TARGETING THE SPLICE FACTOR KINASE CLK1 IN PROSTATE CANCER CELLS

By

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Author's Declaration

"This thesis is submitted in partial fulfilment of the requirements for the award of PhD in Biomedical Science and except where duly acknowledged or referenced it is entirely my own work. It has not been submitted, either in whole or in part, for any other award at UWE or elsewhere."

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Project Supervisor's Declaration

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ABSTRACT

Prostate cancer is the most rampant diagnosed cancer and the second leading cause of cancer related death in men between middle age and old age. There is a need to identify the molecular genetic processes that underpin prostate cancer and to search for new treatments. Alternative splicing affects over 94% of human genes and aberrant splicing is implicated in prostate cancer. Splicing is regulated by splice factors and protein kinases (the latter including SRPK1 and CLK1). The CLK1 protein kinase specifically regulates alternative splicing by phosphorylation of SR proteins within the nucleus, particularly in nuclear speckles (splice factor storage sites). CLK1 is also found to be overexpressed during malignant prostate cell transformation-; therefore targeting the splice factor kinase CLK1 by its specific chemical inhibitor (TG003) could be therapeutically useful. We confirm that CLK1 expression is itself regulated through alternative splicing: skipping of exon 4 or retention of intron 4 results in truncated, inactive CLK1. The aims of this project are to study CLK1 alternative splicing and to explore the effects of targeting the splice factor kinase CLK1 in prostate cancer. Prostate cancer cell lines (androgen independent PC3 and DU145 cells) were treated independently for 24, 48 and 72hrs with varying concentrations (10nM -100μM) of the benzothiazole compound (TG003) a specific inhibitor of CLK1. Results suggest that chemical inhibition of CLK1 with TG003 treatment suppressed growth and induced apoptosis in prostate cancer cell lines, as well as causing decreased cell migration and invasion. Similar effects were observed when CLK1 expression was reduced with an siRNA. There was also increased E-cadherin expression with TG003 treatment; in contrast, vimentin expression was reduced suggesting reversal of endothelial-mesenchymal transition following TG003 treatment. RT-PCR analysis revealed that TG003 treatment altered CLK1's own splicing by altering exon 4 skipping rates in a

dose dependent manner, suggesting that a feedback loop mechanism contributes to the regulation of CLK1 expression. In vivo mouse work with PC3 cell line xenografts showed a highly significant reduction in tumour growth and volume following intraperitoneal TG003 administration (10 and 50μM). In conclusion, the findings presented in this thesis suggest that targeting CLK1 may bring considerable anticancer benefits.

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List of Abbreviations and Units

ABCA1 - ATP-binding cassette transporter 1

ADT- Androgen deprivation therapy

ATMgene - Ataxia-Telangiesctasia mutated gene

BPH – Benign prostatic hyperplasia

BSA- Bovine serum albumin

BT – Brachytherapy

CLK- CDC-like kinase

CLK1- CDC-like kinase 1

cDNA- Complementary DNA

DAPI- 4',6-diamidino-2-phenylindole

DI – Deionised water

DMEM- Dulbecco's Modified Eagle's Medium

DMSO- Dimethylsulfoxide

DNA- deoxyribonucleic acid

dNTP- deoxyribonucleitide

DRE- Digital rectal examination

DYRK1A - Dual specificity tyrosine-phosphorylation-regulated kinase 1A

ECACC- European collection of authenticated cell cultures

EDTA- ethylenediaminetetraacetic acid

EMT- Epithelial-to-mesenchymal transition

EPCA – Early prostate cancer antigen

ERT – External beam radiotherapy

ESE- Exon splicing enhancer

ESS - Exonic splcing silencer

FBS- Fetal bovine serum

FAC- Fluorescence-activated cell sorting

FAS- Fatty acid synthase

FASN – Fatty acid synthase gene-N

FGFR1OP- Fibroblast growth factor 1

GSTP – Glutathione s- transferase

HGPRS – Hutchinson Gilford progeria syndrome

HIFU – High intensity focused ultrasound

hnRNP- Heterogeneous nuclear ribonucleoprotein

HPC1 – Hereditary prostate cancer 1

HRP- Horseradish peroxidise

IGF-1 – Insulin-like growth factor-1

IGFPBs - Insulin-like growth factor binding proteins (IGFPBs)

ISE- intron splicing enhancer

ISS- Intronic splicing silencer

ISUP - International society of urologic pathology

LMNA - Lamin A gene

LXRa - Liver X receptor alpha

LUTs – Lower urinary tract symptoms

mRNA- messanger RNA

NF1 - Neurofibromatosis 1

NMD- Nonsense-mediated decay

PBS- Phosphate buffer saline

PCa- Prostate cancer

PC3 cells- Prostate cancer-3 cells

PCR- polymerase chain reaction

PIN – Intraepithelia neoplasia

PKA- Protein kinase A

PKC- Protein kinase C

PPARy - peroxisome proliferator-activated receptor gamma

PSA- Prostate specific antigen

PSI- Percentage splice index

PSMA- prostate specific membrane antigen

PVDF- polyvinylidene difluoride

RNA- ribonucleic acid

RRMs - RNA recognition motifs

RNASEL - Ribonuclease L (2', 5’-oligoisoadenylate synthetase-dependent

RIPA buffer- Radioimmunoprecipitation assay buffer

ROS- Reactive oxygen species

rpm- revolution per minute

RRM- RNA recognition motif

RT- reverse transcriptase, real time

RT- Room temperature

SDS-PAGE- Sodium dodecyl sulfate Polyacrylamide gel electrophoresis

SE – Standard error

SEER – Surveillance epidemiology and end resort

siRNAs- Small interfering ribonucleic acid

SPHINX-((5-methyl-N-[2-(morpholin-4-yl)-5-(tri-fluoromethyl)phenyl]furan-2 carboxamide)

SR- Serine-arginine rich

SR protein- Serine-arginine rich protein

SRE- Splicing regulatory element

SRPK1- Serine-arginine protein kinase 1

SRSF- Serine-arginin rich splicing factor

SSO- Splice switching oligonucleotide

TAE- Tris-Acetate-EDTA

TBST- Tris-buffered saline with Tween 20

TEMED- Tetramethylethylenediamine

TG003- 1-(3-ethyl-5-methoxy-2(3H)-benzothiazolylidene)-2-propanone

TOES- Targeted oligonucleotide enhancers

TRUS - Transrectal ultrasound

TSS- Transcription start site

USM = Urinary secretary markers

UTR- Untranslated region

VCaP cells- Vertebral-cancer of the prostate cells

Chapter 1

Introduction

1.1 Prostate cancer

There are different forms of cancer including prostate cancer arising from several factors such as chemical, environmental, genetic, immunological, or factors of viral origin (David and Manley, 2010; Ghigna *et al*., 2008). Genetic and epigenetic changes that promote cancer also accumulate over a period of time (Lapuk *et al*., 2014). However, the causes of several forms of cancer (including prostate cancer) are still unclear (Kandoth *et al*., 2013; Augello *et al*., 2013). Prostate cancer incidence has increased dramatically. Apart from family history, one of the notable and strongest prostate cancer risk factors is age and ethnicity including African-American related races (Gann, 2002). These are discussed in detail in other sections. Prostate cancer is an increasing, devastating, deleterious and disastrous pathologic disorder affecting men with over twenty-nine thousand seven hundred and twenty death per annum in the western word (Siegel *et al*., 2013)**.**

Apart from skin cancer, in men between middle and old age, prostate cancer (PCa) is the most rampant diagnosed cancer and the second leading cause of cancer related mortality among men in the U.K and USA (Aragon and Dahut, 2009). In 2013, Siegel and her research team estimated that 238,590 men in the United States are likely to develop prostate cancer per annum and 29,720 will die from the disease (Siegel *et al*., 2013). Prostate cancer is an age-dependent disorder manifesting generally from middle to older age (Watson and Watson, 2010). Other etiological factors that could contribute to prostate cancer risk are dietary fat intake, androgens, race, environmental (such as chemical exposure) and genetic factors (Watson and Watson, 2010). These are further explained in subsequent sections.

Prostate cancer is usually confined to the prostate at the early stage of development. Surgery or radiation therapy could provide treatment at this stage. (Narla *et al*., 2005; Aragon *et al*., 2009; Drake *et al*., 2014), but the disease becomes too devastating and difficult to manage or cure at later metastatic stages. Prostate cancer in most cases grows very slowly at the initial stage of manifestation. At this stage it relies on androgen (male reproductive hormone that seem to fuel prostate cancer growth) to grow, that is why androgen deprivation/depletion therapy (castration) is beneficial for the treatment of early stage androgen dependent prostate cancer (Sha *et al*., 2013). However, the disease at the later stage transforms to androgen independent (castration resistance) impeding effective treatment (Wozney *et al*., 2014). Greater precision and accuracy in the diagnosis, detection and staging of the prostate cancer would help to ameliorate the devastating and deleterious effect of the disease (Aragon *et al*., 2009; Drake *et al*., 2014).

In the U.K. and other parts of the world, prostate cancer is frequently diagnosed when a patient is presenting with lower urinary tract symptoms (LUTS). However, there is possibility of a man suffering from detrusor instability (inability of the bladder muscles to control contraction) or Benign Prostatic Hyperplasia (BPH) resulting in presentation with poor urinary stream, hesitancy and urinary frequency (Verhamme *et al*., 2002). Prostate cancer is mostly asymptomatic at early stages as most of the prostate malignancies stem from the peripheral zone farther from the prostatic urethra. Symptomatic presentation is in most cases associated with prostate metastatic extension to the urethra, bladder neck, neighbouring tissues and glands (Pentyala *et al*., 2016; Litwin and Tan, 2017). Intermittent urinary flow, hesitancy, urinary stream slowing are the obstructive voiding symptoms of prostate malignancy especially during extension to the urethra and bladder neck (Litwin and Tan, 2017; Verhamme *et al*.,

2002). Urgent and frequent urination pose the uncomfortable voiding symptoms though not specific to prostate cancer. With cancer progression, due to obstruction of the ejaculatory duct, sexual symptoms such as slow and low ejaculating volume, and haematospermia may be present. Extension of the prostate metastasis to the neurovascular bundles may lead to erectile dysfunction (Hamilton and Sharp, 2004). Furthermore, metastatic invasion of the skeleton may result to symptomatic bony pains, however, Gohiji *et al.,* (2003) reported that osteoblastic metastases is frequently observed in majority of prostate cancer cases. Other possible symptoms are; vein and lymphatic vessel occlusion causing edema and anaemia due to bone marrow metastatic involvement.

Due to the unprecedented prostate cancer increase across the world, prostate cancer researchers have focused their effort towards valuable and relevant research leading to new prostate cancer therapies. Prostate cancer survival rate has increased thanks to prostate cancer research. However, efforts need to be intensified towards developing new treatment options that could tackle prostate cancer metastases and progression with minimal or no adverse effect.

There is a need to understand the molecular genetic principles that underpin prostate cancer in order to generate molecular minimal toxicity treatment to keep men safe from the devastating effect of prostate cancer. Before now, prostate cancer treatment options include, radiation therapy, surgery, chemotherapy, cryosurgery, hormonal therapy, dietary therapies such as tomatoes, low fat diets and others. These are discussed in subsequent sections. However, apart from dietary therapies, some of these treatment options possess some form of hazardous and cytotoxic effect (Chen and Zhao, 2013). For instance, radical prostatectomy, which is the surgical removal of the cancer affected prostate often goes with the surgery of the surrounding lymph

nodes such as pelvic lymphadenectomy which makes surgery a multimodal (non-monotherapy) strategy with numerous associated side effect such as high PSA levels, risk of lymph node malignancy and exaggerated positive surgical margins (Lawrentschuk *et al*., 2010; Donnelly *et al*., 2010; chen and Zhao, 2013). Hormone therapy for prostate cancer treatment implies the starving of prostate cancer by withdrawal of androgen as androgen presence typically fuels the growth and metastases of prostate cancer. Androgen deprivation therapy (ADT) in combination with surgery seem to be effective and beneficial in the initial treatment of androgen dependent prostate cancer (Perlmutter and Lepor, 2007). Androgen deprivation therapy is a treatment for primary prostate cancer treatment but does not cure cancer when used as a monotherapy (Johnson *et al*., 2010). Commonly applied and combined chemotherapy for the treatment of advanced metastatic prostate cancer include doxorubicin, mitoxantrone, vinblastine, docetaxel, paclitaxel and others. Combination of these therapies has improved survival rate in most cases, but the adverse cytotoxic effect has not been overlooked (Tannock *et al*., 2004). Dietary modification is a double-edged sword, while some diets could help to reduce prostate cancer risk, others could lead to increased risk of developing prostate cancer. For instance, high risks of prostate cancer can be averted by abstaining from high fat diets (Llaverias *et al*., 2010).

Current strategies for prostate cancer treatment also include immunotherapy; for the past few years immunotherapy has remained an essential strategic modality for prostate cancer treatment especially in combination with other strategies for optimal synergistic effect. Studies have reported optimal beneficial synergic effect of immunotherapies with cancer vaccines with minimal or no side effect. One of such benefits is the induction of immune response with huge long-lasting clinical outcome

without significant toxicity (Bilusic *et al*., 2017). In 2010, the U. S. Food and drug authority (FDA) approved the use of an immunotherapeutic drug- Sipuleucel-T (Dendreon Corp) for the treatment of mild symptomatic or asymptomatic castration resistant prostate cancer (Kantoff *et al*., 2010). Another improved treatment option is the use of high-intensity focused ultrasound (HIFU) for prostate cancer treatment. With HIFU average life expectancy and quality of life of men with prostate cancer has dramatically improved with a reduction in prostate cancer age detection and early diagnosis at the stage where curative treatment is possible (Chaussy and thuroff, 2017). High-intensity focused ultrasound is a non-invasive strategy that employs accurate delivery of ultrasound energy to achieve tumour cell death in the absence of surgical excision or radiation.

Furthermore, kinase inhibitors has been suggested to possess effective prostate cancer therapeutic strategy as demonstrated in xenografts. For instance. Wise *et al*., 2017 demonstrated that loss of *PTEN* (tumour suppressor gene) function which leads to phosphoinositide 3-kinase (PI3K) dysregulation is associated with aggressive metastatic prostate cancer development thereby making PTEN a valuable prognostic prostate cancer biomarker to differentiate indolent tumour from the more likely prostatic tumours. BKM120 which is a pan-PI3K inhibitor has been used by Maira *et al.*, (2012) to reduce in vivo prostate cancer (PC3) growth. SPHINX, an SRPK1 inhibitor has been used by Mavrou *et al*., (2014) to drastically reduce tumour volume in prostate cancer induced nude mice.

1.1.1 Structure and function of the prostate gland

The prostate is a male reproductive exocrine gland situated below the urinary bladder posterior to the rectum at the base of the penis (Gupta and McVary, 2017). The prostate gland secrets prostatic fluid stimulated by seminal vesicles which forms part of the semen that nourishes and protects the sperm cells during ejaculation (Levin, 2018). The prostate gland also secretes a special protein PSA (Prostate Specific Antigen) which maintains the liquid state of the semen and is detectable in large amount in the blood stream after recent ejaculation and in disease conditions. One of such disease conditions is prostate cancer and BPH (Fung *et al*., 2004). By definition, prostate cancer is an uncontrolled continuous proliferative growth of the prostate gland (Torre *et al*., 2015).

The prostate gland is a component of male urinary and reproductive system, spherical in shape and measuring about three centimetres thick and four centimetres in diameter. The prostate comprises of two lobes that winds around the urethra. During excretion, the urethra transports urine from the bladder to the penis through the prostate (Ayala *et al*., 1989; Wilson, 2014). Structurally, the prostate gland is made up of prostatic capsule which is a layer of connective tissue covering the prostate. Fibrous cells, muscle cells and gland cells constitute the cells of the prostate gland and are known to provide the gland's structural support, control of ejaculation and urine flow and produce part of the semen fluid respectively. Other structures around the prostate are the seminal vesicles responsible for semen production, nerve bundles located on both sides of the prostate responsible for erectile function control, the *vas deferens* transports sperm to the seminal vesicles from the testes (Wilson, 2014).

There are three zones of the prostate gland which include the peripheral, transition and the central zones. At birth the peripheral zone is the largest zone of the prostate where about 75 percent of prostate tumours are localised, it is the site usually felt by clinicians during digital rectal examination (DRE) due to its proximity to the rectum. The transition zone is located in between the central and the peripheral zones at the middle of the prostate. Before the age of 40, the transition zone makes up about one-fifth of the prostrate and enlarges proportionally with age until eventually becomes the largest part of the prostate at old age leading to benign prostatic hyperplasia (BPH) (Lee *et al*., 2011; Yacoub and Oto, 2018). The peripheral zone is being pushed to the rectum by this enlargement. The central zone is located frontal to the transition zone of the prostrate far from the rectum. Tumours in this zone cannot be diagnosed by DRE because of its distance from the rectum (Yacoub and Oto, 2018).

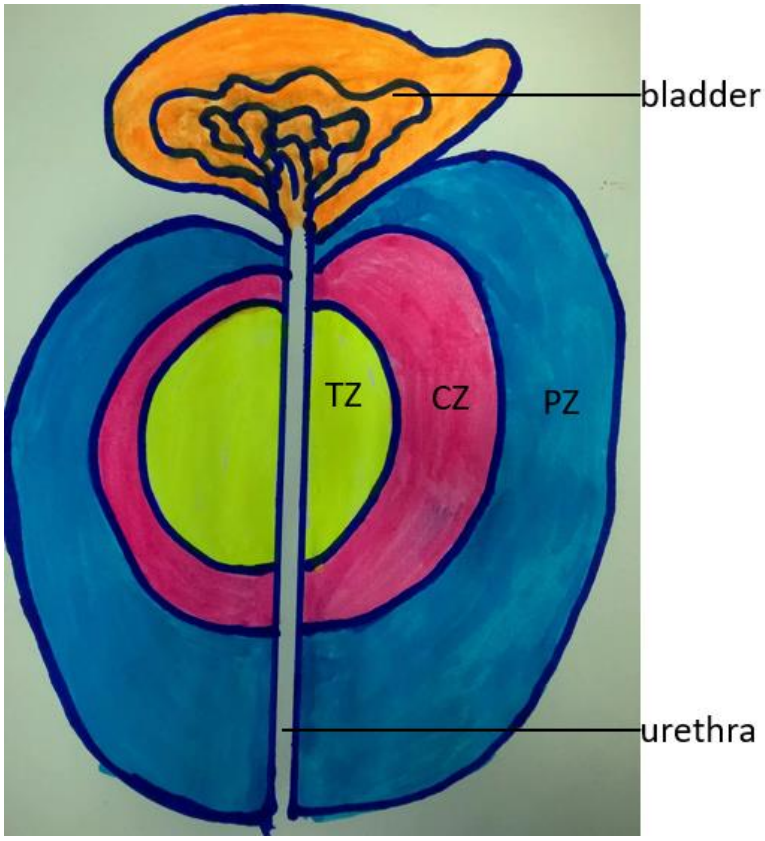

Figure 1.1. Skeletal diagram of the prostate gland. The peripheral zone (PZ), the transition zone (TZ) and the central zone (CZ) of the prostate are duly represented. The prostate gland is located at the base of the bladder. The urethra passes through the prostate. The peripheral zone is the largest zone of the prostate and constitute the site for digital rectal examination. Most prostate cancers are localised at the peripheral zone. The transition zone enlarges with age and later becomes the largest zone at old age. The central zone is located far from the rectum, tumours at the central zone cannot be diagnosed by digital rectal examination due to its distance from the rectum.

1.1.2 Benign prostate hyperplaxia (BPH) and prostate cancer

BPH is a non-metastatic enlargement of the prostate due to prostate cell hyperplasia (Dai *et al*., 2016). This is a rampant disorder of older and frail male population with significant effect on the lower urinary tract (LUT) with or without symptoms (Gupa *et al*., 2015; Wu and Kapoor. 2013). It is the most common diagnosed disease of men over 50 years of age due to prostatic cell proliferation resulting to enlarged prostate

gland, LUT symptoms and urethral occlusion (Homma *et al*., 2011; Skinder *et al*., 2016). Indeed, when men over 50 present with LUT symptoms, BPH becomes a diagnosis of exclusion (Skinder *et al*., 2016). Mobley *et al*., (2015) estimated that one –fourth of male population aged 50 to 65 years are prone to or already have lower urinary tract symptoms. Pathophysiological examinations have described the association of UTI, hormonal dysregulation, genetic predisposition, and inflammation, release of prostate growth factors and pro-proliferative mediators with the development of BPH. In 2018, Chaitoff *et al*., proposed a combination therapy of 5-alpha reductase inhibitor and alphablocker as a potent synergic regimen against BPH (Chaitoff, 2018). Anti- muscarinic drugs (such as cholinolytics), trans urethral resection of prostate, laser therapy and phosphodiesterase-5-inhibitors are also recommended as the current effective therapeutic measures of targeting BPH though most patients do not respond positively to treatment (Rahman, 2016). BPH common risk factors include: advancing age, diabetes, obesity, functioning testicles, family history, race and metabolic syndrome (Sarma *et al*., 2012).

Furthermore, the aetiology of prostate cancer and BPH disorders are yet to be fully understood. Anatomical studies revealed that BPH originates from the prostate transition zone while prostate cancer is an adenocarcinoma which originate from the peripheral epithelial zone of the prostate gland and rarely arise from the transition prostate zone (McNeal, 1998; Miah and Catto, 2014). Autopsy study revealed that about 83.3% of prostate cancer cases occurs concurrently with BPH (Bostwick *et al*., 1992). BPH affects more than 70% of men as they grow older as it is the most common disease of aging men, while prostate cancer is the rampant form of cancer among middle aged and elderly men in UK. It has been statistically proven that the most common cause of lower urinary tract symptom (LUTS) is BPH and not prostate cancer.

However, only few male patients can develop prostate cancer related LUTS. Furthermore, combined results of DRE, PSA and biopsy can serve as a clinical differential diagnosis of BPH and prostate cancer (Konwar *et al*., 2008).

1.1.3 Epidemiology of prostate cancer

In previous centuries, prostate cancer was seen as a very low frequently occurring disorder (Carlsson and Vickers, 2015). Now it is the most common cancer of men all over the globe. In the United States and Europe for instance, prostate cancer is the second most frequent cause of cancer related mortality (with 220,800 estimated new cases and 27, 540 death in 2015) (Siegel *et al*., 2015). In 2018, prostate cancer estimated new cases was 164,690 and 29,430 death (Siegel *et al*., 2018) It is one of the major devastating public health problems of men in developed and developing countries including United Kingdom and United States (Siegel *et al*., 2013; Farlay *et al*., 2013). Autopsy results reveal that approximately 29% of men population already have microscopic evidence of prostate cancer between the age of 30-39 which increases to about 65% at the age of 70 (Chodak and Warren, 2006).

Furthermore, prostate cancer incidence rate varies greatly around the globe probably due to variations in healthcare access, screening potentials and policies. Globally, prostate cancer is the most common third male cancer in the world with about five hundred thousand new cases per annum (Parkin *et al*., 2005; Parkin *et al*., 2001). However, while prostate cancer mortality rate is decreasing in industrial nations such as UK, Canada, Australia, Germany, Italy, France and Spain, its incidence rate is on the increase in the above countries. It has been proposed that the incidence rate of prostate cancer is one in six men, but the mortality rate is one in thirty- six (Gulati *et al*., 2011). This accounts for the age dependency of the disease, occurring between

middle age and the end of life. In most cases symptoms develop before death (Jemal *et al*., 2010; Gulati *et al*., 2011). Therefore, prostate cancer is at times a symptomless progressive disorder. Furthermore, in a global cancer statistics, 2012, Torrre *et al*., (2015) critically analysed the worldwide statistical incidence of prostate cancer as follows: worldwide estimated new cases and prostate cancer mortality as of 2012 are 1,111,700 and 307,500 respectively. In developed countries, the estimated new prostate cancer cases and mortalities are 758,700 and 142,000 respectively. While in developing countries the estimated new cases and death are 353,000 and 165,500 respectively. Diet and environmental exposures account for the higher incident cases in developed countries while in developing countries, the incident is attributed to infections (Torrre *et al*., 2015).

Some researchers have identified genetic and environmental changes as possible cause of worldwide increasing prostate cancer incidence. In USA, the prostate cancer incidence analysis reveals that the prostate cancer incidences of US born Japanese, Chinese and Filipino is little above that of their native immigrants (Shimizu *et al*., 1990). They discovered that the native immigrants' incidences of prostate cancer were about half that of US-born Japanese, Chinese and Filipino. Nomura *et al*., (1991) speculated that prostate cancer disease becomes increasingly common when groups of people migrate from the region of low incidence to the region of high incidence. This correlates the role of genetic and environmental factors.

There is a difference between statistical prevalence and statistical incidence. Statistical incidence provides information of the number of new cases of prostate cancer in a population diagnosed in a specified period, while statistical prevalence gives information of the number or population of people who have prostate cancer in a specific period. However, epidemiological studies of a disease are based on its

incidence (diagnosed new cases) but due to the asymptomatic and latent feature of prostate cancer, many cases of prostate cancer are undetected at the time of diagnosis leading to misleading and untrue prevalence of prostate cancer in a population (Haas *et al*., 2008). This was confirmed by autopsy studies which has reported several latent cancers of the prostate that diagnostic test could not detect (Sakr *et al*., 1994). However, accurate prevalence of prostate cancer disease can be achieved by continuous advances in prostate cancer diagnosis and detection.

Furthermore, the term prevalence also applies to the number of incidence of a known disease condition existing in a given population comprising of diagnosed and visibly present cases. Prevalence of prostate cancer can be evaluated from different sources. Several years ago, transurethral prostatectomy pathological examination was the only means of prostate cancer detection in suspected patients with BPH, on the contrary up to one-fourth of the patients operated for BPH were discovered to have prostate malignancy (Armenian *et al*., 1974; Bostwick *et al*., 1992). However, with the introduction of prostatic specific antigen (PSA) the frequency of such misleading incidence is drastically reduced as most patients booked for BPH operation are tested for PSA (Chou and LeFevre, 2011).

Other sources evaluated the prostate cancer prevalence from the cystoprostatectomy (operation due to invasive bladder cancer) point of view. However, about one-fourth to two-fifth of this category were discovered to suffer prostate cancer and not bladder cancer though it has been investigated that both prostate and bladder cancer share a common route of carcinogenesis (Chun 1997; Abbas *et al*., 1992; Montie *et al*., 1989; Troncoso *et al*., 1989).

In a cancer statistics study published by Siegel *et al*., 2013, the estimated new prostate cancer cases and mortalities in United State is 238,590 and 29,720 respectively. In United Kingdom, in 2015, the incident case was 47,700 and 11,500 deaths per year (Cancer research UK). It has been estimated that above two million men presently living in the United States have prostate cancer and about 16.48% of the entire male population will be diagnosed of the disease in the near future (Jemal *et al*., 2010; Siegel *et al*., 2011). In 2011, over 240,000 men were diagnosed with prostate cancer **(**Howlader *et al*., 2011)**.** In 2006, about 234,460 new cases of prostate cancer was predicted in the USA which is about three times higher than the number of prostate cancer cases that occurred in 1985 (Jemal *et al*., 2006: American Cancer society, 2010). Between 1976 and 1994 prostate cancer mortality increase by 20% but the death rate stabilized and then reduced since 2000 (Ries *et al*., 1997; Oliver *et al*., 1993). Many researchers have proposed possible causes of prostate cancer incidence which include the availability of modern diagnostic and early detecting techniques (PSA and systemic biopsy), increased life expectancy and increased environmental carcinogens (Pienta *et al*., 1993; Pack *et al*., 1993).

