in platelet

aggregation (334) while the GPI gene encodes a glycoprotein coupled receptor which TXA2 binds to also causing platelet aggregation (335). Studies have shown that polymorphisms in these genes result in a reduced response of aspirin to cardiovascular disease but also could affect aspirin response to cancer with aspirins effect on platelets thought to play a key role in its anti-cancer effects. Aspirin is also known to reach a higher concentration in the colon compared to distal tissues such as the prostate, skin and liver (145). Therefore, while plasma concentrations of aspirin have been shown to have an anti-cancer effect in distal tissues, people with colon cancer may receive a greater benefit from treatment than those with other types of cancer. Investigating expression of markers associated with resistance in prostate cancer cell models would help us to determine which patients would most benefit from combined aspirin/metformin treatment in the future.

Finally, it was an aim of this study to examine the effect of aspirin and metformin on an *in vivo* metastasis model to help determine whether the combination has an additive effect on cell migration. Work is currently underway with our collaborator Dr Oltean at Exeter University who is intravenously inoculating nude mice with 1 x 10^6 luciferase-tagged PC3 cells and then treating them with either saline (control), aspirin, metformin or a combination of the two drugs. The degree of metastatic spread will be monitored twice weekly using *in vivo* imaging with an IVIS Xenogen device to determine whether the drugs prevent cell migration within an *in vivo* model. Pilot studies have confirmed the timeline of metastasis showing the formation of lung metastases over time and the experiments determining drug concentrations are currently underway (Figure 6.3).

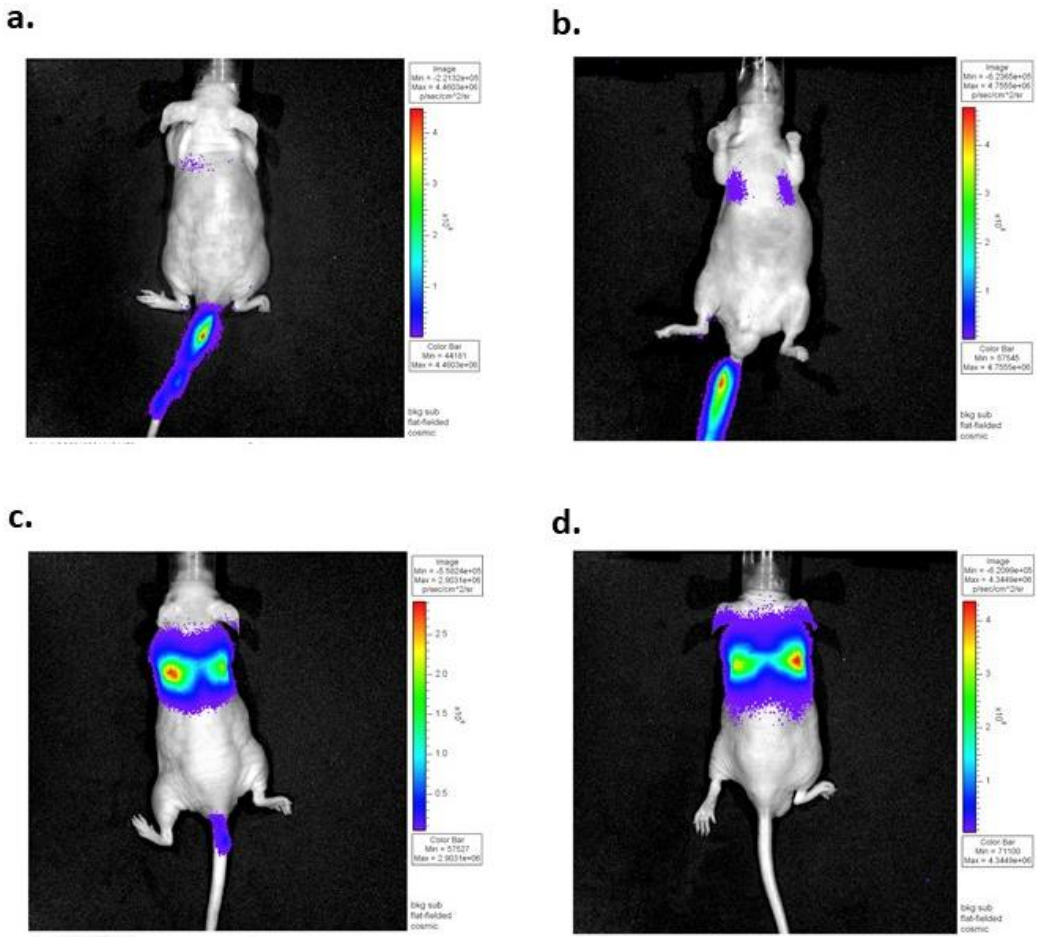


Figure 6.3: Pilot studies of *in vivo* PC3 metastasis.
1 x10^6 luciferase tagged PC3 cells are injected into the tail vein of the mouse and form lung metastases.

6.4 Overall conclusion

The data presented in this thesis suggests that aspirin in combination with metformin may be a valid and novel approach to improve prostate cancer treatment. This work strongly supports findings that aspirin is anti-proliferative with a decrease in cell proliferation observed in all the cancerous cell lines tested. Aspirins effect on apoptosis is dependent on the mutations acquired during tumorigenesis, inducing apoptosis in tumours which retain wild type p53, which could be greater than 80% of prostate cancer cases. Aspirin was also seen to reduce cell migration which suggests that aspirin may be beneficial for both cancer prevention and treatment.

Metformin did not reduce prostate cell proliferation which contradicts studies which do show an anti-proliferative effect. While the short duration of the experiment may have had an impact, many studies use concentrations of metformin which are supra-pharmacological which should be taken into consideration. Despite this, metformin was highly effective at inhibiting cell migration and did so at an earlier time point than aspirin, a feature which is important for the treatment of aggressive cancers.

In terms of the combined effect of the drugs, the most advanced cell line, the PC3s, exhibited an additive decrease in cell proliferation and cell migration, confirming that in combination the drugs could prevent the formation of secondary sites of cancer.

In summary, this thesis does support the use of aspirin and metformin in combination to reduce prostate cancer cell proliferation and migration. Importantly, as aspirin and metformin appear to target two distinct hallmarks of cancer they have a greater chance of efficacy in heterogenous tumours and combined treatment may prevent progression to the metastatic disease.

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