The cause of decreased prostate cancer death is still unclear but can be attributed to early detection though the relationship has not been proven, because increased death rate is observed in Australia despite high PSA screening while declined mortality is observed in United Kingdom despite a relatively low screening rate (Smith *et al*., 1998; Oliver *et al*., 2000). However, the impact of early detection is largely attributed and responsible to a great extent for the diminishing rate of advanced prostate cancer (Hankey *et al*., 1999; Hoedemaeker *et al*., 2000). Nevertheless, researches have suggested that improved advanced prostate cancer treatment, radiotherapy and radical prostatectomy are few factors that could explain decline in prostate cancer

related mortality (Master *et al*., 2005; Galper *et al*., 2006). In the trial by the European prostate cancer screening randomized study to determine if screening has an effect on prostate cancer mortality, out of 38,350 men that were randomly selected and screened based on their DRE and PSA levels 1.4% of the men were actually diagnosed with prostate cancer while others had clinically confined prostate cancer (Andriole *et al*., 2005; Schroder, 2005).

1.1.4 Prostate cancer risk factors

Several factors have been queried as contributing to the development of prostate carcinogenesis. However, epidemiological studies suggest that age, race, family history and lifestyle are the core etiological factors of prostate carcinogenesis (Jemal *et al*., 2002; Carter *et al*., 1993).

Family history: the genetic correlation of prostate cancer with family history has not been well elucidated. However, what was formerly defined as hereditary prostate cancer is a diagnosed prostate cancer in the family that fulfils at least one of the following criteria; prostate cancer affecting at least three relatives, prostate cancer diagnosed in two relatives below or at the age of 55, thirdly, prostate cancer identified in each of three successive generations (Carter *et al*., 1993). Scientists suggest that this definition of hereditary prostate cancer tends to exclude some families with X-linked or autosomal recessive transmission (Schaid, 2004). It has been reported that about 5% to 6% of prostate cancer diseases occur ten years earlier than sporadic prostate cancer comprises hereditary prostate cancer (Carter *et al*., 1993). Hereditary prostate cancer also comprises of about one-third of prostate cancer incidences diagnosed prior to 60years of age (Bratt *et al*., 1999). Based on its earlier onset,

hereditary prostate cancer is accountable to greater prostate cancer mortality in male population than sporadic prostate cancer.

In 1996, genetically related prostate cancer was first linked to chromosome 1q23–25 during which hereditary prostate cancer 1 (HPC1) was identified on chromosome 1(susceptibility locus for prostate Cancer) (Smith *et al*., 1996). Other genes such as *RNASEL* (gene responsible for prostate hereditary carcinogenesis) were discovered and studied within this chromosomal region (Schaid, 2004). Other regions of chromosome such as chromosome X have also been linked to play a role in hereditary prostate cancer. Furthermore, prostate cancer incidence variances between regions and populations could be partially attributed or linked to the susceptibility genes and environmental factors heterogeneity. However, research has to be intensified to identify and determine the role and relevance of susceptibility genes in prostate carcinogenesis (Schaid, 2004; Bratt *et al*., 1999).

Age: prostate cancer has been described as an elderly men disorder. It is a disease of middle men and above with rare diagnosis at ages below fifty and high exponential incidence at fifty years and above (Haas and Sakr, 1997). However, the role of age in prostate carcinogenesis is not yet clear but can be linked to age-dependent sclerotic atrophy of the prostate gland which is considered to be a precancerous alteration (Franks, 1954). However, researchers have proven that cancer of the prostate originates from the glandular active epithelium of the prostate rather than the atrophic glands (Michael, 1969). Other research conducted in African countries suggested the absence of histological correlation between prostate carcinoma and aging (Kovi *et al*., 1982). Other studies suggested that prostate cancer is stimulated from accumulation of cellular events and environmental insults leading to neoplasia and the role of aging in cancer is to provide adequate time for the accumulation (Miller, 1980). This

suggestion explains why prostate cancer incidence increases with increased life expectancy.

Race: prostate cancer age-matched studies confirmed the greater frequency and higher grading stage of prostate cancer disease in African American men over their white counterparts (Brawley, 1998; Littrup, 1997). At the time of diagnosis, African American men are younger in age and have low rate of survival probably due to socioeconomic status (Austin *et al*., 1990; Tewari *et al*., 2005). It has been difficult to understand the cause of the higher incidence of prostate cancer in the black African race. Though not yet proven, a researcher has traced the cause to a combination of genetic and environmental factors since elevated levels of androgen metabolites has been reported in black men over their white counterparts (Elis and Nyborg, 1992). Furthermore, several studies have investigated the impact of vitamin D3 in cellular growth, differentiation and regulation of oncogenes. Therefore, studies suggest that the black race have limited ultraviolet induced vitamin D3 production than whites probably due to huge access to sunlight (Schwartz and Hulka, 1990; Buttyan *et al*., 1987). Reduced ultraviolet induced vitamin D3 production is found in black people than the whites (Gerland *et al*., 2006). Diminished serum level of 25-hydroxyvitamin D (25(OH)D) is significant evidence of lack of vitamin D which is predominantly high in all races especially in black American people (Looker *et al*., 2002; Arya *et al*., 2004). Another suggested reason for early development of prostate cancer in blacks more than the whites is attributed to discrepancies and variations in nutrition as the blacks prefer food richer in animal fats (Mettlin *et al*., 1989).

1.1.5 Genetic and other factors in prostate cancer predisposition

A wide range of genetic factors have been implicated in the aetiology of prostate cancer. For instance, metabolic and androgen related genes have been linked to functional prostate growth (Stanford *et al*., 2002). Steroid hormones have been reported to play a role and deeply involved in the regulation of gene involved in prostate oncogenesis, while oestrogen plays a modulatory function, the androgen is responsible for prostate growth and development. Studies also report that decreased oestrogen level could be a risk factor of prostate cancer. Due to its involvement in the stimulation and synthesis of sex steroid hormone (testosterone), cytochrome P-450 steroid 17, a hydroxylase (CYP17) has been seen as a prostate cancer marker (Sobti *et al*., 2008; Niino *et al*., 2000). Relatives of men with BPH early onset pose about 66% risk of BPH related prostatectomy, while relatives of men who have had prostatectomy possess a four- fold increase in age related prostatectomy for BPH and probably prostate cancer. However, first degree relatives of the above scenarios have a six-fold increased risk. Studies of genetic polymorphism of numerous genes associated with metabolic pathway of steroids have been linked with prostate cancer increased risk (Park and Choi, 2014).

There is rapidly growing research suggesting the association of bladder cancer with prostate cancer. In a study by Chun *et al.*, (1997), the incidence of prostate cancer in men with bladder cancer is far higher than prostate cancer patients without bladder cancer. Furthermore, compared to control population, greater number of bladder cancer patients are also diagnosed with prostate cancer (Chun, 1997). The role of some genetic factors in the development of disease could explain this association as *RB* and *TP53* tumour suppressor genes have been linked to both bladder and prostate cancer development (Singh *et al*., 1999).

There is also evidence of overexpression of stem cell antigen in human cell carcinomas (Amara and Palapattus, 2001). There could be possibilities of immunologic response or induced elevated androgen level with high risk of prostate cancer following vasectomy (Giovannucci *et al*., 1993). There are conflicting studies about induction of prostate cancer by early sexual relationship, numerous sexual partners and sexually transmitted diseases (Oishi *et al*., 1990).

The role of cigarette/tobacco smoking in the development of prostate cancer is not yet fully understood. Studies have linked prostate cancer to a known carcinogen (cadmium) found in tobacco or cigarette (Matzkin and Soloway, 1993: Waalkes and Rehm, 1994).

In a cytokine molecular study by Robbert *et al*., 2009, 81% of 283 of samples suspected of prostate cancer were stained positive for CD3 inflammatory T-cell marker and larger prostate volume and symptoms correlate with patients with higher levels of inflammation (Robbert *et al*., 2009). Studies also suggest that CD8+ T-cells are recruited by BPH or PCa epithelial cells due to low level of prostatic dihydrotestosterone (DHT), therefore CD8+ T-cell fuels proliferation of BPH epithelial cells under diminished androgen level (Yang *et al*., 2017). Numerous studies have also linked the association between prostate cancer development and inflammatory infiltrates, these infiltrates are usually found in prostate specimen of men with prostatic disorder and the degree of inflammation correlate with the prostate weight and volume thereby suggesting that chronic inflammation is a contributing factor to prostatic disorders including prostate cancer (Gandaglia *et al*., 2013; Fibbi *et al*., 2010; Kramer *et al*., 2007). During inflammation, angiogenesis and growth factor production by the inflammatory cytokines are affecting prostate tissue. Furthermore, prostate hyperplasia has been described as an immune inflammatory disorder (Dobrian *et al*.,

2001) due to T- cell increased production of ROS whose deleterious effect is associated with DNA damage, tissue injury, aberrant growth, proliferation and neoplasia (Farooqui *et al*., 2011)

Diabetes has been linked with increased risk of LUTS, BPH and prostate cancer (Tseng, 2011). Epidemiological studies have identified obesity, meat and fat consumption as lifestyle factors that could aggravate risk of LUTS, BPH and PCa, while decreased risks are associated with physical exercise and vegetable consumption (Hsing *et al*., 2011; Porter *et al*., 2005). Obesity itself has been known to potentially weaken the potency of 5α-reductase inhibitors (5-ARI). Studies have confirmed the role of vitamin D in prostate volume reduction and decreased prostate cell proliferation especially when induced by testosterone, dihydrotestosterone and growth stimulating molecules such as IL-8, Des (1-3) IGF-1. Therefore, daily intake of 6000 iu of vitamin D supplement is highly recommended (Tuohimaa *et al*., 2007).

Zinc is a metal ion with reference range of 9.6 - 31.6 µmol/L (Ghasemi *et al*., 2012) required for normal enzymatic activities and transcription factors needed for normal growth and development (Iguchi *et al*., 2004). Studies revealed that highest concentration of zinc is found in the normal prostate with much lower concentration in prostate carcinoma and BPH (Franz *et al*., 2013; Prasad *et al*., 2010; Murakami and Hirano, 2008). However, Zhao *et al*., (2016) reported that highest zinc level was found in BPH than normal cells with much lower concentration in malignant prostate cell. The mechanism by which zinc is gradually lost in progressive prostate cancer is yet unknown (Zhao *et al*., 2016). Zinc in the form of $ZnCl_2$ was reported to inhibit androgen-responsive cell growth which also slowed down prostate cancer growth and proliferation (Liang *et al*., 1999; To *et al*., 2018). Research has examined the effect of zinc on prostatic lesion. Progressive high levels of serum zinc was identified in benign

and malignant prostatic lesions of patients compared to normal and healthy subjects (Zhao *et al*., 2016).

It has been reported that abdominal accumulation of fat is associated with urological disorders such as erectile dysfunction, urinary incontinence, BPH and prostate cancer (Kolonel *et al*., 1999). Fatty acids and prostate disorders have been linked to lipids peroxidation, inflammatory mechanisms, oxidative stress, 8-hydroxy-2'–deoxyguanosine accumulation and increased androgen synthesis stimulating prostate growth and enlargement (Kolonel *et al.,* 1999; Khandrika *et al*., 2009). It has been reported that high fat diet is responsible for metabolically triggered inflammation though the process is still unclear but thought to be through oxidative stress resulting to increased vulnerability of the prostate to several diseases including prostate disorders (Rahman, 2016).

1.1.6 Diagnosis of prostate cancer

Between 1960s and 70s, Dr. Donald Gleason in a prospective study involving 2,900 patients, pioneered the invention of grading and staging of prostate adenocarcinoma based on histological growth patterns in comparison with prognosis and clinical data (Gleason, 1977). In 2004, the world health organisation (WHO), adopted this grading pattern and it was named "Gleason" after the inventor (Eble *et al*., 2004). The Gleason (grading) pattern (figure 1.2) ranges from 1 to 5 which denotes the best differentiation (better prognosis) and the poor differentiation (poor prognosis) respectively. The term Gleason score was developed to facilitate proper, efficient and accurate calculation of the grading (Gleason). The Gleason score is the summation of the Gleason patterns harboured by primary and secondary prostate adenocarcinoma. Where only one histological grading exists, for instance, if the primary Gleason is 3 it is assumed that

both primary and secondary Gleason are same. In this case the Gleason score is 6 (i.e 3+3) (Gleason, 1977; Chen and Zhou 2016; Amin *et al*., 2004).

From the original Gleason classification pattern of Dr. Donald Gleason based on haematoxylin and eosin staining, the following features are linked with Gleason pattern 1: separate, single, uniform round, closely packed glands in a well circumscribed nodular borders (figure 1.2) (Eble *et al*., 2004). However, this pattern is very rare and the features was later discovered to be mistaken features of adenosis, otherwise called atypical adenomatous hyperplasia (AAH) and not prostate cancer (Eble *et al*., 2004). Features of Gleason 2 are almost same as 1 but with less peripheral circumscription, more separated stromal glands (figure 1.2), no stromal infiltration and more variable glandular shapes (Eble *et al*., 2004). This pattern was also suggested to be rare as in 1 above (Humphrey, 2004; Berney, 2007). Gleason grade 3 consists of a poorly differentiated edge with some structures still circumscribed, irregularly separated, closely packed, single and many variable glands, slight infiltration with more loosed nodules, angulated or compressed glands, pseudo atrophic appearance of large glands or undulating luminal contours in minimal sized glands. Gleason grade 3 is the most common Gleason score (McKenney *et al*., 2011; Humphrey, 2004). Features of grade 4 Gleason are as follows: fused glands due to non-existing intervening stroma in adjacent glands, predominant presence of acinar structures with or without formed lumina, presence of papillary tumours with invasive irregular edges, abnormally enlarged cribriformed gland nodules without supporting stroma (Dong *et al*., 2013; Kweldam *et al*., 2015; Amin *et al*., 2004). Gleason grade 5 possesses the following features; presence of comedonecrosis which comprises intraluminal central necrotic cells with papillary spaces, cord formation with vacuoles generally referred to as signet ring cells with absence of glandular lumens and presence of single cells

(Shah *et al*., 2015; Amin *et al*., 2004, Chen and Zhou, 2016). Some of these grading features are depicted in figure 1.2.

The original Gleason pattern has been modified and updated due to results obtained from new testing and diagnostic modalities such as needle biopsy, PSA and surgical procedures. For instance, results from multiple radical prostatectomies and needle biopsies from multiple sites need to be taken into consideration by pathologists and incorporated in the Gleason grading system (Chen and Zhou, 2016). One of the major consensus reached by two-third majority of 70 leading urologist/pathologist during 2005 international society of urologic pathology (ISUP) consensus is that Gleason score 1+1 equals 2 diagnosed by needle biopsy should be stopped, as this is a typical diagnosis for adenosis (Eptein *et al*., 2006; Osunkoya, 2012). Needle biopsy Gleason scores of 3 and 4 from various combinations of primary and secondary Gleason such as 2+2, 1+2 and 2+1 is considered controversial due to their non-correspondence and poor reproducibility with the grading of prostatectomy samples, more so the edge of the nodule from needle biopsies made from the transition zone is difficult to assess. Therefore, the ISUP 2005 recommends that diagnosis involving Gleason score 3 and 4 from needle biopsy be performed in conjunction with expert urologic-pathologist. On occasions where there is moderately or minor irregular nodule edge on Gleason score 2+3 equals 5 or vice versa, diagnosis can only be made by experts (Epstein *et al*., 2005).

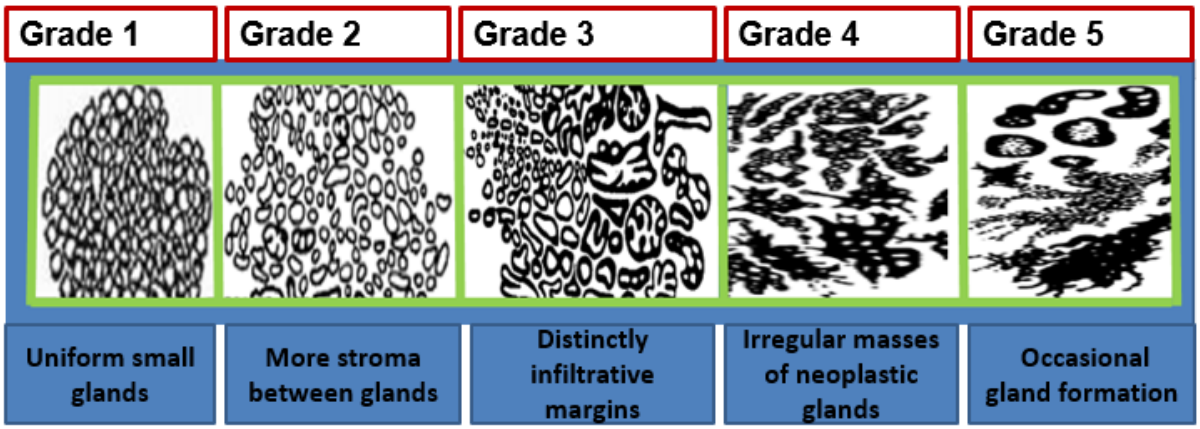

Figure 1.2 Gleason grading of prostate cancer. This depicts some of the features of the various Gleason grading patterns of prostate cancer

Prostatic specific antigen (PSA), otherwise known as human kallikrein 3 (hK3) with molecular weight of 33KDa. It is called (hK3) because it is a serine-protease of kallikrein tissue identified in 1970s from human prostatic extracts and are involved in seminal fluid synthesis (Ablin *et al*., 1970). In 1979, Wang *et al*. discovered PSA in human sera, followed by its isolation from prostate tissue in 1980 by Papsidero *et al* (Papsidero *et al*., 1980) confirming its relevance as a biomarker of PCa that could be used in a screening test (Potosky *et al*., 1995; Stephenson *et al*., 1996). The acinar cells, the ductal cells and the periurethral gland of the prostatic epithelium produce PSA. Produced PSA is subsequently transported into the seminal fluid where it liquefies seminal coagulum during ejaculation as its primary physiological function (Robert *et al*., 1997).

There have been increases in the detection of prostate cancer following the introduction of the PSA test and efforts have been geared towards its improvement as a prostate cancer screening test. However, PSA is not a specific prostate cancer marker since its serum levels are elevated in other conditions such as recent ejaculation, prostatitis, prostate hyperplasia, cigarette/tobacco smoking, digital rectal

examination (DRE), urologic manipulations and medication such as finasteride (Guess *et al*., 1993).

Furthermore, Stamey and research colleagues demonstrated in 2004 that PSA measurement is an accurate biomarker in detection of prostate diseases including prostatitis, prostate cancer and BPH, but not necessarily prostate cancer (Stamey *et al*., 2004). In 2005, Thompson *et al* demonstrated that PSA screening is very sensitive in prostate disease detection (detecting both mortal and non-mortal cancers) but not specific to prostate malignancies (Thompson *et al*., 2005). Notwithstanding these limitations PSA screening has aided the early detection of the disease as it is often diagnosed late due to its asymptomatic during development (Chodak *et al*., 1989). In 1987, there was a substantial increase in PSA screening tests which resulted in a dramatic increase in prostate cancer detection and therefore treatment (Ries *et al*., 1999; Ellison *et al*., 1999). Prostate cancer specific deaths reduced in 1993 as more cancers were detected early and treated (Hankey *et al*., 1999). The improved early detection and screening of prostate cancer resulted in intensified research on prostate cancer chemoprevention and chemotherapy.

Initial evaluation of PSA as a screening test reveals that the best predictive value clear cut off is 4ng/ml. This cut off allows for detection of still confined and curable prostate cancer and reduces rates of unnecessary biopsies in non- prostate cancer patients (Catalona *et al*., 1991). Based on research, this 4ng/ml bench mark is recently in doubt as 48% of patients with PSA Level <4ng/ml suddenly measured >4ng/ml within a space of four years and 13% of these patients were diagnosed of prostate cancer (Smith *et al*., 1996). In similar research, prostate cancer was detected in 15.2% of 2,950 men with normal DRE and PSA level <4ng/ml (Thompson *et al*., 2004). Based on these reports, researchers recommend PSA cut off of 2.6ng/ml as an ideal

reference (Smith *et al*., 1996). However, some authors suggest adjustment of PSA cut off for frail and younger men since serum PSA levels increase with increase in prostate volume and age thereby resulting in low specificity and sensitivity PSA screening values in frail and younger men respectively (Partin *et al*., 1996).

PSA use in prostate cancer screening has gained global preference to TRUS and DRE for economic, specificity, and effectiveness reasons. Therefore, PSA is the most widely recognised prostate cancer screening parameter; but it is also the most controversial prostate cancer marker due to misleading impression of either absence or presence of prostate cancer at varying PSA levels (Shan *et al*., 2017). Researchers have intensified efforts to authenticate the relevance of PSA in early prostate cancer detection. However, it is recommended that its combination with other diagnostic measures for prostate cancer screening will give more specific, efficient and reliable results. Babaian and Camps, (1991) suggested that a PSA value of 4ng/ml in conjunction with positive DRE is diagnostic of prostate cancer. A study also suggested that to maintain accuracy and precision in the use of PSA for prostate cancer screening, men with increased PSA levels should be subjected to at least four kallikrein markers screening test to guide biopsy decisions (Ferro et al., 2016). However, Ferro *et al*., 2016 suggested that prostate cancer screening with PSA should be ideally carried out two or three times in a life time between the age of 55 and 59 as over diagnosis is possible at age 63 and above.HK2 is closely related to PSA with 80% conservation in amino acid sequence. Like PSA, elevated levels of HK2 are most frequently seen in the prostatic tissue (Kumar *et al*., 1997; Yousef and Diamandis, 2001). HK2 is otherwise called free PSA (fPSA) because it is free and unbound in the serum. It is predominantly used to differentiate between normal and cancerous prostate cells (including BPH and prostate cancer) (Kwiatkowski *et al*., 1998; Nam *et*

al., 2000). Elevated levels of free PSA in a total PSA is an indication of BPH, not cancer (Catalona *et al*., 1995). However, it has also been reported that increased total PSA with minimal elevated values of fPSA is suggestive of prostate cancer. In 1999, Partin and his research colleagues reported that prostate cancer is better diagnosed, identified and confirmed when there is elevated levels of HK2 in combination with lower levels of PSA (less than 25%) and when the HK2/PSA ratio is less than 0.18 (Partin *et al*., 1999; Hong, 2014). A comparative study of HK2 and PSA levels in preoperative prostatectomy patients (Haese *et al*., 2000), revealed that the 37% sensitivity and 100% specificity of HK2 in detection of organ confined prostate diseases compared to 14 and 100% sensitivity and specificity (respectively) for total PSA.

In a recent study, Lee *et al*., (2015) reported serum levels of total HK2 detection through effective silicon antibody immunoassay which improves prostate cancer detection with or without PSA. However, Mao *et al*., (2018) suggested that the combination of biomarkers such as HK2, PSA and prostate cancer gene 3 (*PCA3*) provides a greater beneficial effect in prostate cancer detection and diagnosis.

It has been clear for some time that some cases of prostate cancer (latent prostate cancer) are undetected or misdiagnosed. Pathologically, the term latent prostate cancer applies to hidden prostate cancer that can only be identified through post-mortem examination of prostate specimen (Sakr et al., 1994). Denton *et al*., (1965) reported about 25% of patients diagnosed and treated for BPH actually had prostate cancer after transurethral surgery and examination of their specimen (Denton *et al*., 1965). This also implies that the true prevalence and epidemiological studies of prostate cancer is difficult to measure with such dormant/hidden form of prostate cancer. This has declined the use of PSA as the only screening test. Therefore true

prevalence of prostate cancer can only be measured by combining the results of PSA tests, transuretheral examination, crystoprostatectomy, biopsy and autopsy (Draisma *et al*., 2003).TRU is very unspecific to prostate cancer since it has been found that minimal percentage of hypoechoic ultrasound images correlate with prostate cancer (Norberg *et al*., 1993), and one- fifth of normal ultrasound images of the prostate gland are positive for prostate malignancy after biopsies (El-Gabry *et al*., 2001). Because TRU guides systemic biopsies, it is also called guided biopsy. Several studies suggest that far lateral zone prostate biopsy should replace systemic sextant biopsy which had remain the gold standard of prostate biopsies for several years until about 31% false negative results was reported (Epstein *et al*., 1997). Eskew *et al*., reported about 34% increased detection of prostate cancer in far lateral zone which was previously missed by sextant TRU (Eskew *et al*., 1997). Eskew *et al*., 1997 also conclusively reported that the lateral zone TRU involving systematic five region prostate biopsy detects more tumours than the sextant ultrasound, due to its efficacy of prostate cancer detection at significant early stage.

Kong and Byun, (2013 and 2015) reported the relevance of prostatic acid phosphatase (PAP) in prostate cancer prognosis and diagnosis. They reported that PAP levels increase proportionately with increasing prostate cancer progression, thereby making PAP a good prognostic tool in prostate cancer management. Kong and Byun also proposed that prostate cancer progression could be arrested by introducing molecules such as an RNA ligand aptamer that specifically binds to PAP (Kong and Byun, 2015). PAP was the first biochemical marker employed for prostate cancer staging and diagnosis. Inorganic phosphates are produced in a hydrolization reaction of esters by PAP in acidic medium. PAP is not a specific prostatic marker as it is being synthesized in other organs such as the brain, liver and lungs, but majority of it is secreted from

the prostate epithelia cells and transported into the glandular lumen (Kong and Byun, 2013).

Earlier studies have reported increased levels of PAP in patients with BPH and more elevated levels in patients with metastatic prostate cancer (Gytman *et al*., 1938, Pentyala *et al*., 2016). Another study reports that 84% of 102 patients with increased levels of PAP were diagnosed of various degrees of prostate metastases (Bahnson *et al*., 1987). Lowe *et al*., (1993) suggested that surgically curable prostate cancer is difficult in patients with elevated PAP. However, the introduction of PSA has drastically reduced the use of PAP in prostate cancer diagnosis and staging.

In patients with different forms of malignancies such as breast, lung, colon and prostate cancers, insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding proteins (IGFPBs) are important markers for tumour progression and prognosis (Yu and Rohan, 2000). These factors also play vital roles in both malignant and normal cell proliferation and transformation (Zumkeller, 2001). In an earlier study (Djavan *et al*., 1999) detected IGF-1 in prostate cancer patients and recommends the use of IGF-1 with PSA in early prostate cancer detection. However, this was disputed in a study by (Shariat *et al*., 2000) who suggested that there was no correlation between prostate cancer and IGF-1 levels. However, serum concentrations of IGFBP-2 and IGFBP-3 are examined in the assessment of disease progression. For instance, lowest levels of IGFBP-3 were found in bony metastatic patients, relatively higher in confined diseases and highest in healthy individuals (Shariat *et al*., 2002). On the other hand, IGFBP-2 is higher in organ confined diseases and lower in disease progression (Yu and Rohan, 2000). Furthermore, Chan *et al*., (1998) reported that increased levels of IGF-1 is majorly associated with high prostate cancer risk. Harman *et al*., (2000)

also reported that elevated serum levels of IGF-1 and low IGF-II are independently linked with high prostate cancer risk

Furthermore, transforming growth factor-β1 (TGF-β1), TGF-β1 cytokines have been implicated in many stages of tumorigeneses and TGF-β1 elevation have been identified in many cancer patients including patients with prostate malignancies (Blobe *et al*., 2000). TGF-β1 is reported to be involved in numerous cancers (including prostate cancer) progression, proliferation and aggressive malignancies (Fuxe *et al*., 2012). Normally, elevated presence of TGF-β1 in disease is an indication of progressive deterioration and poor prognosis (Wu *et al*., 2015; Drabsch and ten Dijke, 2012). Immunohistochemical studies have been used to differentiate normal prostate tissue from neoplastic prostatic epithelium due to increased expression of TGF-β1 in cancer (Perry *et al*., 1997). Shariat *et al*., (2001) and Adler *et al*., (1999) have confirmed the positive association between TGF-β1 plasma concentrations and prostate cancer progression. However, this assumed correlation was refuted by Perry *et al*., (1997), but rather uphold a strong correlation between urinary levels of TGF-β1 and prostate cancer progression. Shariat *et al*., (2004) reported plasma TGF-β1 elevation in extra prostatic (spread to vesicle and lymph nodes) pre and postoperative patients. In 2015, Wu *et al* conclusively reported TGF-β1 as a potential biomarker and could be targeted suggesting a future potential prostate cancer therapy (Wu *et al*., 2015).

Interleukin-6 (IL-6) is also a prostate cancer biomarker as some studies propose that there are signs of inflammatory response in most prostate cancers and that there is an association between stromal proliferation and T-cell infiltration, suggesting the involvement of IL-6 and IL-8 (Drabsch *et al*., 2012; Handisurya *et al*., 2001). Abbasabad *et al*., (2018) also proposed a strong relationship between inflammation

and the presence of malignant prostate cells. IL-6 is recognised as an inflammatory molecule synthesized and released by malignant cells (Abbasabad *et al*., 2018). IL-6 is an immunological factor that regulates numerous cellular activities such as bone turn over and immune function. Comparative studies have shown 18 and 8-fold increased concentration of IL-6 protein and receptors respectively in confined prostate cancer than normal prostate tissues (Giri *et al*., 2001). Furthermore, Shariat *et al*., (2001) reported elevated plasma IL-6 in metastatic prostate cancer patients and increased IL-6 soluble receptor levels in patients with prostatic bone metastases followed by patients with lymph nodes involvement. Shariat *et al*., (2001) also carried out a research on 309 prostate localized disease patients and reported increased IL-6 and its receptor levels in preoperative patients which drastically decreased after prostatectomy irrespective curative achievement. Giri *et al*., (2001) and Adler *et al*., (1999) detected elevated levels of IL-6 in both human prostate cancer and BPH and suggest that overexpression of IL-6 could be a serious sign of malignant prostate cells. In Northwest population of Iran, Abbasabad *et al*., (2018) identified a mutation (single nucleotide polymorphism-SNP) in interlukin-6 gene (IL-6 -174G>C (rs1800795) and its association with prostate cancer metastasis.

Magita *et al*., (2009) observed that fatty acid synthase gene *(FASN*) is a prostate cancer oncogene which exerts its oncogenic properties by inhibiting the intrinsic apoptotic pathway. Apart from adipose tissues and liver, *FASN* is hardly expressed in normal tissues but highly expressed in tumour cells and its overexpression in tumour is an indication of poor prognosis (Kusakabe *et al*., 2000; Carvalho *et al*., 2008). In 2006, it was discovered through fluorescent in situ hybridization that 25% of human prostate cancer tissues are highly positive for FAS protein (Shah *et al*., 2006). Oncoantigen 519 (OA-519) immunohistochemical staining in combination with FAS

was first used by Epstein *et al* to study the pathological stages of prostatectomy specimens (Epstien *et al*., 1995). They confirmed that QA-519 immunohistochemical staining provides excellent predictive information of the pathological grades and progression of prostate cancer than Gleason scores. Furthermore, Swinnen *et al*., (2002) identified increased FAS signalling from a low to high grade prostatic intraepithelial neoplasia (PIN) in frozen needle prostate biopsies. The study suggests that higher FAS signalling correlates with higher prostate cancer proliferative index and FAS expression is an indicative of early prostate cancer development (Swinnen *et al*., 2002).

Early prostate cancer antigen (EPCA): numerous cancers are clinically diagnosed by critically observing the cell nuclear matrix to determine the shape and the texture of the nucleus under microscope, the nuclear matrix protein of the prostate otherwise known as early prostate cancer antigen (EPCA) has been identified as a potential prostate cancer biomarker. However, the nuclear matrix protein of other cancers constitute their respective specific early cancer antigens (Zhao *et al*., 2010; Leman *et al*., 2008). Immunohistochemistry has been used to identify a nuclear protein linked with prostate cancer (Ohir *et al*., 2004). Ohir *et al*., (2004) also studied the efficacy of EPCA in prostate cancer biopsy specimens and found that EPCA staining possesses 84 and 85% sensitivity and specificity respectively in prostate cancer detection. The study also reports that EPCA immunohistochemistry may provide the opportunity to detect PCa five years earlier than PSA and drastically reduce the number of biopsies due to misleading increased PSA levels (Ohir *et al*., 2004).

Use of urinary secretory markers for prostate cancer detection (USM): the prostate secretes prostasomes and exosomes as two forms of microvesicles; the prostasomes are secreted from the normal prostatic duct which form part of the semen and are

important for male fertility (Burden *et al*., 2006). The exosomes are nanovescicles secreted by both tumour and normal cells. However, exosomes have been found to be over-secreted in urine, effusion and serum of prostate cancer patients (Mitchell *et al*., 2009; Wilkosz *et al*., 2011).

Numerous studies have identified a novel protein called PCA3 as a useful marker that can be used with DRE and PSA due to its specificity to enhance accuracy of prostate cancer diagnosis and grading (Tinzl *et al*., 2004). Number of biopsies performed on patients suspected of prostate cancer have been lowered by the introduction of PCA3 urine test due to its potentials of specific prostate cancer detection (Nakanishi *et al*., 2008). Other conventional prostate cancer urine markers are: 8-hydroxy-2'-deoxyguanosine (8-OHdG), Annexin A3 (ANXA3), δ-Catenin, Endoglin, Oestrogen metabolites, GOLM1, LOH (loss of heterozygosity), Met, Alpha-methylacyl CoA racemase (AMACR) etcetera (Wilkosz *et al*., 2011).

Cancer cells tend to resist therapy due to the expression of numerous splice variants associated with poor survival rate. Research suggest that cancer cells specific splice variants expression are good prognostic and diagnostic markers of cancer including prostate cancer (Pal *et al*., 2012). More so, prostate cancer specific variants of crucial genes such as *CCND1* (a gene whose overexpression is associated with cancer chemo resistance (Noel *et al*., 2010) could be used as a therapeutic measure to target proteins of non-healthy cells. It was suggested that it might be helpful to reduce prostate tumour growth by developing a compound or tool that will preferentially and specifically modulate transcript variants expression of prostate cancer cells at the expense of healthy cells. Zhang *et al*., (2006) was able to differentially separate prostate cancer cell lines from other organ's cancer cell lines using splicing-sensitive microarray that is made up of selected splice variants and genes subset. Using a

splicing-sensitive microarray in biopsy sample, it was possible to specifically distinguish between splicing signatures of neoplastic prostate and normal prostate cells. However, this suggests that during prostate tumorigenesis, there is evidence of splicing changes thereby presenting splicing variants important and precise prostate cancer biomarkers. Numerous groups have performed a comparative analysis comparing cancerous prostate and normal prostate. In general, there would appear to be consistent, changes in the expression of alternative splice variants in prostate cancer (Hagen and Ladomery, 2012; Lapuk *et al*., 2014; Hagen *et al*., 2013; Venables *et al*., 2006).

Li *et al*. (2006) using exon arrays validated by RT-PCR established splicing changes in prostate cancer. About one thousand five hundred mRNA variants from prostate cancer genes were examined using splicing arrays. This approach could help to differentiate prostate cancer cell lines from cell lines of other tumours based on differences in splicing patterns.

Prostate cancer progression has been linked with many specific splicing patterns (Watson and Watson, 2010; David and Manley, 2010). For instance, in prostate cancer, prostate-specific membrane antigen (PSMA) is up-regulated (Watson and Watson, 2010). Four specific splice PSMA isoforms exist which are useful prognostic indicators for PCa. They include: PSM, PSM-C, PSM-D and PSM-E (Watson and Watson, 2010; Chen and Miller, 2013). The progressively increased expression of these antigens (for instance, from PSM to PSM-E) is associated with the highest grade of prostate cancer. The preferential expression of specific isoforms also creates opportunities for detection of prostate tumour grades using immunological techniques (Williams and Kole, 2006; Watson and Watson, 2010; Cao *et al*., 2012).

1.1.7 Established treatments of prostate cancer

Prostate cancer screening and diagnoses has led to early detection of the disease and higher prevention of prostate cancer related death. However increased screening has resulted in prostate cancer over diagnosis and treatment of indolent tumours with the associated chemotherapy toxicity and other side effects (Gulati *et al*., 2011; Etzioni *et al*., 2002). Therefore, instead of chemotherapy, radiotherapy or prostatectomy, active surveillance and monitoring/watchful waiting has become a viable corrective option for patients who have still confined prostate cancer (Lund *et al*., 2014). It is an indirect way to delay aggressive treatment in low risk PCa patients who are unlikely to develop symptoms in a life time. The patients are followed up by PSA, DREs and biopsies and about one-third of the patients out of psychology, anxiety and distress switch to active therapy especially when symptoms are likely to develop following biopsy results (Dall'Era *et al*., 2012). In the 80s active surveillance and watchful waiting are useful approaches to monitor progress of prostate cancer. The former is a situation where suspected confined prostate cancer patients are not treated but monitored until evidence of local metastasis is established, here frequent tests are required to monitor cancer growth. While the later is otherwise called watch and wait, a situation where time is allowed before medical intervention is needed (Bill-Axelson *et al*., 2008). However, Klotz suggested that nowadays due to multiple co-morbidities watchful waiting can only be offered to prostate cancer patients with at most five years life expectancy (Klotz, 2013). Furthermore, both active surveillance and watchful waiting are meant for men with low risk and low grade prostate cancer with Gleason score and PSA less than 6 and 10 respectively. However, while watchful waiting is a viable option for older men, younger men less than 65 years with considerable life expectancy are likely to benefit from active surveillance (Miller *et al*., 2018).

Prostatectomy (surgical removal of part or whole of prostate gland) is the oldest means of treating confined and non-malignant and high risk prostate cancer to avert complications of urinary incontinence and advanced metastases (Meuleman and Mulders, 2003: Petrelli *et al*., 2014). There is usually a high rate of erectile dysfunction following post-prostatectomy though the aetiology and pathophysiology has not been fully established. However, Meuleman and Mulders, (2003) suggested that prostatectomy is not the core cause of erectile dysfunction, that erectile dysfunction following prostatectomy is a coincidental dysfunction often mistaken for already pre-existed erectile dysfunction due to old age (since prostate cancer is diagnosed in between 6 to 8 decades of life). Radical prostatectomy in most cases is not used as a prostate cancer monotherapy because high-risk prostate cancer still require supplementary adjuvant treatment such as androgen deprivation therapy (ADT) and radiotherapy to avert metastasis. However, Petrelli *et al*., (2014) suggested that radical prostatectomy can be used as a single therapy only in patients with quality life expectancy without significant competing death challenges. Heidenreich *et al*., (2011) in line with leading guideline indicated that radical prostatectomy is non-beneficial to candidates with less than 10 years life expectancy; however, it is preferred to radiotherapy for patients with life expectancy from 10 years and above at the time of initial diagnosis. According to the surveillance epidemiology and end resort (SEER) database, radical prostatectomy has been reported to improve survival more than radiotherapy (Sun *et al*., 2013).

Adjuvant radiotherapy is required to significantly minimise risks of disease reoccurrence and PSA relapse (biochemical recurrence) in patients who had radical prostatectomy of pathological advanced prostate cancer (Thompson *et al*., 2006). Radiotherapy involves the use of ionization radiation to kill or disable proliferation of

malignant cells. Two types of radiotherapy exist, they include; Brachy therapy (BT) which is employed for treatment of small and confined prostate tumours by radioactive capsule implantation right inside the prostate to kill tumour cells. External beam radiation therapy (ERT) employs high energy X-rays directed to the prostate to damage and quench cancer cell division while affected normal cells undergoes auto repair (Baskar *et al*., 2014; Bentzen, 2006). Incrocci *et al*., (2002) reported cases of 84% and 51% of erectile dysfunctions following external beam radiotherapy and brachytherapy respectively. ERT in combination with hormone therapy is often a treatment of choice for men with high stage prostate cancer with PSA greater than 10 ng/ml while either BT or radical prostatectomy could be beneficial to men with small prostate tumour who are below 70 years with PSA less than 10 ng/ml in the absence of other risk factors such as pulmonary and cardiovascular disease (Bolla *et al*., 1997; Incrocci *et al*., 2002).

Androgen deprivation therapy was first used in 1941 to supress testosterone release from the hypothalamus in advanced prostate cancer patients. This is achieved either by surgical castration or by administration of diethylstilbestrol (DES) which inhibits the production of luteinizing hormone-releasing hormone (LHRH) from the brain (Perlmutter and Lepor, 2007; Huggins and Hodges, 1941). Androgen deprivation therapy (ADT) is often used as an adjuvant in combination with radical prostatectomy or radiotherapy for prostate cancer treatment to improve survival. For patients with locally advanced prostate cancer, long term ADT administration up to three years is recommended, while six months short term treatment is ideal for patients with localized prostate cancer (Bolla *et al*., 2009; Pisansky *et al*., 2015). ADT treatment accounts for about 90% sexual dysfunction and decreased libido in men receiving treatment, there is also detrimental effect of ADT treatment on mental and bone health, diabetes,

cardiovascular disease, cognition, erection and testicular size leading to distress and regret of treatment (Hadziselimovic *et al*., 1987; Haliloglu *et al*., 2007).

Cryotherapy, a technique that involves the insertion of liquid nitrogen or argon gas to the prostate through a transurethral cryoprobe to ensure the death of localized prostate cancer by extremely low temperature (freezing). Cryotherapy was first introduced in 1966 by the American Urology Association (Han *et al*., 2003; Gonder *et al*., 1966). Third generation cryotherapy was established by monitoring the inserted cryoprobe and the temperature of the surrounding tissues through transrectal ultrasound control (Sverrisson *et al*., 2013). Principally, cryotherapy induces cell death by either apoptosis or coagulation necrosis through dehydration resulting to cell membrane protein denaturation and disruption by ice (Hoffmann and Bischof, 2002). Rodriguez *et al*., (2014) suggested that cryotherapy is a valid option treatment for men with localized tumours who have high surgical risk and complication from radiotherapy, beneficial effects of cryotherapy include: high survival cryosurgery, overall survival rate and rare cases of biochemical recurrence. However, it can be repeated after histological confirmation of biochemical relapse (Rodriguez *et al*., 2014).

The contrast of cryotherapy is high intensity focussed ultrasound (HIFU). This is a non-invasive approach that employs the use of high ultrasound energy (heat) delivered to the prostate gland (without damaging the surrounding tissue) to achieve cancer cell necrosis in the absence of surgical excision and radiation (Chaussy and Thuroff, 2017). This technic is feasible because ultrasound beams transmission are capable of passing through a targeted solid tissue without surgical operation/excision or insertion of probes (Wu *et al*., 2001). Over the years they have been recorded success on the use of transrectal high intensity focused ultrasound for the treatment of prostate cancer and BPH (Nakamura *et al*., 1997). Blana *et al*., (2004) reported the efficacy of HIFU in

the treatment of confined prostate cancer with minimal morbidity and repeatable in cases of biochemical relapse. This procedure is a treatment alternative for men with prostate cancer who lack suitability for radical surgery.

Apart from erectile dysfunction and urinary incontinence, other complications associated with prostate cancer treatment cannot be overlooked. These complications in some cases may lead to a lot of interventions such as surgery, rectal-anal and urologic interventions due to gastrointestinal or genitourinary haemorrhage, infections and urinary occlusion (Wallis *et al*., 2018). Studies suggest though radiotherapy has a minimal risk of invasive procedures yet radiotherapy is hugely associated with high and long term risk of secondary cancer, hospitalisation, major surgical and rectal-anal procedures (Nam *et al*., 2014; Wallis *et al*., 2015). Other complications of radiotherapy that is often associated with morbidity include; chronic tissue ischemia, bowel symptoms and urinary fistulae leading to urinary diversion (Bassett *et al*., 2016). Compared to watchful waiting, it has been reported that radical prostatectomy has low rate of urinary occlusion (Steineck *et al*., 2002).

Research suggests that prostate cancer can be treated with metformin. Metformin is an anti-hyperglycaemic therapy used to lower blood glucose of type 2 diabetic patients (Shaw *et al*., 2005). Apart from the anti-hyperglycaemic property, metformin has been reported to possess the therapeutic advantage of retarding and preventing the risk of cancer growth including prostate cancer (Currie *et al*., 2012; Preston *et al*., 2014). Lipogenesis and androgen receptor pathway inhibition are the possible mechanisms via which metformin inhibits prostate cancer growth, proliferation and migration. Another suggested mechanism is by up regulation of PEDF (pigment epithelium derived factor) secreted in many tissues including prostate stromal and epithelial tissues (Wang *et al*., 2015; Chen *et al*., 2016). It has been reported that PEDF is

downregulated and under expressed during malignant prostate cell transformation. Therefore, Chen *et al*., (2016) hypothesized that the antitumorigenic effect of metformin is dependent on PEDF upregulation and overexpression. Chen and his research team also discovered that PEDF upregulation results to increased apoptosis, reduction in prostate cancer migration and invasion both in vivo and in vitro in a dose and time dependent manner of metformin.

Cell biology, epidemiological and animal in vivo studies suggest the effectiveness of lycopene (which gives tomatoes its characteristic red colour) as an anti-cancer agent including in prostate cancer and also reduces the risks of cardiovascular diseases (Xin *et al*., 2016; Story *et al*., 2010). Epidemiological studies revealed that lycopene blood levels and its dietary intake are linked to prostate cancer reduced risk (Wang *et al*., 2015). Research also suggests that the lycopene content of watermelon is greater than that in tomatoes. Numerous epidemiological studies have reported lower risk of prostate cancer in subjects with increased tomatoes intake than individuals with less tomato consumption (Chen *et al*., 2015; Rao 2004; Shahar *et al*., 2011; Salem *et al*., 2011). Furthermore, Chen *et al*., 2015 reported that tomato consumption plays a preventive and protective role against prostate cancer while others reported negative correlation between tomato intake and prostate cancer risk (Diallo *et al*., 2016; Hadin and Witte, 2011; Takachi *et al*., 2010; Ambrosini and de Klerk, 2008). In a recent study by Xin *et al*., (2016), confirmed that there is a positive association between tomato intake and reduced risk of prostate cancer.

The mechanism through which tomato exerts its protective role has been linked to the properties of lycopene which has been reported to supress prostate cancer proliferation and progression in many studies through ABCA1, LXRa and PPARy activation (Yang *et al*., 2012; Elgass *et al*., 2014). Furthermore, androgen-

independent prostate cancer (PC3, DU145) cell migration and adhesion was also inhibited by the tomato lycopene in a study by Elgass *et al*., 2014. In vivo studies reported a significant effect of dietary tomato and lycopene on mouse prostate carcinogenesis (Wan *et al*., 2014). In summary some natural products (such as lycopene) could provide opportunities for adjuvant therapy of prostate cancer.

1.2 Alternative (differential) splicing and prostate cancer

1.2.1 Pre-mRNA splicing

Pre-mRNA splicing (figure 1.3) is a general term to represent the process which involves intron removal and joining of corresponding exons from pre-rnRNA to form a mature rnRNA capable of generating different kinds of protein from same gene needed for biological activities such as growth, differentiation and apoptosis (Johnson *et al*., 2003). Alternative splicing which I will discuss later, helps produce isoforms proteins from the same single gene (Wang *et al*., 2015). Pre-mRNA splicing is initiated by spliceosome machinery comprising of a large number of associated proteins and five small nuclear RNAs (snRNAs) (Wahl *et al*., 2009). Additional splicing factors such as Ser/Arg-rich (SR) proteins in combination with snRNPs are responsible for exon selection. The spliceosome machinery is recruited to the site of splicing aided by splice factors such as SRSF1 and SRSF2 binding to intronic or exonic splicing enhancers (ISE or ESE) (Malumbres *et al*., 2014).

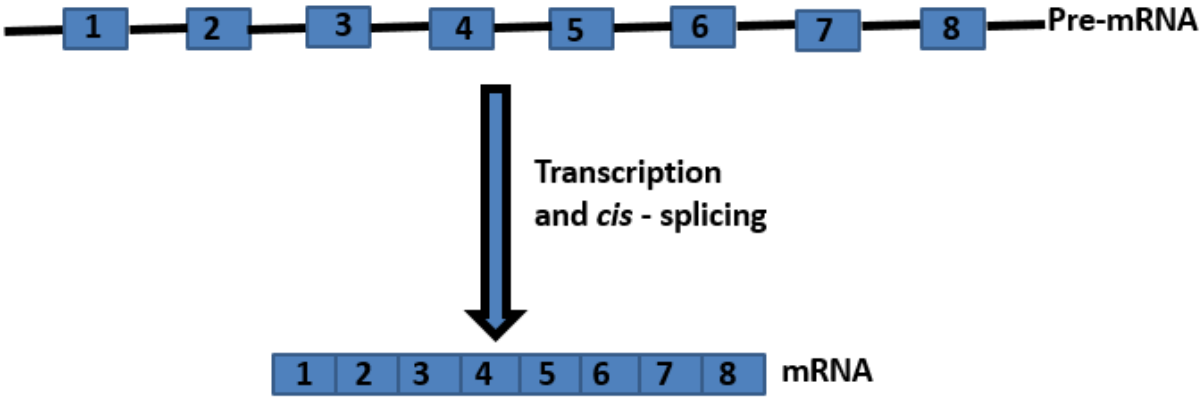

Figure 1.3. Pre-mRNA splicing. In eukaryotic genes, *cis*-splicing is required to decode split genes. The thick lines denote introns while the rectangular boxes represent exons. While the introns are removed, the exons are joined together by the spliceosome to form a mature mRNA.

1.2.2 Pre-mRNA splicing mechanism

Splice sites (ss) are the intron – exon boundaries where the pre-mRNA are cut during splicing. An intron is surrounded by 5' upstream ss and 3' downstream ss (figure 1.4). The 5'ss joins with the 3'ss during splicing, thereby getting rid of the intervening intron. The process involves two transesterification reactions; in the first, the branchpoint A (located in the intron, upstream of the 3' ss) provides a hydroxyl (-OH) groups that attacks the 5' splice site creating a phosphodiester bond through the process of trans-esterification. Figure 1.4 shows the two primary reaction pathways/steps through which splicing occurs. During the first reaction step, the 2' –OH groups of the branch point attack the 5' ss leading to 2'-5' new linkage resulting to lariat intermediate formation. This reaction releases the upstream exon with the terminal 3' –OH group. In the second reaction step, the 3'-5' phosphodiester bond which joins the 3' ss and the intron is attacked by the 3' –OH on the terminal of the upstream exon. The result of this reaction is the formation of brand new 3'-5' phosphodiester bond linking the two exons and getting rid of the lariat intermediate and the intron (figure 1.4). Additional

pre-mRNA *cis*- acting elements are the exonic and intronic splicing silencers (ESSs and ISSs) or exonic and intronic splicing enhancers (ESEs and ISEs) which regulate alternative or constitutive splicing by binding to the modulatory proteins that either repress or stimulate spliceosomal assembly complexes.

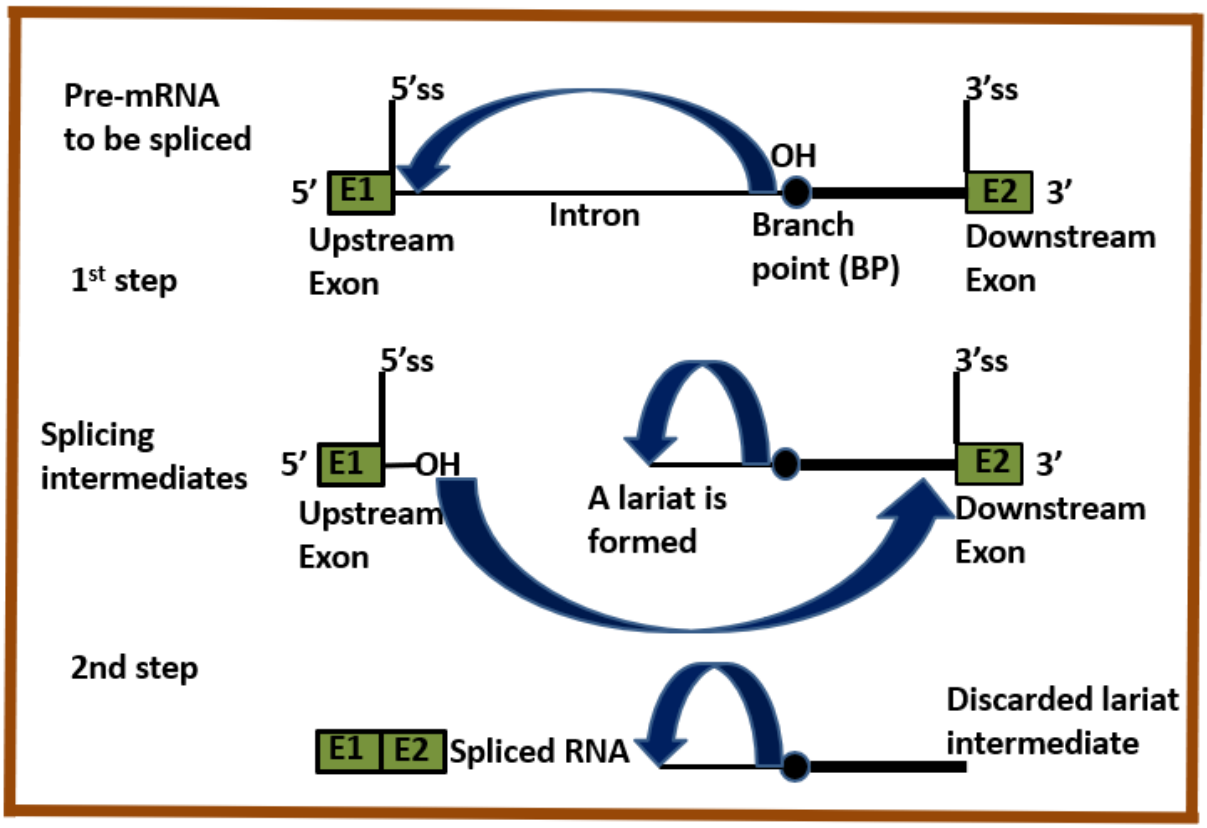

Figure 1.4. Pre-mRNA splicing mechanism and reaction. Pre-mRNA splicing are cut at the intron-exon boundaries which constitute the splice sites. Surrounding the introns are 5' splice site and 3' splice site. The 5' splice site joins with 3' splice site to get rid of the intron at the middle. Trans-esterification reaction initiated by hydroxyl groups (-OH) occurs leading to the formation of phosphodiester bond. Lariat intermediate is formed when the 2' –OH attacks the 5' ss thereby creating a new 2'- 5' linkage. 3'- OH attack the 3'-5' phosphodiester bond leading to the removal of the lariat intermediate with the intron.

If splicing does not occur correctly, incorrect isoform ratios or malfunctioning proteins may be produced (Wang *et al*., 2015). Efficient splicing of pre-mRNA takes place in the spliceosome, a large macromolecular complex consisting of five small nuclear ribonucleoproteins (snRNPs), including U1, U2, U4/U6 and U5 and up to100 non-snRNP splicing factors. The later are mainly RNA binding proteins (RBP) that include the SR (serine-arginine rich) proteins and hnRNP (heterogeneous ribonucleoprotein) (Kramer, 1996). snRNPs are the essential spliceosome building block, the first primary step in the spliceosome cycle (assembly) is the association of proteins with newly transcribed RNA. Newly transcribed RNA associates with hnRNP proteins to form an H complex from RNA-protein interaction (Will and Lu, 2011). The second step is the early (E) complex formation via proteins binding to 5' ss and 3' ss and snRNPs. 5' ss forms a base pair with U1 which is strengthened and reinforced by U1 snRNP protein component known as U1C. Proteins such as U2AF65, U2AF35 and SF1 bind with the 3' ss. U2AF65 interacts with the polypyrmidine tract, while the U2AF35 and SF1 interact with 3' ss and branchpoint respectively. However, U2AF65 interacts with both U2AF35 and SF1.These three proteins tend to stabilize one another on 3' ss (Shi, 2017).

The third step is the formation of A complex. At this stage there is displacement and subsequent replacement of SF1 by U2 via base-pairing with the pre-mRNA branchpoint. The branchpoint and U2 snRNP base-pairing does not perfectly match, this makes the branchpoint adenosine to puff up. The 2' –OH group of the expanded or bulged out adenosine branchpoint kicks off the first splicing catalytic step. U2AF65 interaction bound to the polypyrimidine tract helps to stabilize branchpoint binding to the U2 snRNP (Will and Luhrmann, 2011).

The next step is the pre-catalytic B complex formation by U5.U4/U6 tri-snRNP addition with its associated proteins. This leads to catalytically active spliceosome B* formation which is the fifth step. Here the snRNPs U1 and U4 are released from the spliceosome leading to the first catalytic splicing step. Upon formation of catalytically active B* spliceosome, a pre-formed nineteen complex (NTC) group of protein otherwise called Prp19 is added. The interaction between 5' ss with U5 and U6 snRNP is stabilized by the nineteen complex protein. These rearrangements formulate the catalytic core for the splicing reaction first step occurring in the catalytically active B* complex. As explained earlier, in the first catalytic step a brand new 2'- 5' phosphodiester bond is formed when the phosphodiester bond of the 5' ss is attacked by the 2' –OH group of the adenosine branchpoint (Lee and Rio, 2015).

Next step is the splicing catalysis. This is the splicing catalytic second step which occurs in the spliceosome cycle called the C complex where the remaining snRNPs such as U2, U5 and U6 are contained and in excess of various proteins. Proteins such as U2AF65, U2AF35 and U1 components which are shelved in the earlier spliceosome cycle and are therefore not contained in this step. The next step is the post-splicing disassembly. When splicing attains completion, the spliceosome releases and exports the mRNA to the cytoplasm, the lariat product is discarded and the snRNPs are recycled for further use (Warf and Berglund, 2010).

It is interesting to note that the proteins added at each stage of spliceosome reaction help to drive the spliceosome assembly forward (figure 1.5). Energy is also required in the form of ATP or GTP (depending on the neuclotide) at each major step especially after E complex. It is also important to briefly highlight the specific role of these proteins in the spliceosome assembly. For instatnce, Prp5 plays the role of remodelling binding of U2 to branchpoint, SF1 is displaced by SUB2/UAP56, with Prp28 U1 is

released to enable U6 binding to 5’ ss, in the presence of Brr2 U4 is released, Snu114-GTPase regulates Brr2 and also releases U4, Prp8 is a component of catalytic domain which also controls Brr2, with Prp2 complex B is remodelled into catalytically active B denoted with B*, spliceosome is remodelled into complex C with Prp16, and finally snRNPs are released from excised lariat with Prp43 (Lee and Rio, 2011; Will and Luhrmann, 2011).

1.2.3 Functional role of spliceosome in pre-mRNA splicing

The assembly of spliceosome occurs through an orderly snRNPs binding to the pre-mRNA in a sequential order of U1, U2 and preformed tri-snRNP U4/U6.U5 (Will and Luhrmann, 2011). A commitment complex is formed when U1 binds to the 5’ ss via U1 and 5’ ss base pairs (Seraphin and Rosbash, 1989). U1 binding to the pre-mRNA requires ATP. U2 through base-pairing binds to the branch point to form the pre-spliceosome which is also ATP dependent. Following U2 binding and 5’ ss interaction, the tri-snRNP (U4/U6.U5) (figure 1.5) is recruited to the spliceosome. Upon formation of pre-spliceosome, the branch point-binding proteins (BBP) such as SF1 (splice factor 1) is displaced by U2 in an ATP dependent manner (Rutz and Seraphin, 1999).

Furthermore, after the U4/U6.U5 binding, spliceosome undergoes a series of conformational changes leading to U1 and U4 dissociation from the spliceosome (Lamond *et al.*, 1988). To release U4 and U1, ATP is needed to disentangle U4/U6 duplexes and disorganise U1-5’ ss base-pairing. Brr2 and Prp28 are responsible for the release of U4 and U1 respectively. Brr2 has been identified as a U5 component and has been implicated in the *in vitro* catalysis of unwinding U4/U6 duplexes (Raghunathan and Guthrie, 1998). In human, Prp28 has been linked with U4/U6.U5 tri-snRNP which is dependent on SRPK2 phosphorylation (Mathew *et al*., 2008).

Though the mechanism is not yet entirely clear, genetic studies have suggested that Prp28 is needed to cause destabilisation of U1-5’ base-pairing (Staley and Guthrie, 1999).

New base-pair formation such as base-pairs between U6 and 5’ ss and between U6 and U2 are made possible by the release of U1 and U4. The cross-link of Prp8 to 5’ ss and 3’ ss is an indication that its binding to the pre-mRNA would cause U5 base-pairing stabilization with exon sequences (Wyatt *et al*., 1992).

It has been known that Nine Teen Complex (NTC) is an active integral component of spliceosome which provides spliceosomal snRNP support. After U1 and U4 release, NTC is needed for activation of spliceosome. It has also been reported that the association of U5 and U6 is stabilized by NTC for the formation of active spliceosome, hence base-pairings of U5 and U6 are provided by the presence of NTC (Chan *et al.*, 2003).

As shown in figure 1.5, the first spliceosomal catalytic step is grouped into two stages, the first group is the Prp2-ATP mediated stage with no chemical alterations to the RNA substrate, while the second stage is ATP-independent where additional protein factors are needed to speed up chemical reaction. In the first catalytic reaction, the Prp2 function is unclear but is thought to be associated with the release of SF3a and b (U2 snRNP subunits) from spliceosome (Lardelli *et al*., 2010). SF3b has been found to play essential role during spliceosome assembly by causing U2 branch site stabilization (Gozani *et al.,* 1998). The second catalytic step is all about 3’ ss identification to align with 5’ ss for the ligation of exon. For exon alignment, U5 plays essential role by enabling exons interaction at the splice junction (Crotti *et al*., 2007). Same as the first catalytic step, the second catalytic step is also ATP-dependent

requiring Prp16 protein and ATP for transesterification reaction (Horowitz and Abelson, 1993).

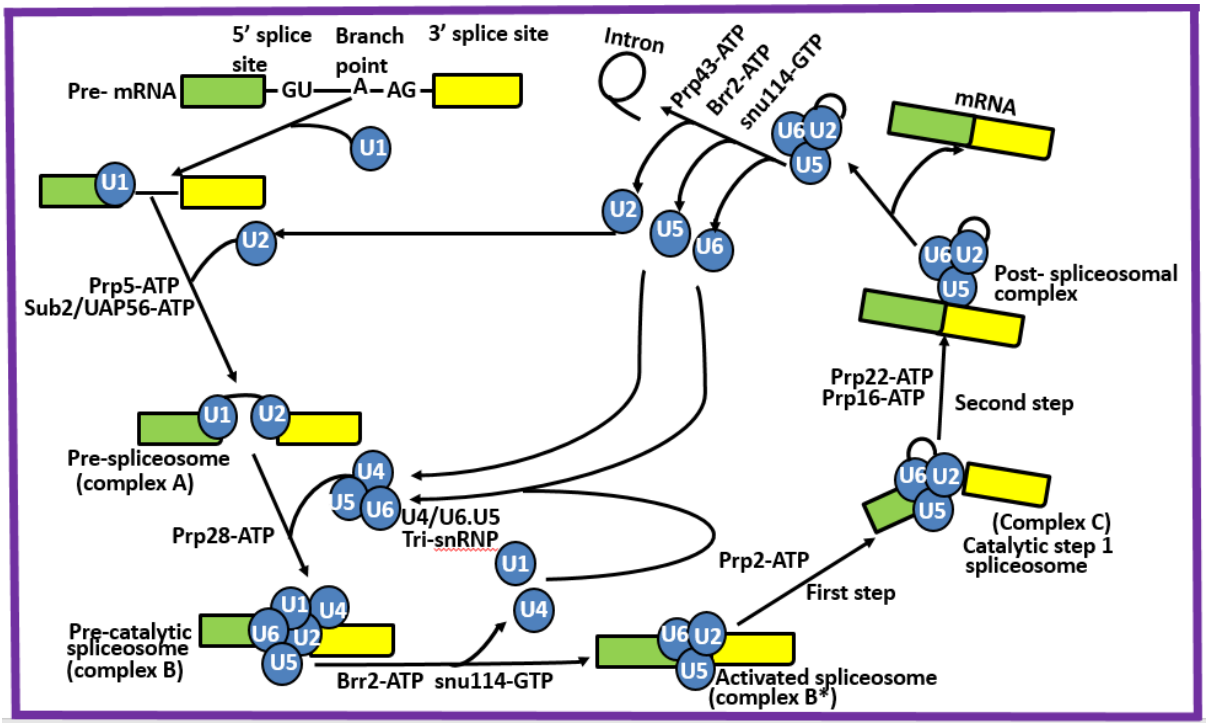

Figure 1.5 Spliceosome structure and function. Circles are used to indicate the snRNPs ordered interactions. Lines and boxes are used to indicate the intron and exon sequences respectively. Also indicated are the various stages of evolutionary conserved RNA ATPases or GTPases snu114, helicaxes such as Prp5, Prp28, sub2/UAP56, Prp2, Prp16, Prp22, Brr2 and Prp43. These ATPases and helicaxes function to enable conformational changes.

1.2.4 Assembly of spliceosomes via intron and exon definition

Intron and exon definitions occur at the very preliminary stages of spliceosome assembly and both are crucial for gene expression. Intron definition implies that the spliceosome first aggregates and clusters around the intron needed to be spliced. Though exons are first recognized by the spliceosome in higher eukaryotes and is

called exon definition. The first basic step in exon definition is the recognition and binding of exons within the pre-mRNA by the early or preliminary spliceosome components (De Conti *et al.,* 2013). These early splicing factors act like punctuation marks informing the spliceosome the location of exons specifically at the 3' ss binding by U2AF and 5' ss binding by U1. U1 binding to to the 5' ss on one exon extreme is stabilized by U2AF binding to the 3' ss on the other extreme of the exon, and vice versa. Exon definition occurs at the early stages of spliceosome assembly while intron removal is associated with the later stages of spliceosome assembly interactions (Barget, 1995; Elliott and Ladomery, 2011).

However, intron definition implies that early spliceosome factors first recognize and cluster around the intron to be removed. Here the molecular interaction between adjoining 5' ss and 3' ss occurs correctly within the intron to be removed. In other words, the intron to be removed is defined by the spliceosome (Elliott and Ladomery, 2011).

1.2.5 Types of alternative splicing

Alternative pre-mRNA splicing is crucial in the regulation of gene expression as 94% of human genes are alternatively spliced (reviewed in Ladomery, 2013). There are four major types of alternative splicing which include: (i) exon skipping which is also called cassette exons (figure 1.6A); (ii) alternative 5' and (iii) 3' splice sites, which occurs at the end of the exon at two or more different positions (figure 1.6B and 1.6C respectively) (Kim and Goren, 2008). In alternative 5' ss a choice of exon selection is made between 5' ss position resulting to shorter or longer version of same exon being spliced into mRNA. (figure 1.6B). Likewise, in the 3' ss, a choice of exon selection is made between 3' ss resulting to longer or shorter version of same exon being spliced

out into mRNA. The fourth type is intron retention, where an intron is retained (not spliced) in the mature mRNA (figure 1.6D). Another category is mutually exclusive exons (figure 1.6E). This is a case where options are made between exon(s) to be included. One is choosen in the place of the other. (Kim and Goren, 2008; Ghigna *et al*., 2008).

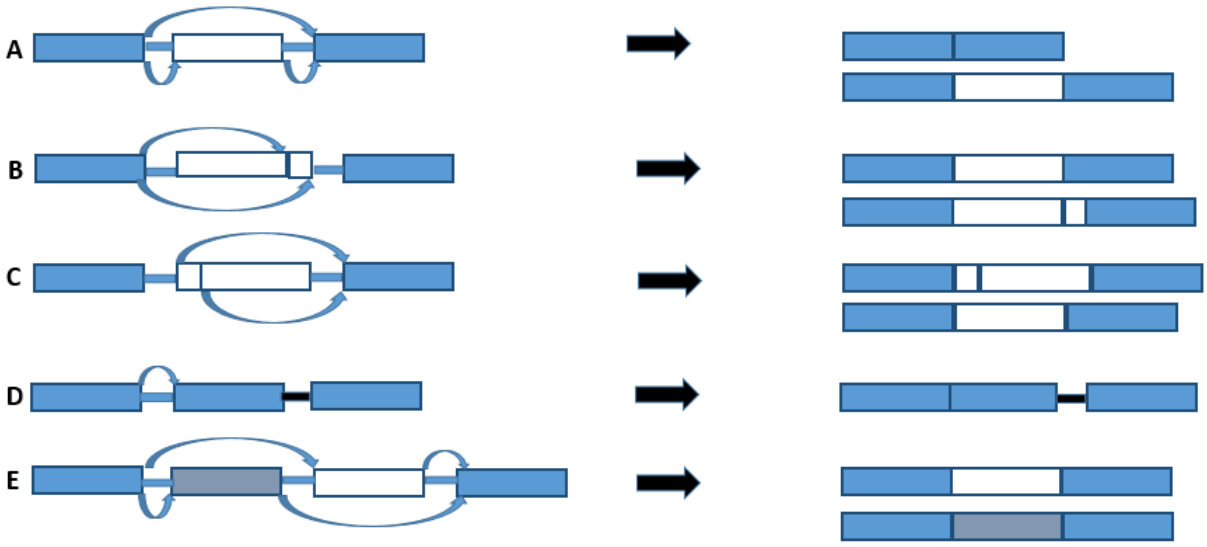

Figure 1.6. Five major modes of alternative splicing. (A) Cassette alternative exon (exon skipping) which is the most wide spread (>30%) form of alternative splicing in vertebrates and invertebrates involving skipping of exon(s) (B) Alternative 5' splice site (5' end of the splice junction is used while the 3' end boundary of the upstream exon is changed). (C)Alternative 3' splice site (use of 3' end of the splice junction while the 5' boundary of downstream exon is changed. (D) Intron retention. Though very rare in vertebrate and invertebrate (<5%) (Kim *et al*., 2007), but the predominant form of alternative splicing in lower metazoans involving retention of one or more intron(s) which is an indication of mis-splicing linked to weaker splice sites. (E) Mutually exclusive exons. This involves retention of 1 out of 2 consecutive exons.

An understanding of alternative splicing is crucial due to increasing numbers of human diseases being associated with aberrant splicing events. Likewise, frequently occurring hereditary diseases have been linked to splicing mutations in both exons and introns. Mutations of any sort could in most cases cause alterations in pre-mRNA

sequences and structures which will prevent the spliceosome from properly decoding the exon-intron boundaries (Faustino and Cooper, 2003; Will and Luhrmann, 2011).

1.2.6 Alternative splicing mutation and disease development

Alterations in the splicing programme also called aberrant or variant alternative splicing have been implicated in the several types of cancer and other diseases (Brinkman, 2004; Pajares *et al*., 2007; David and Manley, 2010; Ahn *et al*., 2011). Brinkman, (2004) suggested that since aberrant splicing is involved in many oncogenic disorders including prostate cancer, a better knowledge of the mechanism will provide opportunities for drug development. Apart from prostate cancer, aberrant splicing has also been investigated and documented in other malignant and non-malignant diseases such as lung cancer, cancer of the liver, colorectal cancer, breast cancer, ovarian cancer, and skin cancer, to mention but a few (Venables, 2004). Non-malignant diseases include; neurodegenerative disorders, diabetes, aging and vascular diseases. Alternative splicing programme changes in breast and prostate tumours compared to normal tissues. Notably, most changes in alternative splicing correlate with changes to proteins linked with survival and increased proliferations (Venables *et al*., 2008). All mutations affecting pre-mRNA splicing result in exon skipping, full length protein is not usually expressed (truncated protein) leading to varieties of human diseases such as Huntington's disease, muscular dystrophy, progeria and cancers of various types including prostate cancer (Duncan *et al*., 1997; Mavrou *et al*., 2014). Furthermore, many forms of genetic disease could result from mutations affecting the intronic and exonic splicing enhancers (ISE, ESE) (Lapuk *et al*., 2014).

It is still not entirely clear how aberrant splicing could initiate the development of cancer and its progression. However, there is an association between mutations of the splicing regulatory factors that alter splice site selection and cancer development. Mutations in the long run lead to the formation of non- functional tumour suppressor genes which causes cell exposure to cancer. The proportion of disease-causing mutations that affect splicing remains controversial. It has been postulated that this proportion is as high as 50%; a more conservative estimate is 15%; regardless of the correct percentage, the proportion is undoubtedly high (Reviewed in Ward and Cooper, 2010). Mutations can also occur at other points such as splice enhancers and silencers, branch points and pyrimidine tracts. It has been reported that exonic splicing enhancer point mutation in the breast cancer gene *BRCA1*) could lead to breast cancer (Welcsh and King, 2001). *BRCA1* encodes a specialised protein responsible for DNA repair; a point mutation in exon 18 causes exon skipping leading to breast cancer (Walsh *et al*., 2006; Tazi *et al*., 2012; Generali *et al*., 2007; Buleje *et al*., 2017.). It has also been reported that AA > AG mutation forms a pseudo 3' ss which creates 11 additional neoclotides to the mRNA of *BRCA1* resulting in truncated protein in breast cancer (Hoffman *et al.,* 1998). This mutation causes alteration of the splice factor SRSF1 (ASF/SF2) binding site. SRSF1 itself has been referred to as proto-oncogene due to its overexpression in cancer (Karni *et al*., 2007). SRSF1 has many targets and affects the alternative splicing of numerous cancer-associated genes. Apart from the splice sites and splice factors (splicing regulators), mutations can also occur and affect the expressions and activities of the regulatory sequences of the core splicing machinery (Ward and Cooper, 2010).

Also, point mutations in the gene encoding $U4_{ATAC}$ has been linked to a diseased condition known as microcephalic osteodysplastic primordial dwarfism as $U4_{ATAC}$

constitutes part of the minor spliceosome machinery and exons identified by this minor spliceosome results in developmental impairments such as growth defects (He *et al*., 2011). Furthermore, mutation in adenomatous polyposis coli (*APC*) gene at intron 4 results to exon 4 skipping which is the leading cause of familial adenomatous polyposis (FAP) (Neklason *et al.,* 2004). Point mutation (AT →GT) on intron 5 of estrogen receptor leads to pseudo 5′ ss formation in intron 5 (Wang *et al*., 1997). Double point mutation on exon 7 of *neurofibromatosis 1* (*NF1*) causes in-frame deletion due to ASF and SF2 ESE binding site disruption (Colapietro *et al*., 2003).

About 185 splicing mutations have been identified in cystic fibrosis transmembrane conductance regulator (CFTR) leading to its loss of function for mucus hydration which is the major cause of cystic fibrosis. 165 of these mutations are intron localized while 20 are located in the exon. In healthy individuals, exon 9 and 12 are alternatively spliced though there are variations of ratio of exon inclusion per individual which explains the variations in the trans-acting elements concentration per individual. Complete skipping of these exons (9 and 12) due to mutation leads to full clinical manifestation of cystic fibrosis presenting with digestive and respirartory tract impairement. Also affected are the sweat gland, biliary, intestinal, male ganitalia and pancreatic systems.

Spinal muscular atrophy (SMA) is a neurodegenerative disorder and the most common cause of infantile mortality due to loss of spinal cord alpha- motor neurons. SMA occurs during infantile stage of life due to mutation leading to the diminution of survival motor neuron gene 1 (*SMN1*) encoding SMN protein that modulates snRNP assembly (Zhang *et al*., 2008).

Tauopathies referes to numerous disorders of the CNS with abnormal accumulation of intracellular microtubule associated protein tau encoded by microtubule associated protein tau gene (*MAPT*) found on chromosome 17. About eight of sixteen exons of *MAPT* gene are alternatively spliced. Four and three microtubule repeats of this gene called 4R and 3R respectively are created by exon 10 inclusion and skipping respectively. An example of disease emalnating from dominant mutation of tau gene is frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). A mutation in exon 10 called N279K which causes pre-mRNA alteration of 4R and 3R tau isoforms has been linked with FTDP-17 (Gallo *et al*., 2007; Andreadis, 2006). Research suggests that alterations between 4R and 3R tau isoform ratio due to mutation is the leading cause of FTDP-17 (Dawson *et al*., 2007). There is also a speculation that changes in the ratio between 4R and 3R tau isoforms could be found in other tauopathies such as Alzheimer's disease.

Furthermore, mutation leading to a disease condition could also occur at the intron. One of the intronic mutation diseases that activate pseudo splice site is Hutchinson-Gilford progeria syndrome (HGPS). In Hutchinson Gilford progeria syndrome (HGPS), instead of splice site mutation, a brand new 5' splice site is created within an exon 11 in the *LMNA* gene encoding Lamin A/C. Lamin proteins are evenly distributed in the nucleoplasm and are responsible for DNA transcription, replication, nuclear assemble and disassemble. Individuals suffering from HGPS have accelerated aging with a maximum of thirteen years life span and frail features such as baldness, midface hypoplasia, loss of subcutaneous fat, osteoporosis, wizened skins and clogged arteries. In contrast, deletion of the entire *LMNA* gene gives rise to muscular dystrophy (Kim *et al*., 2011).

There is an association between hypercholesterolemia (leading to arteriosclerosis) and a mutation (single neuclotide polymorphism - SNP) at exon 12 of low-density lipoprotein receptor (LDLR). LDLR physiologically plays the role of removal of low-density lipoprotein (LDL) that would cause hypercholesterolemia from the blood stream. This suggests that skipping of exon 12 by SNP is linked with cholesterol level in the blood (Zhu *et al*., 2007).

A mutation at the 5' ss leading to a disease called familial dysautonomia (FD) has been identified (Cooper *et al*., 2006). FD is generally caused by complete loss of i-kappa-B kinase complex associated protein (IKBKAP) function leading to nervous system development impairment in children with symptoms of demyelination, volmiting crisis and reduced pain perception. The loss of (IKBKAP) is caused by a point mutation of 5' ss at exon 20 where T replaces C at position 6 of inton 20 which causes the interruption of U1snRNA base-pairing. This mutation definitely prompts exon 20 skipping as the intronic 5' ss is being weakened (Carmel *et al*., 2004).

Medium-chain acetyl-CoA dehydrogenase (MCAD) deficiency is another form of splicing mutation resulting to mitochondrial β-oxidation defect with symptpms of metabolic crisis such as high blood glucose, seizure and lethargy. Under normal physiological condition, this enzyme (MCAD) is responsible for medium chain length fatty acid degradation. Its deficiency is caused by missense mutation (362C->T) at exon 5 of *MCAD* gene leading to exon 5 skipping and mRNA degredation by NMD (Nielson *et al*., 2007). Some of the splicing mutation diseases and features are depicted in table 1.

Table 1. Examples of splicing mutation diseases and features (*Tazi et al*., 2009; Anna and Monika, 2018)

Disease	Gene	Accession Number	Specific exon(s) where changes occur	Features
Hutchinson-Gilford progeria syndrome	*LMNA*	BC014507	Exon 11 skipping	GGGCGGA to GGGTGGA or GTCACTCGCA to GTCATTCGCA Activates cryptic splice site
Spinal muscular atrophy	*SMN1*	U18423	Exon 7 skipping	TTTAGA to TTCAGA promotes exon skipping
Hypercholesterolemia		AY114155		CAACGGG To CAATGGG Causes skipping
Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency	*ACADM (MCAD)*	M16827	Exon 5 skipping	GACTGC To GATTGC causes exon skipping, ACAGGG to ACCGGG promotes inclusion
Hypercholesterolemia	*LDL*	AY114155		CAACGGG To CAATGGG Causes skipping
Frontotemporal dementia with parkinsonism linked to chromosome 17	*MAPT*	J03778	Exon 10 skipping	AATAAGA to AAGAAGA causes inclusion
neurofibromatosis 1 (NF1)	*NF1*		Exon 15 and 16 skipping	Double point mutation resulting to in frame deletion due to ASF/SF2 and SC35 ESE binding site disruption.
Autosomal dominant congenital cataract	*MIP*		Exon 3 skipping	c.606+1G>A. Canonical splice site point mutation leads to the entire exon skipping.
Becker muscular dystrophy (BMD)	*DMD*		Exon 25 skipping	c.3432+1G>A. Canonical splice site point mutation leads to the entire exon skipping.
Haemophilia B	*F9*	P00740	Exon 3 skipping	c.253-19_253-16del. The polypyrimidine tract is shortened from 24 nucleotides to 20 by mutation. This leads to exon 3 skipping and inefficient splicing.

Other diseases that may be affected by aberrant splicing due to mutation include: Myotonic dystrophy (DM), Leukaemia and sarcomas, Alzheimer disease (AD), paraneoplastic syndrome, stickler syndrome, retinitis pigmentosa, azoospermia, hepatocellular carcinoma, papillary renal cell carcinoma, fabry disease, X-linked spondyloepiphyseal dysplasia tarda, hay-wells syndrome, Ehlers-Danlos syndrome, androgen insensitivity syndrome, xeroderma pigmentosum and so on (Garcia-blanco et al., 2004; Anna and Monika, 2018).

1.2.7 Regulation of alternative splicing by splice factors

SR proteins are essentially involved in spliceosome maturation and are therefore required for efficient pre-mRNA splicing. Components of the SR proteins include RNA recognition motifs and RS domain otherwise known as the C- terminal domain rich in Arg-Ser repeats. In the RS domain, SR proteins are phosphorylated at many serines by SRPKs. Phosphorylation of the RS domain facilitates SR protein entry into the nucleus and also play crucial role in both mRNA export and alternative splicing (Mueller and Hertel, 2012; Ghosh and Adams, 2011). Serine – argenine rich proteins called SR proteins are members of nuclear factors with numerous essential roles in both constitutive and alternative RNA splicing. They participate in many stages of splicing regulations by binding exonic splicing enhancers (ESEs) via RNA recognition motifs (RRM) and by moderating protein-RNA and protein-protein interactions through their RS domain (Shen and Green, 2006; Busch and Hertel, 2012). Specific RNA binding proteins are identified and recognised by splice factors. RNA recognition motif (RRM) is often encountered as the RNA binding domain. This motif identifies generally the targeted single stranded RNA sequences (Pedrotti *et al*., 2012).

In the past, several names have been given to same SR protein making it difficult to understand in literatures. For clarity, a more standardized nomenclature has been proposed for twelve core members of SR proteins. For instatnce, ASF/SF2 or SF2/ASF is presently named SRSF1; SC35 is now named SRSF2; SRp20 is renamed SRSF3; SRp75 is same as SRSF4; SRp40 equals SRSF5; SRp55 equals SRSF6 and 9G8 is renamed SRSF7 (Manley and Krainer, 2010). These SR proteins are the key substrates of protein kinases activity which are vital in the regulation of alternative splicing (Amin *et al*., 2011; David and Manley, 2010).

In constitutive splicing, SR proteins such as SRSF1 and SRSF2 play essential regulatory roles, for instatnce, SRSF1 and 2 facilitate binding of U1 snRNP to 5' ss and U2 snRNP to 3' ss and subsequently stop the association between the initial splice site recognition steps in the pre- and mature spliceosomes (Cho *te al.,* 2012). The best known function of SR protein is its ability to promote selection of splice site by binding to the ESEs. The choice of splice site regulation and selection is usually initiated and determined by the exonic and intronic splicing enhancers (ESEs, ISEs) for exons and introns respectively. Weak splice sites are actively promoted by the splice enhancers, conversely, strong splice sites are repressed by the splice silencers. Splice factors such as SR proteins and RNA binding proteins such as hnRNP are typical examples of splicing activators and suppressors respectively (Cooper *et al*., 1997). Therefore, alternative splicing can be actively regulated by the interaction of the specific regulatory RNA sequences such as ESE or ISE and the binding splice factors. Regulatory sequences can also repress splicing; these are known as ESS and ISS (exonic and intronic splice silencers). A study suggest that SR proteins do not only enhance exon inclusion but also facilitate induction of exon skipping depending on the pre-mRNA interactions. For instatnce, binding of SR protein to exon promotes exon

inclusion while exon skipping is the resultant effect when SR protein binds to the intronic sequence (Erkelenz *et al.,* 2013). In 2011, Han *et al* demonstrated that SR protein binding to a flanking exon resulted to internal alternative exon skipping where as exon inclusion is promoted when SR protein binds to the alternative exon (Han *et al.*, 2011; Stanford *et al.*, 2009). Other roles of SR proteins in splicing include: RNA stability regulation through nonsense mediated RNA decay (Zang and Krainer, 2004), chromatin interaction and nuclear export of mRNA ((Loomis *et al.*, 2009; Huang and Steitz, 2005), transcription machinery coupling and translational control by cytoplasmic shuttling of SR proteins (Sanford *et al*., 2004; Michlewski *et al*., 2008).

Another form of alternative splicing regulation is through promoters. This was indicated in a minigene construct where the type of promoter used typically influenced the expression of the splice isoforms ratio. Alternative splicing is regulated by promoters through the mechanism of transcriptional elongation speed by the RNA polymerase II. For instance, spliceosomes tend to choose stronger splice sites at fast transcription. However, weaker splice sites take more time to be recognised by spliceosomes when the transcription is slow. This was typically illustrated in 2003 by Mata *et al* in EDI exon skipping in the fibronectin gene due to faster transcriptional elongation initiated by RNA polymerase II. Increased exon inclusion was observed when a transfection to cause mutation and slow down the RNA polymerase II was initiated. Furthermore, alterations of RNA polymerase II elongation in different part of the gene affects the specificity of exons alternative splicing (Mata *et al.,* 2003).

For more information. It is worthy of note that alternative splicing factor; ASF/SF2) now called SRSF1) overexpression has been identified in numerous cancers due to its pro-oncogenic potential (Das and Krainer, 2014). Upregulated SRSF1 is associated with increased cell transformation, proliferation and invasion (Olshavsky *et al*., 2010).

SRSF1 expression mediates switching of caspase 9 and BIN1 splicing to produce pro-oncogenic isoform that supress apoptosis in many cancer cells including prostate cancer (Anczukow *et al*., 2012). In the RON and MNK2 genes, increased SRSF1 levels was found to promote the pro-oncogenic isoforms thereby enhancing the invasive and migratory processes of cancer cells including prostate cancer. This is thought to occur due to the impairment of signalling pathways of mammalian target of rapamycin (mTOR) and p38-mitogen-activated protein kinase (MAPK) (Ghigna *et al*., 2005; Maimon *et al*., 2014).

1.2.8 SR protein kinases

The first SR protein kinase was discovered in 1994 and named SR protein specific kinase 1 (SRPK1) (Gui *et al.,* 1994). Other homologues such as SRPK2 and SRPK3 were subsequently discovered in human and mice. While SRPK1 is generally expressed, SRPK2 and SRPK3 are majorly expressed in the nervous system and muscle cells respectively (Nakagawa *et al*., 2005; Wang *et al.*, 1998), suggesting that individual members of SRPK family may possess unique function during development and in various cell types.

Apart from SRPKs, there are several other kinases capable of phosphorylating SR proteins in vitro. Some of which include: Akt (Patel *et al.,* 2005), protein kinase A (PKA), protein kinase C (PKC) (Colwill *et al.*, 1996), dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs) (Aranda *et al.,* 2011) and cyclin-dependent like kinases otherwise called CDC-like kinases (CLK1-4) (Duncan et al., 1998). However, among these kinases, only CLKs and SRPKs are capable of in vivo phosphorylation of SR proteins as demonstrated through either direct chemical inhibition or genetic ablation (Zhong *et al.,* 2009; Yomoda *et al.,* 2008).

1.2.9 Regulation of splice factors (SR proteins) by splice factor kinases (SRPK and CLK)

Over ten years ago, it was discovered that SR specific protein kinases such as SRPKs (SR protein kinases) and CLKs (CDC2-like protein kinases) are responsible for SR protein phosphorylation causing changes in SR protein functions and intracellular distribution. Studies have shown the implication of splicing factors in various forms of cancer. For instance, in colorectal cancer, SRSF1 expression is found to be upregulated. In breast, prostate, pancreatic, and colorectal cancers, splice factor kinases such as SRPK1 and CLK1 expressions are reported to be upregulated (Zhou *et al*., 2012). Mermoud *et al*., (1994) suggested that phosphorylation is an essential process for assembly of spliceosome whereas dephosphorylation is a critical catalytic process of spliceosome assembly. It is important to understand the phosphorylation mechanism especially how the SR proteins are recognised and phosphorylated by the SR protein kinase (Zhou and Zu, 2013). Structurally, an SR protein is composed of C- and N-terminals. The C-terminal is otherwise called the RS domain, and is rich in Arg-Ser repeats. A representative example of this domain is ASF/SF2 now called SRSF1. RS domains of SR proteins are phosphorylated by protein kinases such as CDC-like kinases (CLKs) and serine-arginine protein kinases (SRPKs) thereby modulating their interactions with exonic or intronic splicing enhancers of pre-mRNAs as well as their subcellular localizations (Twyffels *et al*., 2011; Long and Caceres, 2009). The N-terminal of SR proteins is composed of RNA recognition motifs (RRMs) which determine the targets of the SR proteins by binding sequences such as ESE splicing enhancers (Zuo *et al*., 1994)

SR proteins can be phosphorylated or dephosphorylated at multiple sites in the SR domain leading to the functional alteration of the SR proteins. For instance, to ensure

the recognition of an appropriate splice site, the splice factor SRSF2 cooperates with U1 snRNP facilitating its interaction with the 5' splice site and the interaction is phosphorylation dependent (Kohtz *et al*., 1994). Two of the best studied splice factor protein kinase families (CLK and SRPK) are responsible for RS domain phosphorylation thereby altering the cellular distribution of the SR proteins (Colwill *et al*., 1996). The impact of RS domain phosphorylation in alternative splicing has not been fully elucidated. The extent of RS domain phosphorylation in SRSF1 regulates the alternative splicing of apoptotic genes such as *Bcl-x* and *caspase-9* favouring expression of their antiapoptotic isoforns (Massiello and Chalfant, 2006). Both families of kinases cooperate to regulate the function of the SR proteins in the cell.

SRPK is diferent from CLK due to its subcellular localization. However, SRPK is detectable in both cytoplasm and nucleus (figure 1.7) with a greater proportion found in the cytoplasm. All members of CLK are nuclear localised and co-localized in the nuclear specles with SR proteins (figure 1.7) (Naro and Sette, 2013). These differences in SRPKs and CLKs localization suggest a synergic phosphorylation regulation partern of SR proteins. For instance, SRPKs in the cytoplasm play the role of initial phosphorylation of SR proteins to promote their nuclear import while the CLKs tune up and improve the SR protein phosphorylation state in the nucleus (figure 1.7). Therefore both SRPKs and CLKs work in a complementary and synergic manner to bring about splicing regulation by SR protein phosphorylation and other processes such as RNA export (Ghosh and Adams, 2011).

Stamm *et al*., 2008 suggested that splice factor activities can be regulated by phosphorylation through the following mechanisms: altering their RNA binding capacity or ability to interact with other proteins (i.e biochemical properties alteration) and or manipulating their intracellular localization. Splice factors are either nuclear or cytoplasmic localized. For instance, SRSF1 has both nuclear and cytoplasmic functions. It is imported into the nucleus when phosphorylated by the SR protein kinases (SRPKs), Phosphorylation of the SR proteins by the SR protein kinases causes increased nuclear concentration of the SR proteins which makes them available to participate in the splicing reaction (Duncan *et al*., 1997; Zhou *et al*., 2013; Araki *et al*., 2015), whereas its dephosphorylation by protein kinase inhibitor promotes its nuclear export (Giannakouros *et al*., 2011). SRSF1 accumulate in the nuclear speckles (splice factor storage site) and the CLK kinases otherwise called CDC2-like 'LAMMER' kinases hyperphosphorylate SRSF1 in the nucleus (figure 1.7). The hyperphosphorylation of SRSF1 (and of other splice factors) by the CLK kinases facilitate its release from the nuclear speckles to the location where splicing takes place.

To understand the biological importance of SRSF1 phosphorylation, it is worth noting that SRSF1 is involved in the alternative splicing regulation of vascular endothelial growth factor A (VEGFA). The two isoforms of VEGF-A that arise through alternative splicing are pro and antiangiogenic isoforms. The pro-angiogenic isoform is being promoted by SRSF1 due to the use of exon 8 (VEGFA terminal exon) 3' proximal splice site. SRSF1 nuclear accumulation is due to high levels of SRPK1 leading to high expression of proangiogenic VEGFA. However, cytoplasmic accumulation of

SRSF1 results from chemical inhibition or knockdown of SRPK1 resulting in higher antiangiogenic VEGFA isoform expression (Amin *et al*., 2011).

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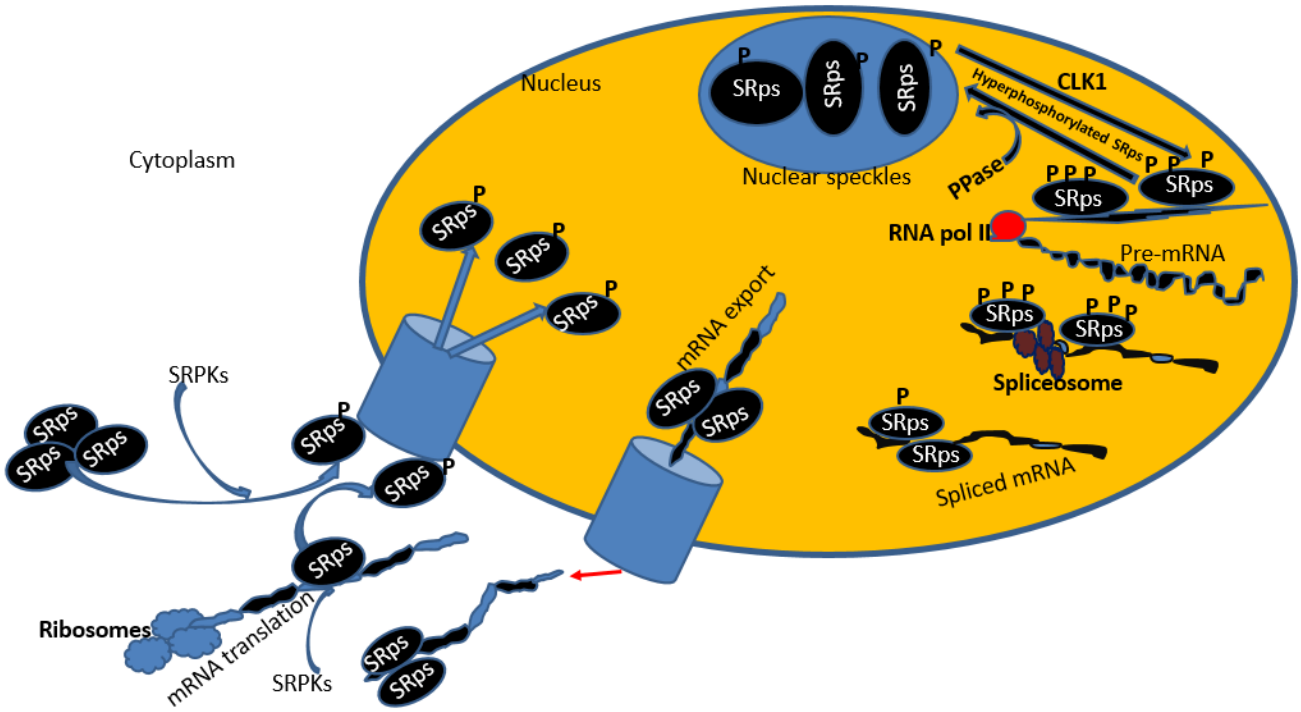


Figure 1.7 Regulation of splice factor activities by splice factor kinases. SRPKs and CLKs families mediates the phosphorylation of splice factors (SRps SR- proteins) and other components of spliceosome. The SRPK located in the mammalian cytoplasmic cells mediates the transport of newly synthesized SRps into the nucleus by phosphorylating them. SRPKs are said to have dual localization due to their ability to also translocate into the nucleus during osmotic stress or G2/M cell cycle phase and therefore has the potential of phosphorylating SRps both in the cytoplasm and nucleus (Naro and Sette, 2013; Gui *et al*., 1994). Cytoplasmic phosphorylation of the SRPs by the SRPK ensures their nuclear import. While in the nucleus the SRPK mediates the SRps release from the nuclear speckles. The CLKs are localized with the SRps in the nucler speckles. Hyper phosphorylation of the SRps is initiated by the overexpression of CLKs which automatically leads to disassembly of the nuclear speckles (Ghosh and Adams, 2011). CLKs nuclear localization distinguishes it from SRPKs which is mostly localized in the cytoplasm. This localization difference makes it possible for their interaction to

regulate subcellular SRps localization. It has also been reported that CLKs have wider substrate specificity while SRPK's substrate specificity is confined to phosphorylation of ser-arg-sites (Colwill *et al*., 1996).

Both in vivo and in vitro studies suggest that CLK family have the ability to modulate splicing (Ninomiya *et al*., 2011; Araki *et al*., 2015; Muraki *et al*., 2004). Duncan *et al*. also investigated the role of CLKs on splicing and concluded that the CLK1 protein kinase specifically regulates alternative splicing by phosphorylation of the SR protein (within the nucleus, particularly in nuclear speckles (splice factor storage sites; Duncan *et al*., 1997). Ninomiya *et al*., (2011) carried research on the effect of the benzothiazole compound TG003 on the phosphorylation of SR proteins, specifically SRSF4, by CLK1. They discovered that TG003 administration to cultured cells causes dephosphorylation of SRSF4 after one hour and re-phosphorylation of SRSF4 occurred after the removal of the compound. Furthermore, researchers have also looked at the effect of TG003 on viruses. TG003 inhibits proliferation of influenza virus both in vivo and in vitro (Karlas *et al*., 2010 and Nishida *et al*., 2011).

These SR proteins are known to be localized within the nucleus where they play crucial role in the alternative splicing programme. These protein kinases are crucial factors that determine exon selection during splicing by binding to either exonic or intronic splicing enhancers of pre-mRNA (Naro and Sette, 2013). Notably, both over phosphorylation and under phosphorylation of SR proteins by the protein kinases serves as a regulatory function which can prevent splicing (Duncan *et al*., 1995). In addition to its active role in the pre-mRNA splicing in the nucleus, SR proteins also participate in posttranscriptional processes such as translation, nonsense-mediated mRNA decay (NMD) and nuclear export of the mature mRNA (Liu *et al*., 2013; Prasad

and Manley, 2003) and these additional activities are undoubtedly also affected by SR protein phosphorylation by CLKs.

CLKs do not only phosphorylate the classical SR proteins. Other splice factors such as SPF45, overexpressed in cancer, are phosphorylated by CLK1. The inhibition of CLK1 drastically reduced SPF45 induced exon skipping (Liu *et al*., 2013). Chemical inhibitors of splice factor kinases have been developed by researchers. Such inhibitors include TG003, a specific CLK1 inhibitor and has also been reported to modulate alternative splicing of pre-mRNAs of CLK and SRSF2 (Muraki *et al*., 2004). Similarly, natural chemical compounds such as herboxidiene, spliceostatin A (SSA) and E7107 has been reported as anti-tumour agents targeting core splicing components such as SF3B1 (Kotake *et al*., 2007; Webb *et al*., 2013).

Araki *et al*., (2015) conducted investigation of the potential inhibitory effect on protein kinases on 870,000 known compounds at a concentration of 1 µM. About 319 of the compounds met the criteria to inhibit 30% of the in vitro kinase activity of protein kinases such as SRPK1. Three compounds identified as compound (Cpd1-3) were investigated in the in vitro kinase assay using CLKS and SRPKS. Cpd -1, Cpd-2 and Cpd-3 were identified to possess inhibitory effect at varying capacities against CLK1, CLK2 and SRPK1 respectively. Cellular activities of the above Cpds were also investigated using anti- pan- phospho- SR antibody. It was identified that the phosphorylation levels of SRSF4 (SRp75) was drastically reduced in a dose dependent manner by the three Cpds. Furthermore, TG003 is a chemical and specific inhibitor of CLK1, Chemical inhibition of CLK1 with TG003 reduced the phosphorylation levels of splice factors such as SRSF1 and SRSF4, in a dose dependent manner. Phosphorylation levels of all SR proteins was reduced upon

treatment of DU145 cells with alkaline phosphatase, whereas phosphatase inhibitor treatment of same cells had a minimal effect confirming that phosphorylated SR proteins was selectively detected by the anti-phospho SR antibody (Araki *et al*., 2015).

The selection of splice site can be altered by many extra cellular factors such as osmotic shock, growth factors, cytokines, depolarization and UVC irradiation through phosphorylation causing changes in SR protein localization (Stamm, 2002). Although the full physiological role of the extensive phosphorylation of the RS domain of the SR proteins is not yet known (Gui *et al*., 1994), it is clear that the phosphorylation affects the protein-protein and protein-RNA interactions of the SR proteins (Xiao and Manley, 1997). Several kinases including SRPK, CLK, hPRP4, LAMMER family kinases has been reported to phosphorylate SR proteins (Rossi *et al*., 1996; Kojima *et al*., 2001). (Nayler *et al*., 1997) reported in vitro and in vivo CLK family kinase phosphorylation of SR protein and SF2/ASF respectively. (Duncan *et al*., 1997) reported that CLKs over expression in disease (cancer for instance) interrupts pre-mRNA splicing site selection (of its own transcript and adenovirus E1A transcript). Though the signal, functional or biological pathway is not yet clear, but these findings have confirmed that alternative splicing is regulated by CLK family through phosphorylation of the SR proteins.

1.2.10 Alternative splicing and epithelial mesenchymal transition (EMT)

Interactions between epithelial and mesenchymal cells are essential in embryonic stages and in foetal organ development. The epithelial and the mesenchymal cells form two transitions (epithelial mesenchymal transition- EMT and mesenchymal epithelial transition - MET) both required for normal development (Larue and Bellacosa, 2005; Peng *et al*., 2014; Pei *et al*., 2019). EMT has been linked to

metastatic disease development and this is linked to alternative splicing changes (Warzecha and Carstens, 2012). During EMT, several known genes with alternative splicing changes include: *p120- catenin* which controls activities of cadherin, *MENA* (*ENAH*) which is a cell motility regulator, CD44 which regulates cell adhesion, and the fibroblast growth factor receptor 2 (FGFR2) (Warzecha and Carstens, 2012, Xu *et al*., 2015).

1.2.11 Identification of mutations causing splicing defects

DNA sequencing is a critical tool for the identification of disease-causing mutations (Foley *et al*., 2015; Mahdieh and Rabbani, 2013). Direct genetic comparison between normal and abnormal tissues through biopsy would have been the best mutation detection method but it is difficult to extract affected tissue for instance heart and brain tissues without harming the subject (Jung *et al*., 2013).

When analysing mutations, software such as ESEfinder can be employed to determine if the mutation might affect an ESE or splice factor binding sites (Cartegni *et al*., 2003). Regardless of predictive software, it is important to assess the potential effect of the mutation on splicing directly by analysing patient RNA (Morlan *et al*., 2009). Minigenes derived from genomic DNA can also be employed in the analysis of mRNA splicing mutations. Minigenes with or without the mutations can be transfected into cell line models to determine the effect of the mutation on splicing.

1.3 Targeting the splicing machinery in prostate cancer

1.3.1 Therapeutic manipulation of alternative splicing:

There is a lot of interest nowadays in manipulating alternative splicing in a way that could be therapeutically useful (Hagen and Ladomery, 2012; Ghigna *et al*., 2008; Antonopoulou and Ladomery, 2018). RNA targeted therapies have been a growing and promising approach to curb the devastating effect of cancer including in prostate cancer (Bennett and Swayze, 2010, Havens *et al*., 2013). There are several strategies that can be employed to manipulate alternative splicing towards therapy.

Antisense oligonucleotides (ASOs) these are short oligonucleotides with 15 to 25 bases targeting specific RNA transcripts. The functional mechanism of ASOs is the formation of Watson-Crick base pairs with targeted RNA. ASO directed to a splice site will inhibit access of the splicing factors to RNA sequence at that site thereby blocking splicing at that point. Splicing may also be redirected to another splice site. Conversely, splicing can either be promoted or blocked when trans-acting regulatory splicing factors are inhibited due to ASOs targeted to splicing silencers or enhancers. Furthermore, the ASOs specific sequence permits them to bind accurately and precisely at the endogenous RNA. More importantly, ASOs can be designed to target specifically to mutated genes. These qualities make ASOs a universal tool for targeting RNA expression for therapeutic purposes (Bennett and Swayze, 2010). The ultimate goal of utilising ASOs as a therapeutic potential is either to eliminate harmful proteins or restore the functional expression of a vital protein that is not expressed or made silent. Therefore, a functional protein that was lost due to mutation is restored by ASO. ASOs restore a dormant or lost protein by either inhibiting the pseudo (cryptic) splice site or by blocking exon skipping via targeting regulatory splicing sequences.

Antisense oligonucleotides that modify splicing have been called ‘Splice-Switching Oligonucleotides’ (SSOs) (Gleave and Monia, 2005; Chan *et al.,* 2006).

Another strategy is targeting the alternative splicing regulatory machinery using compounds that specifically target and inhibit splice factors or components of the core spliceosome. Chromatin remodelling factors that are functionally involved in exon definition can also be chemically targeted. Recently, trans-splicing otherwise called spliceosomal mediated RNA trans-splicing (SMaRT) has been subjected to detailed studies for development of specific therapies against diseases including prostate cancer (Wally *et al*., 2012). SMaRT is the most effective and efficient means of correcting gene expression with mutations at 5’ or 3’ splice sites. For example, mutations prevent U1 snRNA binding to 5’ss which in turn inhibit spliceosome assembly leading to splicing failure. In order to restore normal splicing, snRNAs (modified version of spliceosome) have been developed with sequence alterations to restore mutated 5’ss base-pairing (Schmid *et al*., 2012).

Another promising approach is to target splice factor kinases; the inhibition of SRPK1 with the compound SPHINX has been reported as potentially useful in blocking the growth of prostate cancer cells (Mavrou *et al*., 2015). Previously in 2011, Amin *et al* showed that SRPIN340, an earlier SRPK1 inhibitor also developed in the Hagiwara lab, blocks angiogenesis and therefore tumour growth in mouse xenografts. The mechanism of action is through altering SRSF1-regulated alternative splicing of VEGFA. Active, phosphor-SRSF1 promotes the expression of pro-angiogenic VEGFA. Whereas much attention has focused on the effects of SRPK1 inhibition, less is known about the potential of CLK inhibition.

1.3.2 CLK inhibition in prostate cancer

CLKs otherwise called dual specificity protein kinases (CDC2-like protein kinases), as discussed, are major regulators of pre-mRNA splicing. During pre-mRNA processing, the CLKs phosphorylate the SR proteins in the nucleus triggering their release into the nucleoplasm where they can phosphorylate their substrates and contribute to splicing regulation (Aubol *et al*., 2014)

The CLK family consists of four related genes named *CLK1* to *CLK4*. Expression of CLK3 is seen in mature spermatozoa suggesting a role in fertilization; whereas CLK1, 2 and 4 are widely expressed in several tissues (Menegay *et al*., 1999). The biological function of CLK1, 2 and 4 is not yet entirely clear. There is increasing evidence that CLKs are involved in cancer. It has been found that during malignant cell differentiations, some SRPKs and all members of the CLK family are overexpressed (Zhou *et al*., 2013).

As discussed previously, CLK1 substrates include non-SR proteins such as SPF45. Overexpression of SPF45 triggers cell proliferation, migration and invasion through exon skipping in many target mRNAs. Multidrug resistance was also reported following overexpression of SPF45 In both ovarian (A2780) and cervical (HeLa) cancer cells (Sampath *et al*., 2003). Interestingly, CLK1 mediated phosphorylation increases SPF45 protein expression while CLK1 inhibition stimulates SPF45 degradation (EIHady *et al*., 2017). CLKs are also likely involved in viral replication. Reducing CLK2 activity causes reduction of HIV-1viral gag protein while overexpressed CLK1 increases it (Wong *et al*., 2011).

CLK1 and 4 maintain cell growth and viability in hypoxic cancer cells (Jakubauskiene *et al*., 2015; Eisenreich *et al*., 2013). It was found that hypoxia inducible factors (HIFs)

contribute to increased CLK1 and CLK4 expression. Bowler *et al* (2018) report that hypoxia causes upregulation of SRPK1, and also CLK1 and CLK3, but not CLK2 or CLK4 in hypoxic PC3 prostate cancer cells.

EIHady *et al*., (2017) also observed a related cell growth retardation following CLK1 inhibition. It was further reported that the chemistry behind the slow growth and anti - cancer effect of CLK1 inhibition on tumour cells is because CLK1 inhibition leads to nonsense mediated decay of mRNAs of genes associated with cell viability, growth and survival. Therefore, treatment based on CLK1 inhibition could also work by preventing translation of mRNAs that promote cell proliferation (EIHady *et al*., 2017). EIHady *et al*., (2017) also verified the lack of cytotoxicity potential of CLK1 and -4 inhibitors using normal cells such as human embryonic kidney cells (HEK293), normal peripheral blood lymphocytes (PBLs). There was no evidence of cytotoxic effect of CLK inhibitors on the proliferating and non-proliferating HEK293 and PBLs respectively.

In search of chemical compounds that could target splice factor kinases, Muraki *et al*., in 2004 screened several compounds discovered TG003, an inhibitor of CLK1 and its close relative CLK4 (Muraki *et al.*, 2004). Muraki *et al*, 2004, tested the specificity inhibitory effect of TG003, a benzothiazole, on CLK1/STY with other protein kinases such as SRPK1, SRPK2, PKC, PKA, CLK2 and CLK3, and discovered that TG003 inhibits most efficiently CLK1 and its closest relative CLK4. 10nM of TG003 is able to inhibit IC50 of CLK1 activity (Li *et al*., 2013).

Apart from the benzothiazole TG003, other specific CLK1 inhibitors have been reported, they include: the indole KH-CB19 (IC50, 20 nM for CLK1) (Fedorov *et al*., 2011), the quinazoline NCGC00010037 (IC50, 37 nM for CLK1) (Rosenthal *et al*.,

2011). In vitro and in vivo studies have confirmed CLK1 inhibitors as antiviral agents against influenza virus (Zu *et al*., 2015). For the purposes of this study, and proof-of-principle, and because it is a well established and widely used CLK inhibitor, and commercially available, we decided to focus on TG003.

1.4 Aims and Objectives

1.4.1 Hypothesis

There is therapeutic potential in targeting CLKs such as CLK1 in the context of prostate cancer.

1.4.2 Aims

Aim 1. To gain a better understanding of the biological function of CLK1.

Aim 2. To explore the potential benefit of targeting of CLK1 in vivo.

1.4.3 Objectives

Aim 1.

(i) To study the effect of CLK1 inhibition with TG003 or knockdown with siRNA on cell proliferation and apoptosis in prostate cancer cell lines.

(ii) To study the effect of CLK1 inhibition with TG003 or knockdown on alternative splicing of cancer-associated (eg apoptosis related) genes.

(iii) To study the effect of CLK1 inhibition and knockdown on cell migration and invasion and EMT marker expression.

Aim 2.

(i) To study the effect of CLK inhibition with TG003 on the growth of prostate cancer cells subcutaneously injected into nude mice.

Chapter 2

2.0 Materials and Methods

2.1 Cell culture

Prostate cell cancer lines (androgen independent PC3 and DU145 cells; and androgen dependent VCaP cells) and the immortalised normal prostate epithelium cell line PNT2 obtained from ECACC were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5mM glucose and 5mM L-glutamine, 10% fetal bovine serum (FBS) and 1% sodium pyruvate were added to make complete media. The PC3 cell lines and all the reagents mentioned above were purchased from Sigma Aldrich, USA.

2.2 Cell treatments with TG003

The benzothiazole compound TG003 that inhibits CLK1 was bought from Sigma Aldrich UK. 50mM stock solutions were prepared in dimethylsulfoxide (DMSO) solvent. $1x10^6$ Prostate cancer PC3 and DU145 cells were treated independently with various concentrations (10 nM -100 µM) of TG003 for 24-72 hours. Phase contrast microscopy (using a Nikon Inverter, Cat No. TE 300, UK) was used to check cell morphology.

2.3 Adherent cell trypsinisation

All adherent cells used in this study underwent trypsinisation as soon as they were about 80% confluent. This was important to avoid excess cell mass accumulation in T_{75} used for cell culture. In T_{75} culture flask containing 80% confluent adherent cells, 5ml of phosphate buffer saline (PBS) was added and mechanically agitated to wash the cells. The process was repeated twice before 1ml of 1x trypsin EDTA (Sigma Aldrich) was added and rocked to cover the flask and incubated at 37^{o}C for 5 minutes. This process detached the cells from the bottom of the flask and made them float; the flask was checked every 2 minutes to ensure complete detachment. 5 ml of fresh complete DMEM was added to the flask and the entire content of the flask was carefully pipetted into 15 ml tube. The 10% FBS content of the DMEM inactivated the

trypsin. Tubes containing the cell mixture was centrifuged at 1,000rpm for 5 minutes at room temperature using Beckman coulter Allegra x-22R centrifuge (Germany). The supernatant was discarded while the cell pellets were re-suspended in fresh DMEM for further use (counting or splitting).

2.4 Cell cryopreservation

Cells were preserved in liquid nitrogen to ensure availability of early passages. Confluent cells were trypsinised with 1x trypsin EDTA (Sigma-Aldrich) and incubated for 5 minutes until the adherent cells detached from T_{75} flask and floated. 5 ml of complete DMEM was added into the flask and the cell mixture pipetted into 15ml tube. The tube was centrifuged at room temperature at 1000 rpm for 5 minutes. The supernatant was discarded and the cell pellets were counted after being re-suspended in freezing media containing 20% (v/v) FBS, 10% (v/v) DMSO and 70% (v/v) Dulbecco's Modified Eagle Medium (DMEM). Freezing media was adjusted to obtain a concentration of $1x10^6$cell/ml. Aliquots of 1ml containing 1 million cells in cryovials were inserted into a cryopreserving chamber containing isopropanol and left overnight in -80^{o}C before the cryovials were stored in a liquid nitrogen chamber for future use.

2.5 Thawing cells from liquid nitrogen

Cells stored in cryovials in liquid nitrogen were thawed in water baths under sterile conditions at 37^{o}C. Thawed cells were transferred into T_{75} culture flask in 10ml of complete DMEM and incubated in CO_2 incubator at 37^{o}c. After 8 hours when the cells were already attached to the bottom of the flask, the media was removed and replaced with 10ml of fresh complete DMEM in order to get rid of DMSO (contained in the freezing media) as much as possible. The flask was incubated and split when 80% confluence was attained.

2.6 Cell proliferation measure *via* trypan blue assay

Three hundred thousand ($3x10^5$) prostate cancer cells were independently seeded in six-well plates containing 4ml of Dulbecco's Modified Eagle's Medium (DMEM) with the following additives: 5 mM glucose and 1% L- glutamine, 10% fetal bovine serum (FBS) and 1% sodium pyruvate to make complete media. The reagents mentioned above were purchased from Sigma Aldrich, USA. The cells were treated with various concentrations of TG003 with controls and incubated in a 5% CO_2 incubator for 24, 48 and 72 h. At each time point, the cells were harvested and diluted with trypan blue. Trypsinised cells were counted in the haemocytometer after being re-suspended in an appropriate volume of media containing equal amounts of 0.4% (w/v) trypan blue. Cell survival and percentage viability of each of the prostate cancer cell lines were counted and determined using a haemocytometer (Neubauer chamber). Stained blue cells were considered dead, while the nucleus of live cells were not stained by the trypan blue.

2.7 Cell proliferation by Ki67 assay

Three hundred thousand ($3.0x10^5$) prostate cancer cells (PC3, DU145) and normal prostate epithelia cell line (PNT2) were seeded on cover slips in six-well plates and serum starved in 4ml of DMEM without fetal bovine serum for 8 hours. Starving media was carefully removed after 8 hours of starvation and subsequently replaced with 4ml of complete DMEM. Cells were immediately treated with chosen concentrations of TG003 (1 µM, 10 µM and 50 µM) and incubated with the controls in 5% CO_2 incubator for 48 hours at 37^0C. After 48 hours incubation, media was removed from each of the wells and cells washed twice with 2 ml of warm PBS. Washed cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes and permeabilized in 0.25% (v/v) triton in

PBS. Fixed cells were blocked in 10% FBS-PBS for 1 hour and incubated overnight at 4^{o}C in 1:1000 rabbit polyclonal anti-Ki67 primary antibody (Abcam, Cambridge, UK). After overnight incubation in primary Ki67 antibody, cells were washed 3 times for 5 minutes each in PBS before incubation for 1 hour at 37^{o}C in the secondary antibody- 1:500 of Alexa flour 488/568 anti- rabbit. At this stage, cells were washed 3 times to remove residual secondary antibody for 5 minutes each. Cells were counterstained with DAPI (3 µl/mL) for 4 minutes and washed 2 times for 5 minutes each. Signal images were obtained using Image Proplus (Nikon TE 300 Japan). The percentage of proliferating cells was measured and calculated using Image J.

2.8 Spectrophotometric measurement of colour changes and media depletion following TG003 treatment (indirect proliferation assay)

Cells (1x10^{6}) were seeded and treated with TG003 in six-well plates. After 96 hours incubation in a 5% CO_2 incubator at 37^{o}C, the media from untreated, DMSO, 1µM, 10 µM and 50µM wells was analysed in the spectrophotometer at 540nm wavelength using PBS and fresh DMEM as blanks and standards respectively.

2.9 Acridine orange assay

Cultured TG003 treated cells were thoroughly vortexed and about 150µl of 20,000 cells in PBS suspension dispensed into a cytofunnel with ethanol cleaned slides and centrifuged in a Shandon Cytospin 4 (No. A78300101, Serial No. CY94701405, Thermo Scientific UK) at 1,500 rpm for 8 minutes. Slides were left to air dry completely after being removed from the Cytospin and then fixed in 90% methanol for 10 minutes. Phosphate buffer made from 0.66% w/v KH_2PO_4 and 0.32% w/v Na_2HPO_4 was freshly prepared and slides dipped into it for 2 seconds and stained in acridine orange solution (24 mg of acridine orange / 200 ml of phosphate buffer) for 45 seconds in a dark box.

Slides were removed from the stain solution and dipped into another dark box containing phosphate buffer for 10 minutes and 15 minutes respectively. Slides were analysed microscopically using a fluorescence microscope (Nikon Eclipse 80i-upright) with BG-12 excitation filter and 0-530 barrier filter.

2.10 Caspase 3/7 apoptosis assay

Three hundred thousand cells of human prostate cancer cell lines (PC3 and DU145) and normal prostate epithelia cell line (PNT2) were independently seeded in six-well plates containing 4 ml of complete DMEM. Cells were treated for 48 hours at 37^{o}C in a 5% CO_2 incubator with varying concentrations of TG003 ranging from 1 µM to 50 µM, while the untreated received no treatment but DMSO alone was used as a solvent control. DMEM was removed from each well and replaced with 1 ml of warm PBS. Then two drops of caspase-3/7 green detection reagent cat 10723 bought from Thermofisher Scientific-UK were added to each well, plates wrapped with aluminium foil and left in the dark incubator at 37^{o}C for 45 minutes. Images were obtained with a fluorescence microscope (using Image Proplus) usig the green channel (Flou-488). Matched images were obtained by switching to the light phase contrast microscope. The percentage of caspase 3/7 positive cells was determined using Image J software.

2.11 RNA extraction

RNA extraction was performed using the Absolutely RNA miniprep kit from Agilent technology Ltd, USA. The reagents include; RNase- Free DNase 1, high salt wash buffer, low-salt wash buffer, 70% ethanol, β-Mercaptoethanol (β-ME), lysis and elution buffers. These reagents were prepared and used according to the manufacturer's protocol. Three hundred and fifty microliter (350 µl) of lysis buffer was mixed with 2.5 µl of (β-ME) to lyse cell pellets containing between $1x10^6$-$5x10^6$ cells. After cell lysis,

the procedures were followed strictly according to the kit protocols until the RNA was finally eluted in 30 µl of the elution buffer.

2.12 RNA quality (assessed by nanodrop)

RNA quality was checked on 1% agarose gels; the concentration and purity of the extracted RNA was determined using a nanodrop device (Spectrophotometric, UK). Firstly, the nanodrop sample dropping point was cleaned thoroughly with a wipe. The device was initialized with water and blanked with elution buffer before taking measurements. About 1.25 µl of the eluted RNAs were measured and the result was displayed on the screen.

2.13 cDNA synthesis

cDNA synthesis was performed using reverse transcriptase and a mixture of oligo (dT) and random primers bought from Promega company Ltd, USA. The procedures for cDNA synthesis, following manufacturer's protocol, is summarised in Table 2.

Table 2. protocol for cDNA synthesis

	Untreated	DMSO	10 Nm	100 nM	1 µM	10 µM	50 µM	100 µM
RNA concentration (ng/µl)								
3µg RNA (µl)								
0.5µl Oligo	0.5	0.5	0.5	0.5	0.50.	0.5	0.5	0.5
0.5µlRandom Primers	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Water (Make up to 5 µl)								

Three microliter (3 µl) RNA was mixed with 0.5 µl of the oligo and 0.5 µl random primers. The reaction was made up to 5.0µl with nuclease free water. The samples were placed in a heat block at 70^{o}C for five minutes, chilled on ice for another five minutes and centrifuged at 12,000 rpm for 10 seconds.

Table 3. The RT reaction mix was prepared as below on ice.

Component	**For 1x reaction**	
Goscript 5x reaction buffer	4.0 µl	
$MgCl_2$	2.4 µl	
PCR Nucleotide Mix (dNTP)	1.0 µl	
RNasin Ribonuclease inhibitor	0.5 µl	
RT	1.0 µl	
Water	6.0 µl	

To each 5 µl RNA mix, 15 µl of RT mix was added. The mixtures were placed on a heat block at varying temperatures and time to fulfil the following conditions: annealing at 25^{0}C for 5 min, extension at 42^{o}C for 1 hour and inactivation of the reverse transcriptase at 70^{o}C for 15 min.

2.14 DNA quantitation

During the DNA quantitation, the same procedures for RNA quality (nanodrop) were used with the same device. The only difference was that DNA parameters were selected from the drop down menu instead of RNA.

2.15 *CLK1* primer design

CLK1 primers targeting exon 4 and intron 4. At exon 4, 1FB and 3RB (forward and reverse primers) were designed; see Appendix I. At the intron 4, 2FB and 3RA (forward and reverse primers) were used. The designed primers were purchased from Eurofins Ltd.

Table 4. *CLK1* primers

	Forward primer (intron 4)	Reverse primer (intron 4)	Forward primer (exon 4)	Reverse primer (exon 4)
Name of primer	2FB	3RA	1FB	3RB

2FB = 5'-GGAGGTCACCTGATCTGTCAG-3'
3RA= 5'-CTGCTACATGTCTACCTCCCGC-3'
1FB=5'-CAAGGATGTGAACCTGGACATCGC-3'
3RB= 5'-CTCCTTCACCTAAAGTATCAAC-3'

2.16 Gel extraction (DNA purification)

For gel extractions the QIAquick gel extraction kit, USA was used. Using a clean sharp scalpel, the DNA fragment was excised from agarose gel and the sliced gel was weighed in a colourless eppendorf tube. Three volumes of QG buffer was added to one volume of gel. The tube containing the gel was incubated at 50^{o}C on a heat block until the gel was completely dissolved. Isopropanol was added to one gel volume and the mixture was properly vortexed. The mixture was placed to a QIAquick spin column over a 2 ml collection tube and centrifuged at 13,000 rpm for 1 min until the samples have passed through the column. The flow-through was discarded and the QIAquick spin column replaced over the tube. To same QIAquick spin column, 0.5 ml of QG buffer was added and centrifuged as above. The flow-through was also discarded and the column replaced to the tube. The QIAquick column was washed with 0.75 ml of PE buffer, centrifuged and allowed to stand for 2-5 min. Over the 2 ml collection tube, the QIAquick spin column was centrifuged to remove traces of residual wash buffer. The QIAquick spin column was placed over 1.5 ml and 50µl of elution buffer (EB) was added at the center of the QIAquick membrane, centrifuged for 1 min and allowed to stand for 4 min. The purified DNA was analysed on agarose gel with a loading dye.

2.17 DNA sequencing

Purified PCR products were sent to Eurofins company Ltd. UK for sequencing. Sequencing reactions were assembled as per Eurofins instructions, Mix2Seq reads. Premixed samples were prepared consisting of 15 µl template (1 ng/µl) DNA plus 2 µl of the relevant primers at 10 µM.

2.18 RT-PCR

cDNA was amplified with *CLK1* primers (forward primers in exons 3 and 4, and reverse primers in exon 5). PCR cycles were as follows: 95^{o}C for 2 minutes (initial denaturation), 95^{o}C for 1 minute (denaturation), 59^{o}C for 1 min (annealing), 72^{o}C for 1.5 min (extension), denaturation step repeated for 34 times followed by a final extension step at 72^{o}C for 5 min.

2.19 Protein isolation

Cells were trypsinized, washed twice with phosphate buffer solution (PBS) and transferred to eppendorf tubes placed on ice. Two hundred microliters (200µl) of cold RIPA+PI buffer was added to the cells while still on ice for 20 min. The cells were freeze-thawed 3 times and spun down for 15 min at 13,000 rpm at 4^{o}C. The supernatant (proteins) were recovered and quantified.

2.20 Determination of protein concentrations using the Bradford assay

Standard BSA protein concentration (2 mg:1 mg: 0.5mg: 0.25 mg: 0.125 mg) were prepared. Protein stock 50µl was diluted with equal volume of water. Twenty five microliter (25 µl) of the mixture was taken for protein concentration measurement according to BCA/ Bradford assays protocol (Fluostar Optima, BUG Labtech, UK). A standard protein curve was generated.

2.21 SDS- PAGE

Ten percent (10%) acrylamide (separating and stacking) gels were prepared according to table 5 below.

Table 5. Ten percent (10%) acrylamide gel preparation

	Separating gel	**Stack gel**
Acrylamide concentration	10%	
MW Range (KDal)	16-70	
30% acrylamide mix (29:1 acrylamide: bis-acrylamide)	3.3 ml	0.67 ml
Buffer A (1.5M Tris pH 8.8)	2.5 ml	_
Buffer B (1.0M Tris pH 6.8)	_	0.5 ml
Water	4.1 ml	2.4 ml
10% Ammonium persulfate	100 µl	100 µl
TEMED	40 µl	40 µl

The separating gel was made first and gently poured into 1.5 mm gel tray in a vertical position. Isopropanol was overlaid on the gel to shield the gel from air which could inhibit polymerisation. When gel has polymerised, the isopropanol was rinsed off and drained out with water. The stack gel was overlaid on the separating gel and 10 well comb was inserted immediately. Twenty micrograms (20 µg) protein in 1:1 ratio of Laemlli buffer was denatured at $100^{O}C$ for 5 min and cooled on ice. Samples (30µl each) were loaded to wells along with protein ladder and controls. Protein gels were run in 1x running buffer at 70V through the stack gel for 30 min, and 90V through the separating gel for 1 hour.

2.22 Buffer preparation

To prepare 1 L of 10x running buffer, the following were added and made up to 1 L of deionised water: 250 mM Tris Base (pH 8.3), 1.9M glycine and 1% (w/v) SDS. While 700 ml with 1x running buffer was made by adding 70 ml of 10x running buffer and 630 ml of deionised water. 10x transfer buffer was prepared with same concentration and pH of tris Base and glycine as in 10x running buffer without SDS. However, 1 L of 1x transfer buffer was made by adding 100ml of 10x transfer buffer to 200 ml of methanol and 700 ml of deionised water. 1 L of 10x TBS was made by adding the following and made up to 1 L with deionised water: 0.5 M Tris HCl (pH) 7.6 and 1.5 M NaCl

2.23 Western blotting by wet transfer

Six blotting papers and 1x PVDF membrane were cut to 9x6cm and the membrane marked for identification. The membrane was activated in methanol for 5 min and washed 3 times along with blotting papers and cassette sponges in ice cold transfer buffer. Stack gel was removed from the separating gel and the cassette assembled as follows: black side of cassette, sponge, 3x pads of blotting papers, gel, membrane, 3x pads of blotting papers, another sponge and red side of the cassette. This assemblage was run in a transfer tank with ice cold transfer buffer and freezer pack at 50v for 2 hours.

2.24 Antibodies

Anti-CLK1 polyclonal antibody from rabbit bought from Sigma Aldrich (cat No. R1471-1S) was used as primary antibody. Membrane was blocked in 5% blocking solution made from 1g of BSA and 20 ml of 1x TSB-T buffer and allowed on Stuart Gyro-rocker (SSL3, Biocote ltd, UK) for 1 hour. And incubated overnight with 1:5000 CLK1 primary

antibody. The secondary antibody is an anti-rabbit purchased from Sigma Aldrich (cat No. 7074S) dissolved in 1:5000 blocking solution. The membrane was washed 3 times for 5 min in 1x TBST and incubated in secondary antibody for $1^1/2$ hour at room temperature on a shaking machine. Membranes were washed three times for 5 min in 1X TBST before taken to the dark room for development.

2.25 Film development

Using tweezers, the membrane was picked from 1x TBST and the corners dripped onto filter or tissue paper to get rid of excess TBST. The membrane was placed on acetate paper, 2 ml of chemiluminescent substrate (Luminata forte, cat No. WBLUF0100, Millipore ltd, UK) was added and rocked for 2 minutes. Excess substrate was dripped from the corners of the membrane on paper. The membrane was transferred flat into the polypocket acetate sheet of cassette. Indications were made with a tick pen on the position of the molecular markers. Light was turned off and film purchased from Sigma Aldrich was laid over the membrane, the position of the markers noted and the cassette closed tightly for 5 minutes exposure. Film was rocked in a developer (Harman technology ltd, UK, cat No. 1757314) until bands were seen, dipped into water for 1 seconds and transferred to the fixer (Harman technology ltd, UK, cat No. 1758285) for 30 seconds and finally washed in water. Images were also captured using Li-Cor Odyssay (UK).

2.26 Wound healing-scratch assay

A yellow 200µl pipette tip guided by a clean glass slide was used to make a straight line scratch in a confluent prostate cancer cells seeded in six well plates. Floating scratched cells were gently removed with warm PBS while a complete DMEM containing TG003 treatment was used to incubate the cells in a 5% CO_2 incubator at

37ºC. A set lumascope was inserted in the incubator which records the extent of scratch closure every 2 hours for 72 hours. The time dependence of scratch closure was compared between TG003 treated cells and the untreated controls. Images were obtained every two hours using the phase contrast lumascope under x4 objective. Image j was used to analyse the length of scratch closure in both controls and TG003 treated cells.

2.27 Cell migration and invasion assay

For invasion assay, prostate cancer (PC3) cells were treated for 48 hours with various concentrations of TG003 ranging from 1 µM to 50 µM in 10% foetal bovine serum medium after being subjected to 8 h pre-starvation in serum free DMEM. All equipment and reagents such as 24 well plates, Transwell chambers, serum free DMEM, pipette tips and micropipettes were kept overnight in a fridge prior to the assay. Stock 7.9mg/ml extra cellular matrigel (ECM) (Sigma-Aldrich E6909) was defrosted overnight at 4ºC. Serum free DMEM was used to dilute the stock matrigel and the transwell chambers suspended on 24 well plate were coated with100 µl of the mixture. The plate was kept for gelling overnight in incubator at 37ºC. Into the lower chamber, 500µl of complete DMEM containing 10% FBS were added as a chemoattractant, while 300µl containing $1.0x10^5$cells/ml in a starving DMEM were added to the transwell chamber suspended on 24 well plate and incubated at 37ºC for 24 h. The cells were washed twice in PBS after the media from each of the wells were removed with pipette. 4% paraformaldehyde (PFA) was used to fix the cells at room temperature for 10 minutes. Non-invasive cells that were still retained inside the insert were carefully scraped off with a swab. Cells were washed twice in PBS and permeabilized in 100% methanol for 20 minutes at room temperature and subsequently washed twice after methanol removal. A sterile scalpel guided with a forceps was used to cut the

membrane containing the invasive cell and turned upside down on clean slide. The slides were stained and incubated with Hoechst for 1 h on coverslip at room temperature in the dark. Invasive cells were imaged and counted using image proplus (Nikon TE 300 Japan). The same material and procedure was applied for cell migration assay, except that the transwell inserts were not coated with matrigel and the equipment were not refrigerated overnight.

2.28 Heat shock assay

Half a million prostate cancer cells (PC3, DU145) were seeded independently in a T25 flask and allowed to settle overnight in incubator at 37^{o}C. Cells were subjected to heat shock at 42°C for 1 h, 3 h and 6 h. RNA was made from the cell pellets and cDNA synthesized for PCR amplification. Protein from the portion of the pellets was quantified and run in a western blot to determine the effect of heat shock on CLK1 protein levels.

2.29 Osmotic stress induction and harmine treatment

Induction of osmotic stress in prostate cancer cell line DU145 was achieved by cell line treatment with 200 and 400mM of sorbitol S0900 bought from Sigma Aldrich and incubated at 37^{o}c for 3 hours. Untreated cells were used as control. For harmine treatment, 50% ethanol was used to dissolve 98% harmine bought from Sigma Aldrich, prostate cancer (DU145) cell lines were treated with 1, 3, and 5 µM of harmine concentration for 6 hours at 37^{o}c while 10µM ethanol treated and untreated cells were used as controls.

2.30 CLK1 siRNA knockdown

PC3 cells ($5.0x10^{5}$) were seeded in six well plates with penicillin free DMEM and allowed to settle overnight. Eppendorf tubes were labelled A and B. Tube A according

to manual contains appropriate volume of Optimem minimal medium and lipofectamine RNAiMAX transfection reagent bought from Fisher Scientific UK Limited. Tube B according to manual contains appropriate volume of Optimem and diluted CLK1 siRNA bought from Santa cruz Biotechnology, USA. Contents of Tubes A and B were thoroughly mixed together and pre- incubated at room temperature on RT for 20 minutes. The mixture was transferred to the cells, topped up to 1ml with Optimem and incubated for 4 hours at 37ºC in a 5% CO_2 incubator. After this step the mixture was topped up to 2ml with complete DMEM void of antibiotics such as penicillin and re –incubated in a 5% CO_2 incubator at 37ºC for 48 hours. Cells were lysed with RIPA+PI. Proteins were harvested and quantified using Bradford method and finally run in SDS PAGE. The same procedures were applicable to the scrambled- control siRNA bought from Santa Cruz, USA.

2.31 EMT marker expression

Three hundred thousand cells were seeded and treated with varying concentrations of TG003 on cover slips in six well plates and incubated in 5% CO_2 incubator at 37ºC for 48 hours. Cells were washed three times with warm PBS and fixed in 4% PFA. Cells were permebilized in 0.25% (v/v) Triton-PBS for 10min on RT after being washed three times in PBS. Cells were blocked on RT for 1 hour in 10% FBS/PBS. E-cadherin and vimentin primary antibodies were diluted 1:150 and 1:400 respectively in 10% FBS-PBS and incubated overnight on RT at 4ºC. After three times wash with PBS, Goat anti-rabbit Alexa flour-568 1:750 dilution was used for E-cadherin secondary antibody, while anti-mouse Alexa flour-488 1:1000 dilution was used for vimentin secondary antibody and incubated at room temperature on RT for 1 hour. Cells were

washed three times in PBS and counterstained in Hoechst (1 µl/ml) for 10 min. Images were obtained using fluorescence microscope and analysed in image j.

2.32 PC3 xenografts

Xenograft experiments were conducted at the University of Exeter in collaborator Dr. Sebastian Oltean's laboratory. Due to my lack of licence to work on live animals, Dr. Sebastian Oltean and Li Ling performed the entire experiment while I participated in tumour measurement. For treatment studies, PC3 human prostate cancer cells were detached from culture flasks and diluted in pre-cold PBS to a concentration of $1x10^7$/ml and placed on ice. 100 µl of cell suspension was subcutaneously injected into each right flank of CD1- nude mice (Charles River), 12 mice in total. Tumour sizes were measured by calliper two times per week. When the tumour sizes reached 3mm x 3 mm, 50 µM TG003 or DMSO were injected intraperitoneally two times a week, 6 mice per treatment group. Mice were culled by cervical dislocation (Schedule 1) when the tumour size reached maximum (12 mm x 12 mm) and the tumours were extracted. Images of each tumours were taken and weighed. Tumours were flash frozen in liquid nitrogen for further analysis. Same procedures were repeated for 10 µM TG003. Tumour volumes were calculated using formula volume= [(length+width)/2]*length*width.

2.33 Statistical analysis

All results were subjected to statistical analysis using graph pad prism version 7.03 after pre-testing data for normality of distribution and between data group homogeneity of variances. Parametric tests were applied for data with both normal distribution and of equal variances, taking sample sizes into consideration. One way variance analysis

(ANOVA) was used to determine the statistical differences among the means of independent groups (multiple comparison). In cases where the effect of two variable factors were to be determined both separately and as groups, two-way variance analysis was applied. Non-parametric tests such as Kruskal-Wallis were used for data showing non-normal distributions. *Post hoc* between data group pairwise comparisons were subsequently made by either 2-sample t-test, Tukey Kramer's test, Mann Whitney U-test or Dunn's test depending on data normality. The statistical package used for each data analyses are indicated in the respective figure legends, n.s depicts not significant, *=p≤0.05, **=p≤0.01, ***=p≤0.001, ****=p≤0.0001.

Chapter 3

Regulation of CLK1 via alternative splicing through intron retention and exon skipping

3.1 Aim

The importance of splice factor protein kinases in the regulation and control of alternative splicing is gradually gaining global recognition. The aim of this chapter is to examine CLK1 expression and alternative splicing through exon 4 skipping and intron 4 retention in a range of human cancer cell lines including prostate cancer. To address this aim we examined the effect of heat shock, osmotic shock and exposure to the drug harmine on CLK1 alternative splicing in prostate cancer cells.

3.2 Design and evaluation of primers to measure *CLK1* alternative splicing

The *CLK1* human is structurally very similar to the corresponding mouse gene which was previously analysed by Duncan *et al*., 1997 and Ninomiya *et al*., 2011. There are reports of alternative splicing of *CLK1* related to mouse *CLK1* (both skipping of exon 4 and intron 4 retention; Ninomiya *et al*., 2011). The alternative splicing of the equivalent exon and intron of human gene has not been examined. We focused on human *CLK1* exon 4 and intron 4 (figure 3.1A). Exon 4 is 91bp with a short intron (intron 4) separating it from exon 5. When exon 4 is skipped, CLK1 is truncated to a 120 amino acid protein described by Duncan *et al*., (1997) as $CLK1^{T}$ which we refer to as CLK^{T1}. However, intron 4 retention also results to truncated protein which we refer to as $CLK1^{T2}$ (figure 3.1A and B). In summary, premature stop codons arising from exon 4 skipping and intron 4 retention result in truncated proteins which we call $CLK1^{T1}$ and $CLK1^{T2}$ respectively.

We designed human specific primers (see the *CLK1* alignment in the appendix I). The best combinations of primers were determined. To measure exon 4 inclusion rates we combined 1FB/3RB (5’-CAAGGATGTGAACCTGGACATCGC-3’/5’-

CTCCTTCACCTAAAGTATCAAC-3’ forward and reverse primers respectively. To determine intron 4 retention the best combination was 2FB/3RA (5’-GGAGGTCACCTGATCTGTCAG-3’/5’-CTGCTACATGTCTACCTCCCGC-3’ forward and reverse primers respectively). In order to check that the PCR products were correct, they were excised out of 2% gels and sent to Eurofins for sequencing.

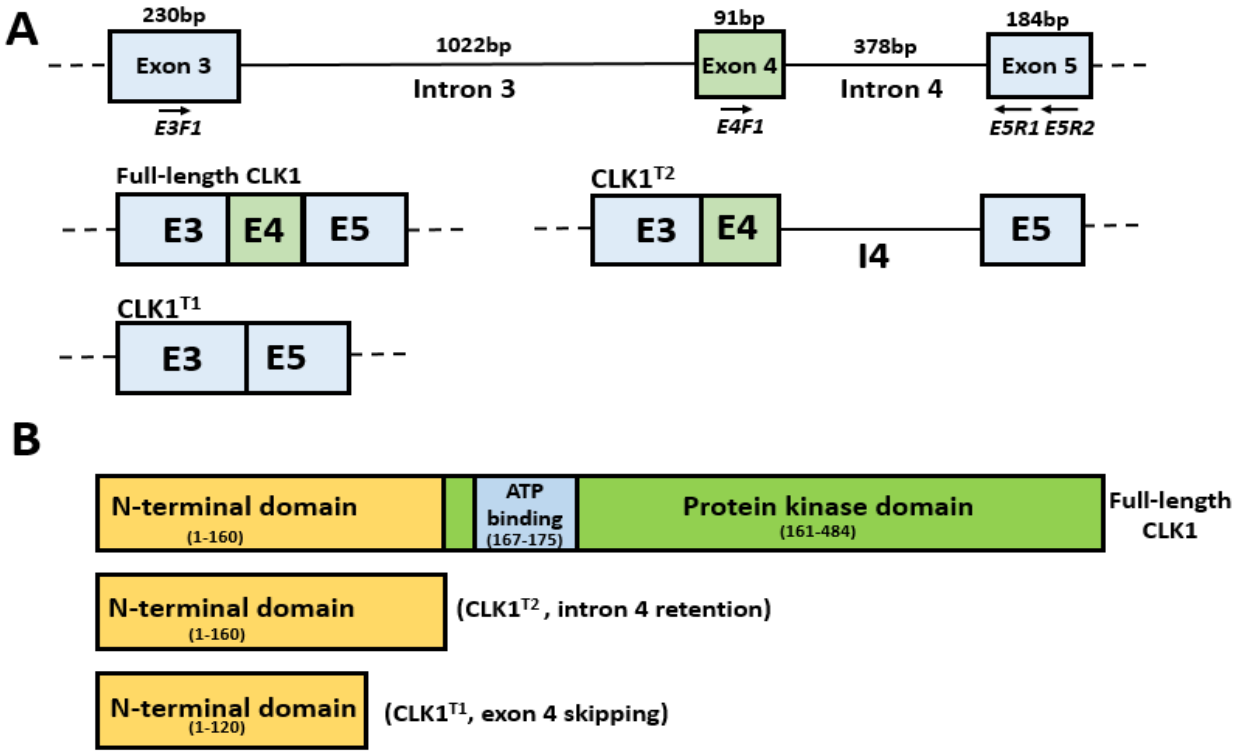


Figure 3.1 A description of *CLK1* alternative splicing of cassette exon 4 and retained intron 4. (A) A schematic of *CLK1* splice isoforms depicting exon 4 skipping and intron 4 retention. The position of the PCR primers used are indicated. (B) *CLK1* protein structure indicating its splice isoforms. (C) The sequence shows the position of putative potential exonic splice enhancers (green) in CLK1 exon 4 (capital letters) and an intronic splice silencer (red), predicted using SROOGLE (Schwartz *et al*., 2008). This figure is adapted from Uzor *et al*., (2018).

3.3 Alternative splicing of human *CLK1* in cancer cell lines

CLK1 expression (in particular its alternative splicing) has not been studied in human cell lines. Here we considered whether or not human *CLK1* is similarly alternatively spliced. Its alternative splicing was surveyed in a panel of cell lines (PC3, DU145 and VCaP prostate cancer), prostate epithelium cell line (PNT2), in leukemic cell lines (K562, TK6, HL60, CMK, MOLT4 and RPKV8226), cervical cancer cell line (HeLa) and foetal kidney cells (HEK293) (Figures 3.2A and B). It is clear that prostate, leukemic and other cancer cell lines (figures 3.2A and B) show varying degrees of intron 4 retention and exon 4 skipping. Of interest there is more intron 4 retention in prostate cancer cell lines compared to leukemic cell lines. Among the prostate cancer cell lines there was less intron 4 retention in PC3 cells compared to DU145 and VCaP. *CLK1* alternative splicing was also visible with a higher degree of intron 4 retention in the normal prostate epithelia cells (PNT2). In foetal kidney and cervical cancer cell lines (HEK293 and HeLa respectively), we also observed alternative splicing of *CLK1*. *CLK1* alternative splicing was also observed in variety of leukemic cell lines with varying exon 4 skipping and intron 4 retention; the latter was at a lower level in acute lymphoblastic leukaemia cells (MOLT4) (figure 3.2B). In summary, although these PCR are semi-quantitative, there is evidence of alternative splicing of *CLK1* in a range of cancer cell lines studied with various degrees of exon 4 skipping and intron 4 retention. More accurate quantitative measurement, for example using qPCR are needed to measure the degree of alternative splicing precisely.

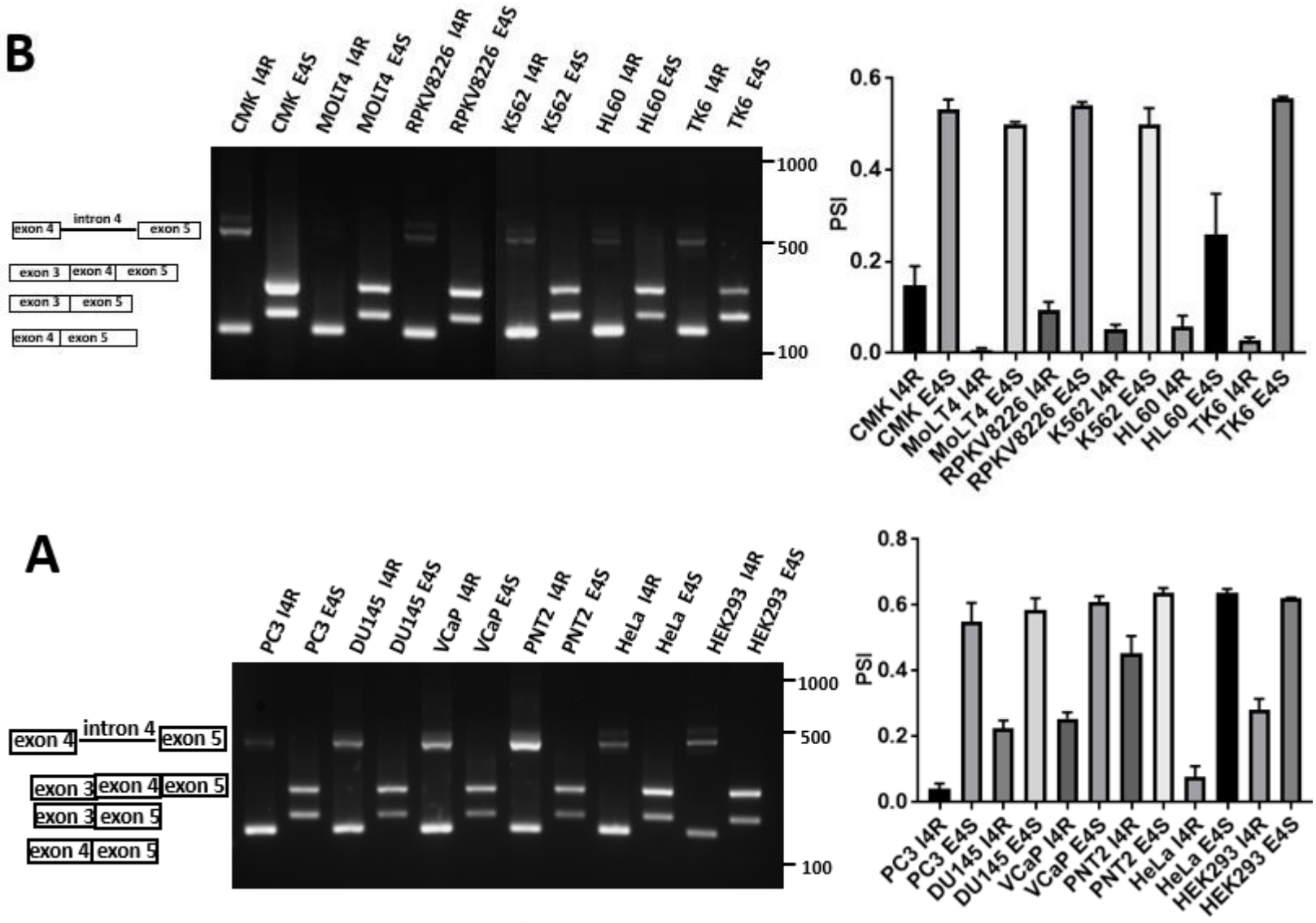
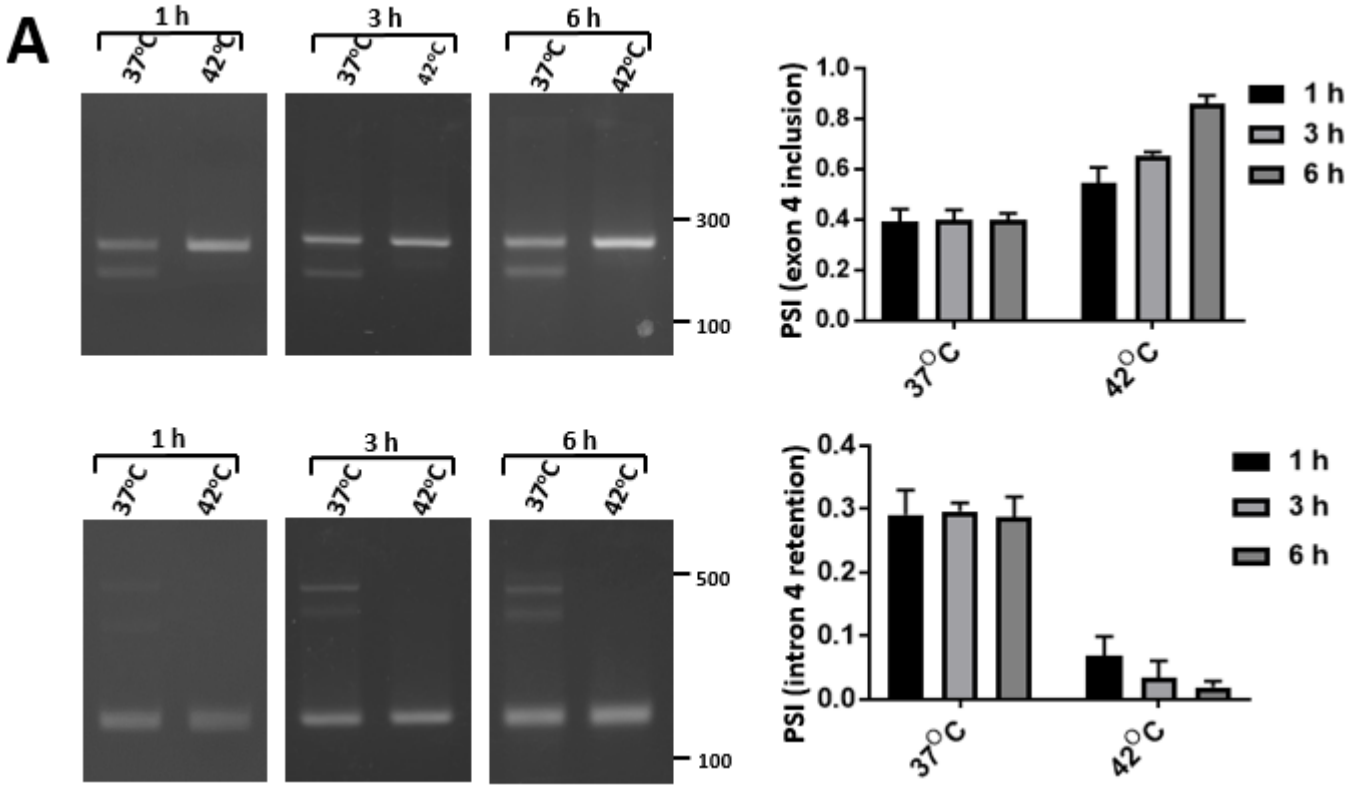

B
CMK I4R
CMK E4S
MOLT4 I4R
MOLT4 E4S
RPKV8226 I4R
RPKV8226 E4S
K562 I4R
K562 E4S
HL60 I4R
HL60 E4S
TK6 I4R
TK6 E4S
exon 4
intron 4
exon 5
exon 3
exon 4
exon 5
exon 3
exon 5
exon 4
exon 5
1000
500
100
PSI
0.6
0.4
0.2
0.0
A
PC3 I4R
PC3 E4S
DU145 I4R
DU145 E4S
VCaP I4R
VCaP E4S
PNT2 I4R
PNT2 E4S
HeLa I4R
HeLa E4S
HEK293 I4R
HEK293 E4S
0.8

Figure 3.2 *CLK1* alternative splicing in a randomly selected cancer cell lines. To determine exon 4 skipping (E4S) E3F1 and E5R2 forward and reverse primers were used, while E4F1 and E5R1 forward and reverse primers were used to determine the extent of intron 4 retention (I4R). The position of the individual splice isoforms are indicated. (A) Alternative splicing of *CLK1* in PC3, DU145 and VCaP (prostate cancer cell lines), for comparison PNT2 (normal prostate epithelium), HeLa (cervical cancer) and HEK293 (human embryonic kidney). (B) Alternative splicing of *CLK1* in CMK (acute megakaryocytic leukaemia); MOLT4 (acute lymphoblastic leukaemia; RPKV8226 (myeloma); K562 (chronic myelogenous leukaemia); HL60 (promyelocytic leukaemia); and TK6 (hereditary spherocytosis). These are leukemic cell lines. Indicated by the right corner are the PSI (percentage splice index) values of exon skipping and intron retention. N = 3 repeats. Individual error bars are indication of standard errors (SE). This figure is adapted from Uzor *et al*. (2018).

3.4 Effect of environmental stress on *CLK1* alternative Splicing.

Based on previous work by Ninomiya *et al*., (2011) on CLK1 alternative splicing in mouse cells, we wished to determine if there is a similar effect driven on human CLK1 alternative splicing. To do this, PCa (DU145) cells were heat-shocked at 42 °C for 1 h, 3 h and 6 h. We observed that there is a simultaneous significant reduction of intron 4 retention and exon 4 skipping after 1 h of heat shock treatment (figure 3.3A). We then performed western blot to determine if the increased *CLK1* exon 4 inclusion and diminished intron 4 retention corresponds to changes in CLK1 protein levels. As expected CLK1 protein expression was increased due to increased exon 4 inclusion (figure 3.3B). We then subjected the PCa (DU145) cells to osmotic stress (200 mM and 400 mM sorbitol treatment). Here we also observed decreased *CLK1* exon 4 skipping and intron 4 retention disappearance (figure 3.3C). The same result was observed following alkaloid (harmine) treatment with 1 µM – 5 µM concentration for 3 h (figure 3.3C). Hamine is a protein kinase (DYRK1A) specific inhibitor. DYRK1A overexpression has been linked with neuronal developmental impairment seen in down syndrome (Gockler *et al*., 2009). At 1 µM harmine concentration, there was complete loss of both *CLK1* exon 4 skipping and intron 4 retention (figure 3.3C). We conclude that like in mouse CLK1 (Ninomiya *et al*., 2011), exposure of cells to heat shock, osmotic stress and alkaloid treatment results to production of full length catalytically CLK1 due to increased *CLK1* exon 4 inclusion (decrease exon 4 skipping) and decreased intron 4 retentio


A
1 h
3 h
6 h
37°C
42°C
300
100
500
100
PSI (exon 4 inclusion)
PSI (intron 4 retention)
1.0
0.8
0.6
0.4
0.2
0.0
0.4
0.3
0.2
0.1
0.0
1 h
3 h
6 h

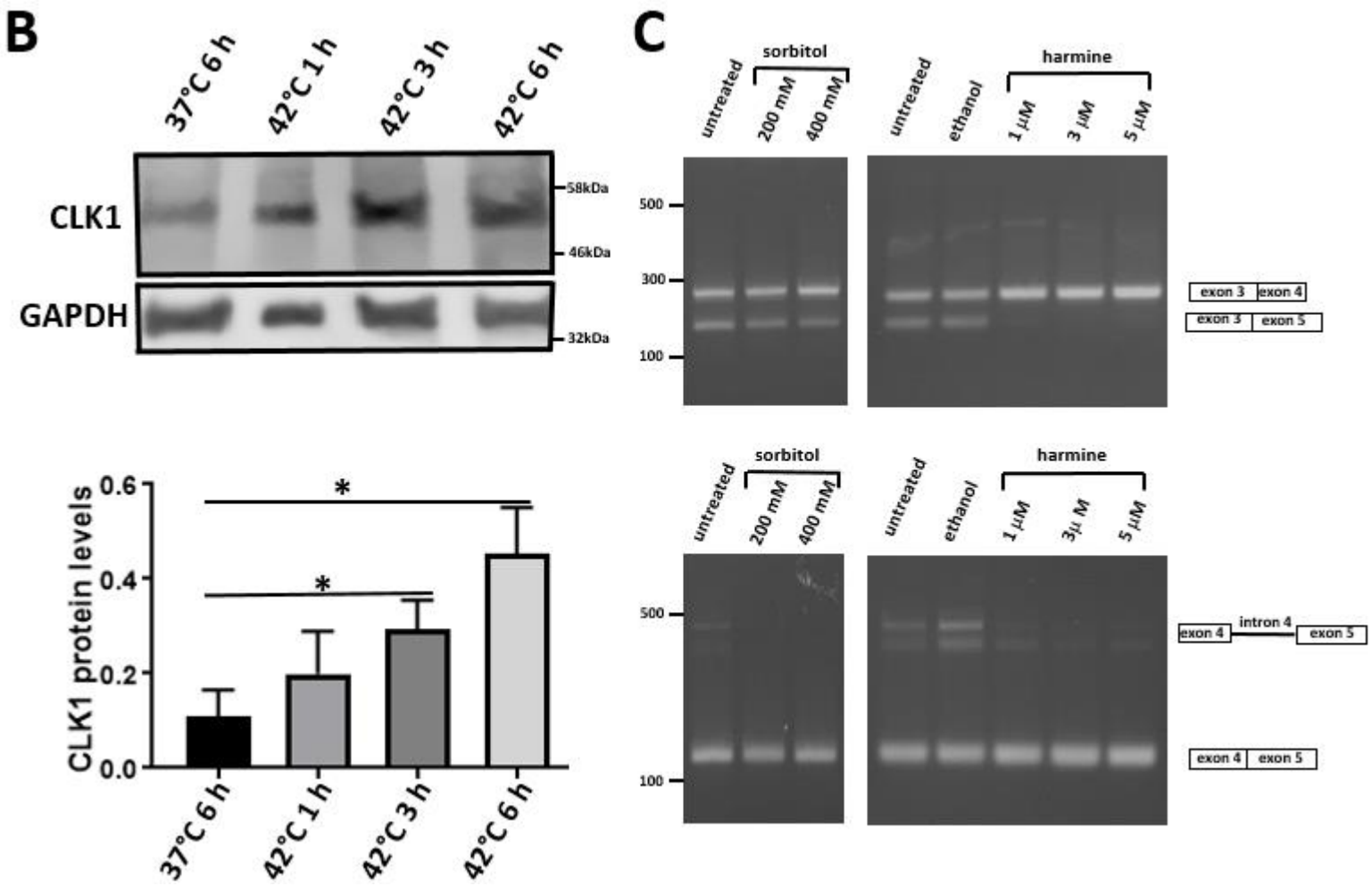


Figure 3.3 Effect of environmental stress such as heat shock, osmotic shock and chemical treatment (harmine) on alternative splicing of *CLK1*. (A) Prostate cancer cells (DU145) were exposed to heat shock-treatment at 42 °C for 1 h, 3 h and 6 h. DU145 cells cultured at 37ºC for 1 h, 3 h and 6 h were used

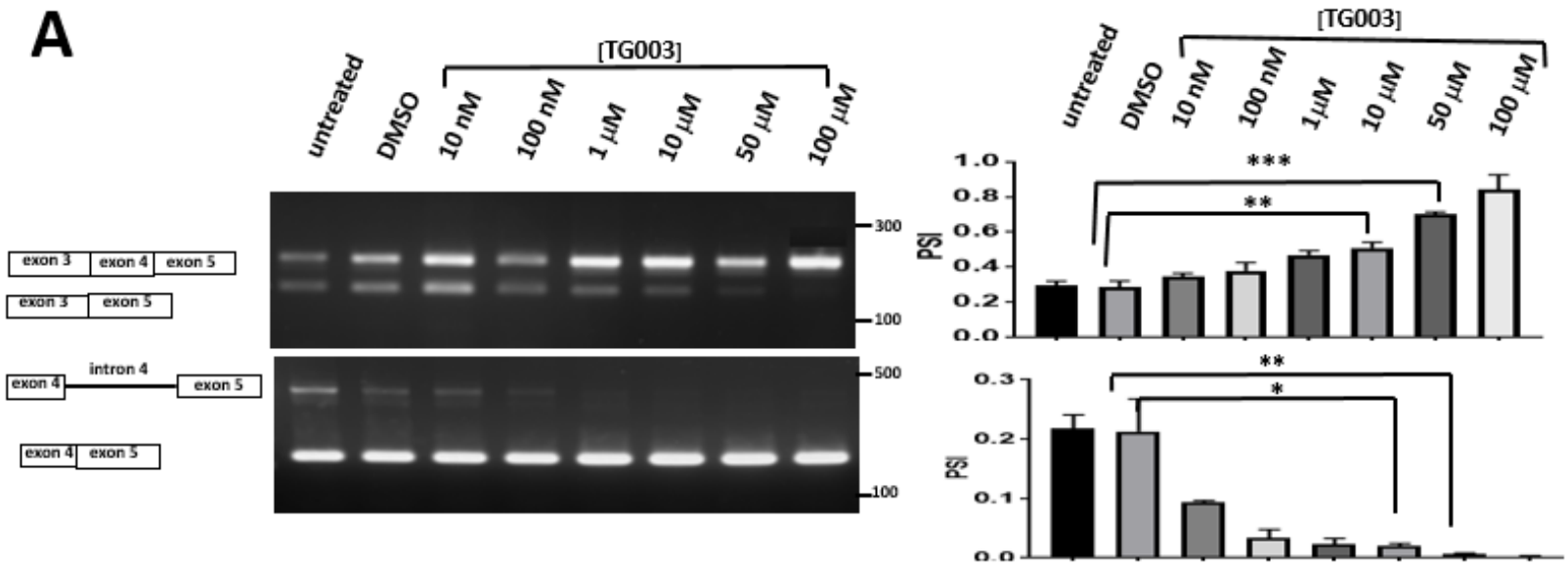
as controls. (B) a gel and graph showing CLK1 protein level in heat shock relative to GAPDH. (C) DU145 cells treated with 200 mM and 400 mM concentrations of sorbitol for 3 h, or treated with the alkaloid harmine (1–5 μM) concentration for 6 h. The PSI values for (A) showing rate of exon 4 and intron 4 retention is indicated by the right hand corner. N = 3, Kruskal-Wallis, *=p≤0.05. The error bars are indication of SE. This figure is adapted from Uzor *et al*., (2018).

3.5 Effect of *CLK1* inhibition with TG003 on alternative splicing

Next, we looked at the effect of *CLK1* inhibition on *CLK1* alternative splicing. Prostate cancer cell lines (DU145 and PC3) were treated with various concentrations of TG003 ranging from 10 nM to 100 µM for 48 h. There was a clear and visible *CLK1* alternative splicing starting from 100 nM (DU145) and 10 µM (PC3) TG003 treatment as shown in figures 3.4A for DU145 and 3.4C for PC3 cell lines respectively. There was clear reduction of exon 4 skipping and intron 4 retention in both cell lines at the highest concentration of TG003. In other words, results clearly show that *CLK1* inhibition by TG003 causes both reduced intron 4 retention and increased exon 4 inclusion (Figures 3 .4A, 3.4C). Both of these are consistent with generating 'productive' *CLK1* mRNA leading to the translation of full-length CLK1, and this is confirmed by a higher level of CLK1 protein (Figure 3.4B). Furthermore, Bowler *et al*., (2018) have shown that CLK1 might be involved in cellular adaptation to hypoxia as it is induced in hypoxic cells. In that study, *CLK1* was also inhibited with TG003. One of the genes whose alternative splicing was altered in hypoxia and also affected by *CLK1* inhibition is *FGFR1OP* (FOP). *FGFR1OP* is a fibroblast growth factor receptor 1 involved in myeloproliferative diseases. *FGFR1OP* alternative splicing was also determined in the same samples (figure 3.5). Consistent with previous findings (Bowler *et al*., 2018), here TG003 induced exon skipping in the *FGFR1OP* gene (Figure 3.5).

Having observed that there is a remarkably different rate of exon 4 skipping and intron 4 retention in several cell lines, the next question was to see if, as was reported in mouse cells, *CLK1* inhibition by TG003 results in increased expression of *CLK1* through altered splicing (in other words, indicating a potential auto-regulatory feedback mechanism). TG003, the compound used in the study is a synthetic specific CLK

kinase inhibitor developed by Hagiwara in 2005. Ninomiya *et al*., (2011) reported that the levels of *CLK1* and CLK4 mature mRNAs are elevated with the administration of TG003. Interestingly they determined that the reason behind increased *CLK1* expression was reduced intron 4 retention and increased exon 4 inclusion. After 48 h of TG003 treatment, protein was extracted and observed by western blotting. Figure 3.4B shows that there is an apparently increase in CLK1 protein following 48 h of TG003 treatment in PCa cells (DU145).


A
[TG003]
untreated
DMSO
10 nM
100 nM
1 μM
10 μM
50 μM
100 μM
exon 3
exon 4
exon 5
exon 3
exon 5
intron 4
exon 4
exon 5
exon 4
exon 5
300
100
500
100
PSI
1.0
0.8
0.6
0.4
0.2
0.0
***
**
PSI
0.3
0.2
0.1
0.0
**
*

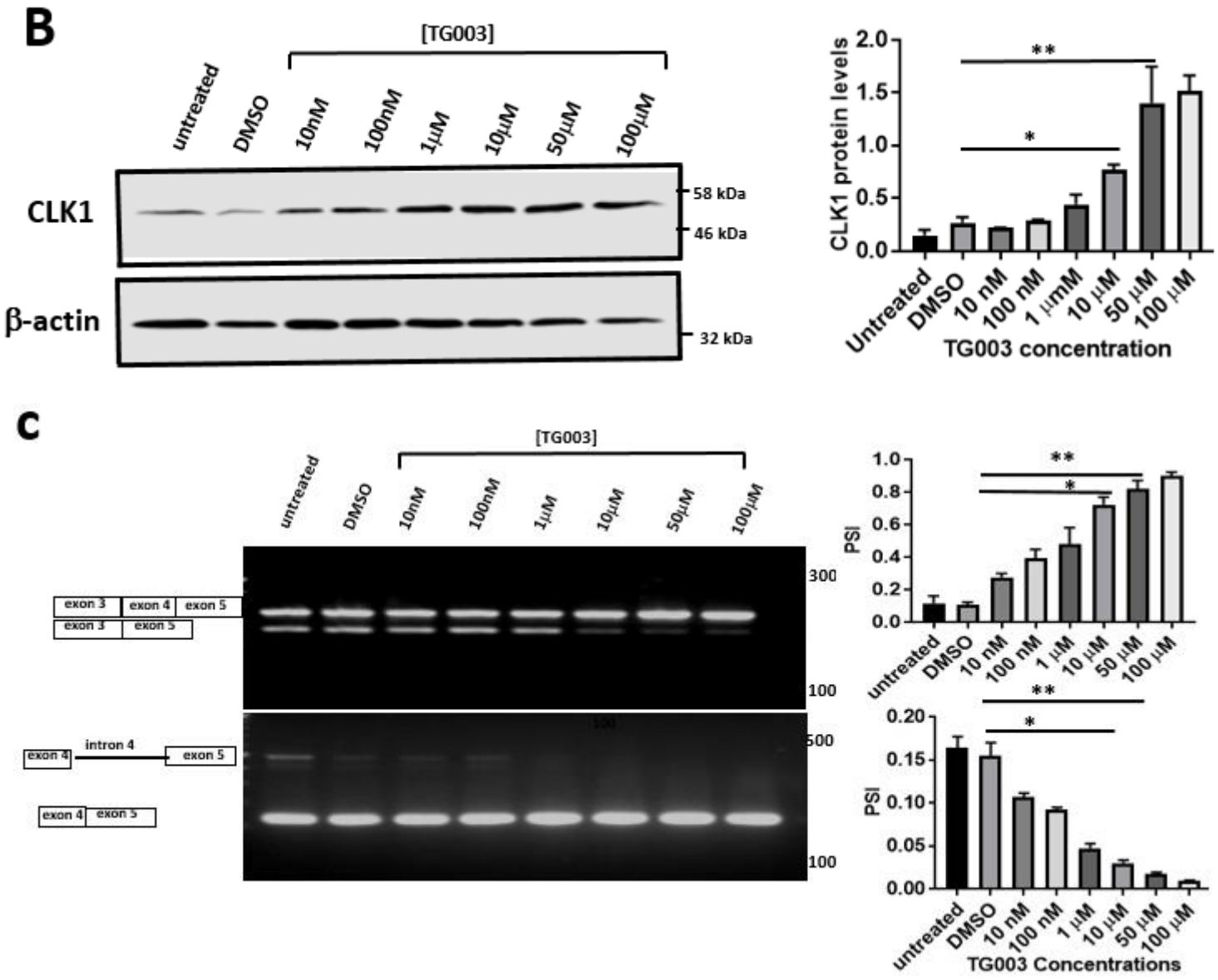

B
[TG003]
untreated
DMSO
10nM
100nM
1μM
10μM
50μM
100μM
CLK1
β-actin
58 kDa
46 kDa
32 kDa
CLK1 protein levels
2.0
1.5
1.0
0.5
0.0
**
*
Untreated
DMSO
10 nM
100 nM
1 μmM
10 μM
50 μM
100 μM
TG003 concentration
C
[TG003]
untreated
DMSO
10nM
100nM
1μM
10μM
50μM
100μM
exon 3
exon 4
exon 5
exon 3
exon 5
300
100
intron 4
exon 4
exon 5
exon 4
exon 5
500
100
PSI
1.0
0.8
0.6
0.4
0.2
0.0
**
*
untreated
DMSO
10 nM
100 nM
1 μM
10 μM
50 μM
100 μM
PSI
0.20
0.15
0.10
0.05
0.00
**
*
untreated
DMSO
10 nM
100 nM
1 μM
10 μM
50 μM
100 μM
TG003 Concentrations

Figure 3.4 Effect of benzothiazole TG003 (specific *CLK1* inhibitor) on *CLK1* alternative splicing. Prostate cancer cells (DU145) were treated with various concentrations of TG003 for 48 h. Untreated cells and cells treated with dimethylsulfoxide solvent (DMSO) were use as controls. (A, C) Effect of *CLK1* inhibition on splice isoforms (intron 4 and exon 4 of prostate cancer cell lines-DU145 and PC3 respectively). (B) Effect on CLK1 protein levels: Western blot determination of CLK1 protein concentration in TG003 (10 nM, 100 nM, 1 µM, 10 µM, 50 µM and 100 µM) treated DU145 cells. Untreated and DMSO controls. The β- acting (loading control) is indicated. The PSI values of both intron 4 and exon 4 rate of inclusions and statistics are indicated by the right hand corner. N = 3, error bars are indication of SE. Kruskal-Wallis, *=p≤0.05, **=p≤0.01, ***=p≤0.001. Figure adapted from Uzor *et al*., (2018).

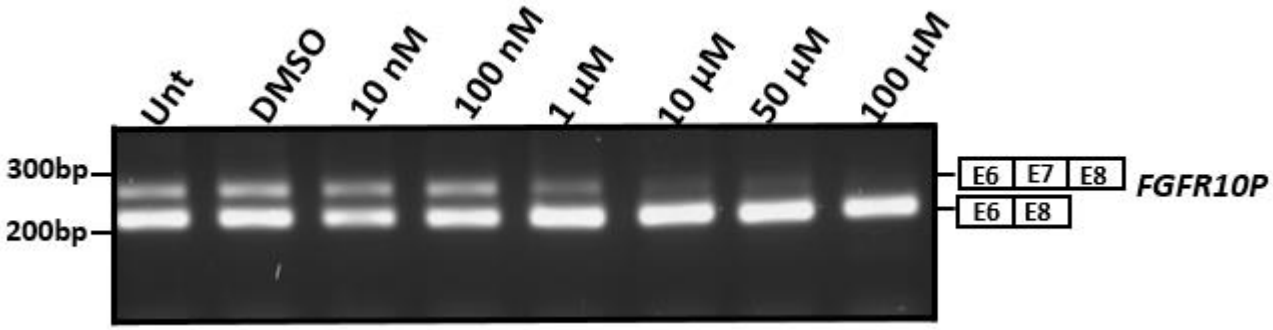


Figure 3.5. Effect of *CLK1* inhibition on *FGRF1* oncogene (*FGFR10P*) alternative splicing: Prostate cancer DU145 were treated with TG003 concentrations ranging from 10 nM – 100 µM. PCR products were optimised using *FGRF1* forward and reverse primers. The position of exon skipping is indicated by the right hand coner.

In summary, we have obtained evidence of extensive CLK1 alternative splicing in a range of human cancer cell lines with varying degrees of exon 4 skipping and intron 4 retention. We found that exposure of cells to environmental stress such as heat shock, osmotic stress and harmine treatment favours the expression of CLK1 mRNAs that encode for full length catalytically active CLK1 as was also found in mouse CLK1 by Ninomiya *et al*., (2011). Treatment of prostate cancer cell line DU145 with benzothiazole compound (CLK1 specific inhibitor) also resulted to a significant decline in exon 4 skipping and intron 4 retention favouring full length *CLK1* expression, an indication of *CLK1* auto regulatory mechanism (figures 3.4 and 3.6). We observed that alternative splicing of *FGFR1OP* were also modified by TG003 treatment (figure3.5) (Uzor *et al*., 2018).

Figure 3.6 proposes a model for the autoregulation of *CLK1* expression through modification of its own alternative splicing. The splice factors involved in the regulation of *CLK1* alternative splicing (exon 4 skipping and intron 4 retention) are not yet known, but it is tempting to speculate that they will be affected directly by *CLK1* phosphorylation.

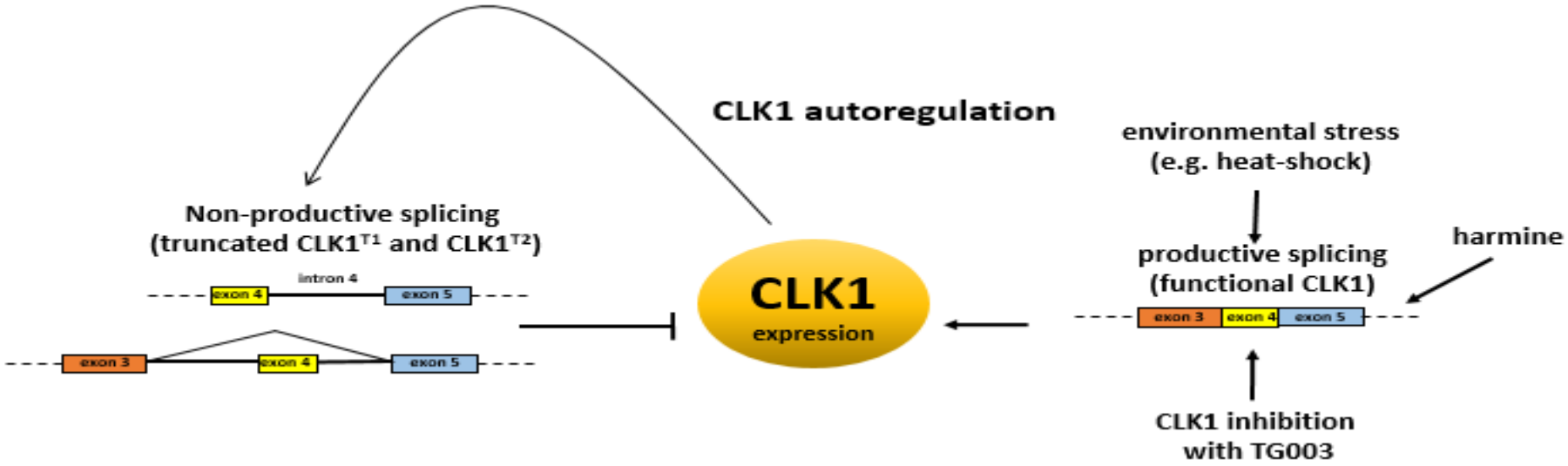


Figure 3.6. Summary of CLK1 auto-regulation: CLK1's own productive splicing is suppressed under physiological condition through exon 4 skipping and intron 4 retention. However, pre-mRNA of CLK1 is more productively and efficiently spliced under heat shock treatment and exposure to other environmental stress such as osmotic shock, harmine (alkaloid) treatment and CLK1 inhibition via TG003 treatment (Uzor *et al*., 2018).

Chapter 4

Treatment of prostate cancer cell lines with TG003 induces apoptosis and decreases prostate cancer proliferation, migration and invasion

4.1 Aim of the chapter

Having established that TG003 is exerting an effect by altering pre-mRNA splicing in prostate cancer cell lines, the aim of this chapter is to see if it has an effect on prostate cancer cell biology such as prostate cancer cell proliferation, apoptosis, migration, invasion and on scratch closure.

4.2 The effect of TG003 treatment on SRSF protein phosphorylation

We first wanted to confirm that CLK1 inhibition by TG003 could reduce splice factor phosphorylation. PC3 cells were treated with various concentrations of specific CLK1 inhibitor for 48 h, after which protein from the treated and untreated controls was extracted and quantified using Bradford assay. SR phosphoprotein levels of both treated and controls were determined using western blotting with an antibody that specifically detects phosphorylated SR proteins. The SR phosphorylation levels (figure 4.1) declined in a dose dependent manner following TG003 treatment.

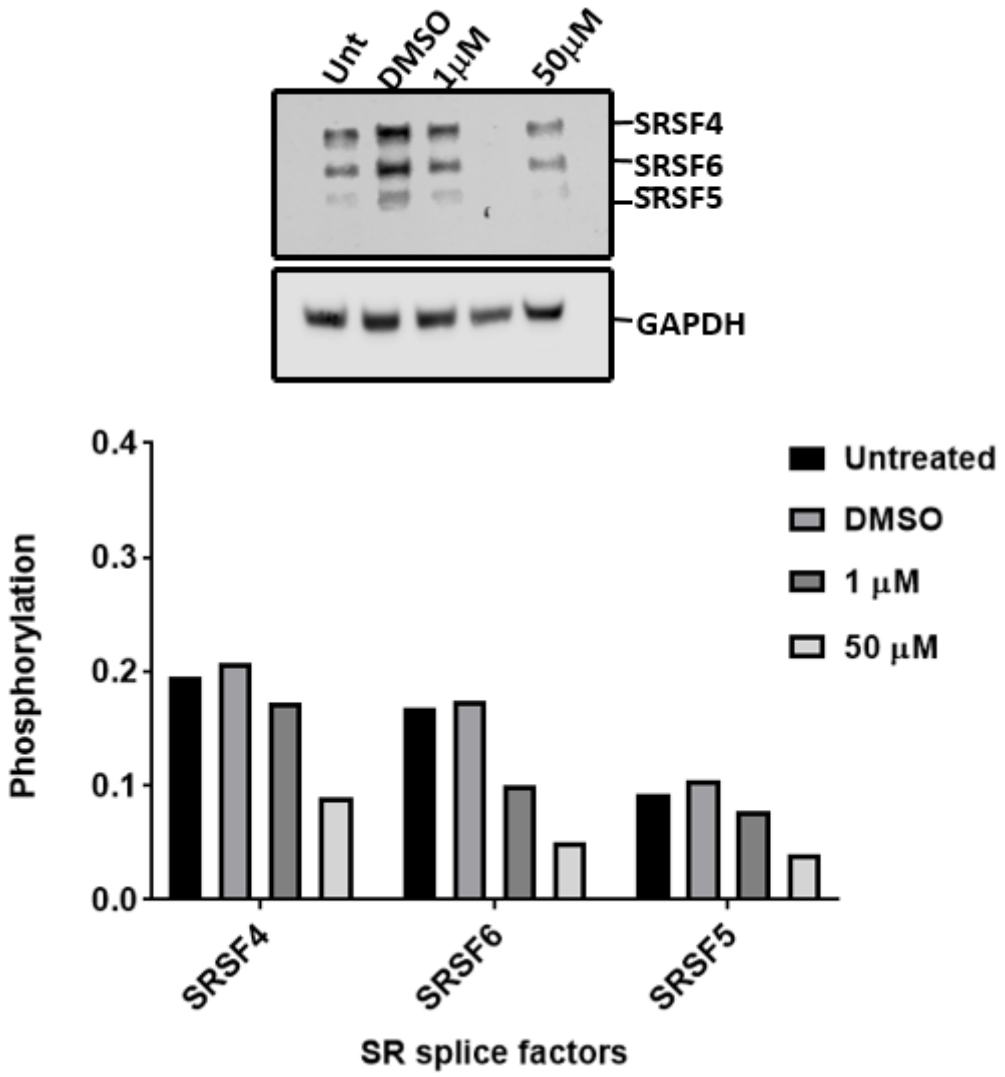


Figure 4.1 Effect of CLK1 inhibition on SRSF protein phosphorylation. Western blot analysis showing the SRSF protein levels after TG003 48 h treatment, probed with pan phospho SRSF antibody and normalised with GAPDH. N=2 repeats.

4.3 Effect of TG003 on prostate cancer cell growth and proliferation

$3x10^5$ of adherent PCa cells (PC3 and DU145) were initially seeded in six well plates. Before treatment with TG003, cells were allowed to adapt and settle eight hours after splitting. After 24-72 h of treatment with various concentrations of TG003 (1 µM- 50 µM), the cells were washed with PBS and trypsinized. Live cell numbers were counted and determined using the trypan blue assay. Cell growth and proliferation was supressed in a TG003 dose and time dependent manner of treatment in both PC3 and DU145 prostate cancer cell lines (figure 4.2). The effect was particularly prominent in PC3 cells (figure 4.2A). Cells did not appear significantly affected at 24 h following 1 µM TG003 treatment of both cell lines. However, at 48 to 72 h, there was evidence of apoptosis and decreased proliferation at concentrations > 1 µM. In contrast continuous growth and proliferation was observed in the untreated and DMSO solvent controls.

At 24, 48 and 72 h, cell numbers increased approximately by $2.0x10^5$ in the controls (untreated and DMSO) and there was a gradual reduction in cell number per TG003 concentration with time, but not below the original seeded number of cells. At 48 h, continuous proliferation was seen in controls, but cell proliferation inhibition was observed in a TG003 dose-dependent manner up to 10 µM. This suggests that chemical treatment with TG003 over a prolonged period at higher concentrations up to 50 µM could induce cell death. However, at lower concentration (1 µM), the cells appear not to be proliferating. In contrast cell numbers increased to $1.0x10^6$ in the controls (untreated and DMSO).

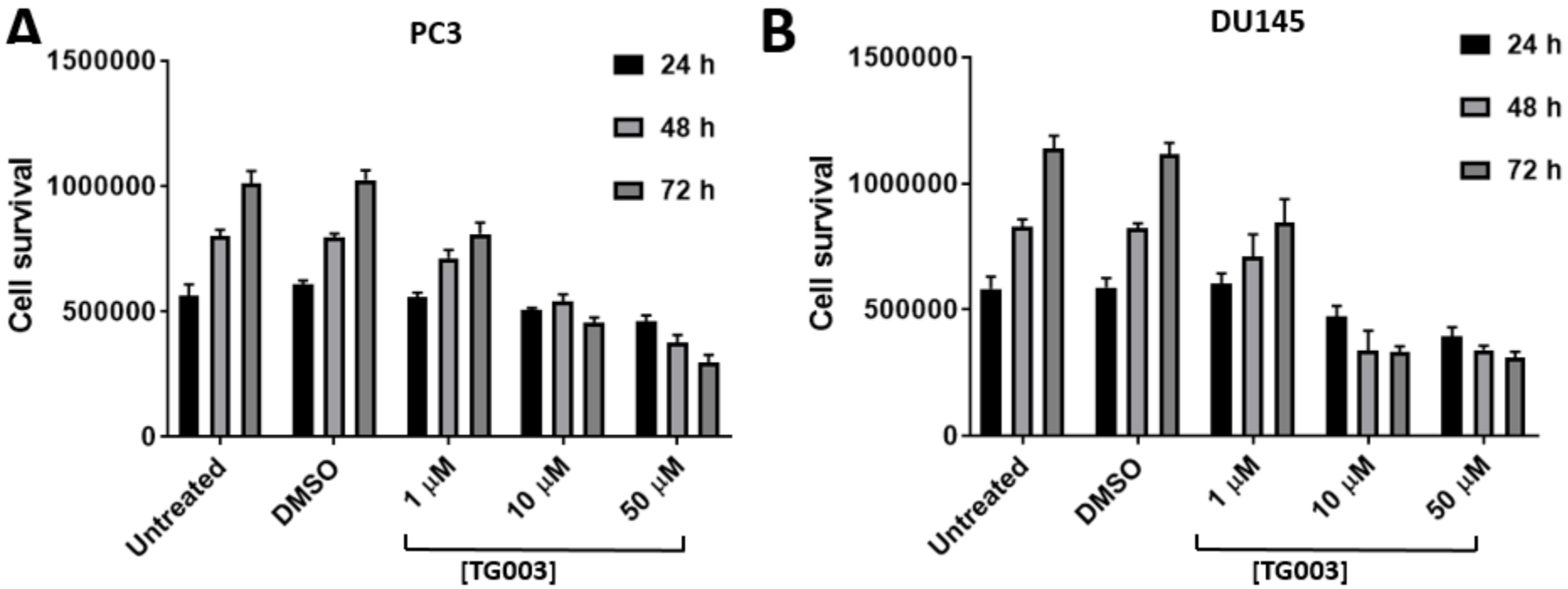


Figure (4.2) Prostate cancer (PC3, DU145) cell survival following 24-72 h TG003 treatment. PCa cells were treated with various concentrations of TG003 (1 μM to 50 μM) for 24, 48 and 72 h. Live cells were determined by trypan blue assay. A and B PC3 and DU145 respectively cell survival after TG003 treatment at various TG003 concentrations. Time legends for 24, 48 and 72 h are indicated by the sides. Untreated and DMSO are controls. N=5 repeats. Error bars are indication of SE.

Percentage viability was also determined in both prostate cancer cell lines following 24 to 72 h TG003 treatment. The untreated and DMSO solvent controls resulted in increased percentage viability with time. However, percentage viability drastically decreased in a dose and time dependent manner suggesting that CLK1 inhibition with TG003 suppresses viability and survival of both PC3 and DU145 prostate cancer cells (figures 4.2, 4.3).

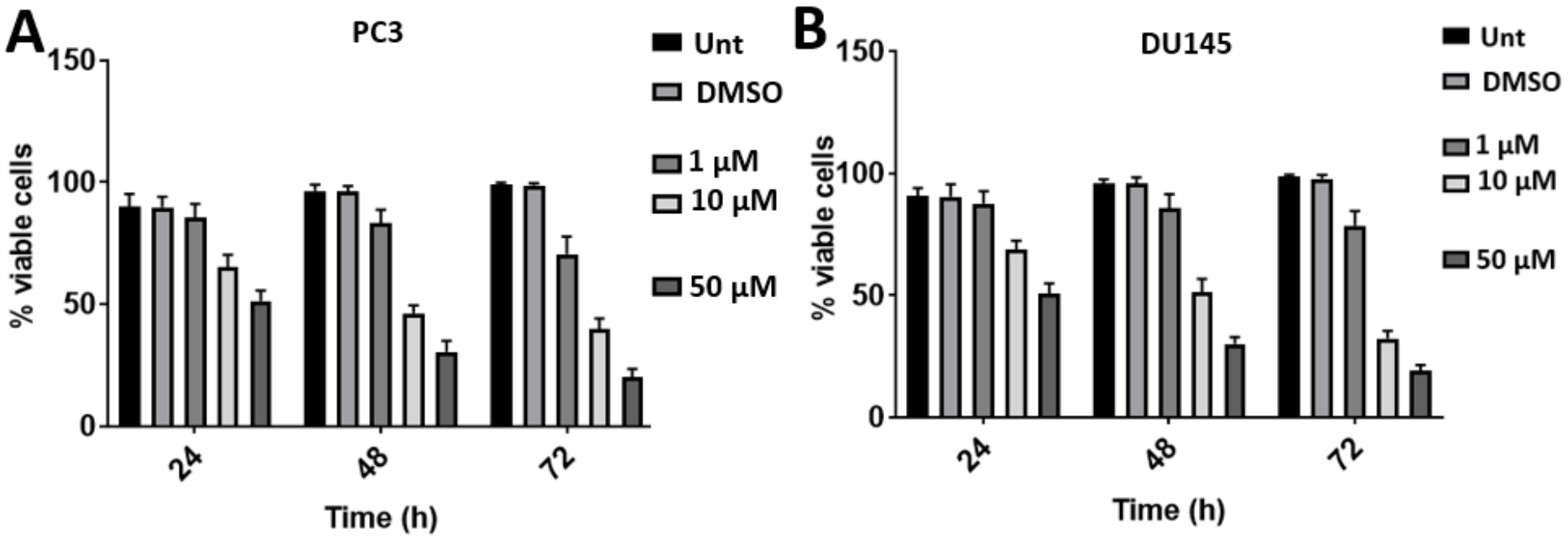


Figure 4.3. PCa (PC3, DU145) percentage viability after TG003 treatment. PCa PC3 and DU145 cells were treated with TG003 (1 µM to 50 µM) up to 72 h. Percentage viability of the PCa cell lines were determined with trypan blue assay with multiple repeats. Untreated (Unt) cells and cells treated with DMSO are controls. N=5 repeats. Error bars are indication of SE.

As an indirect measurement of cell proliferation, the change in colour of the growth medium following TG003 treatment was measured (figure 4.4). The colour change was higher in the untreated and DMSO controls, probably due to continuous cell growth and proliferation in the controls leading to media depletion. The colour changes were measured spectophotometrically at 540 nm and graphically represented (figure 4.4B). The colour changes effect decreased in a concentration dependent of TG003 compared with the control media, an indication of less cell growth and proliferation in the treated cells especially 10 and 50 µM TG003 concentrations.

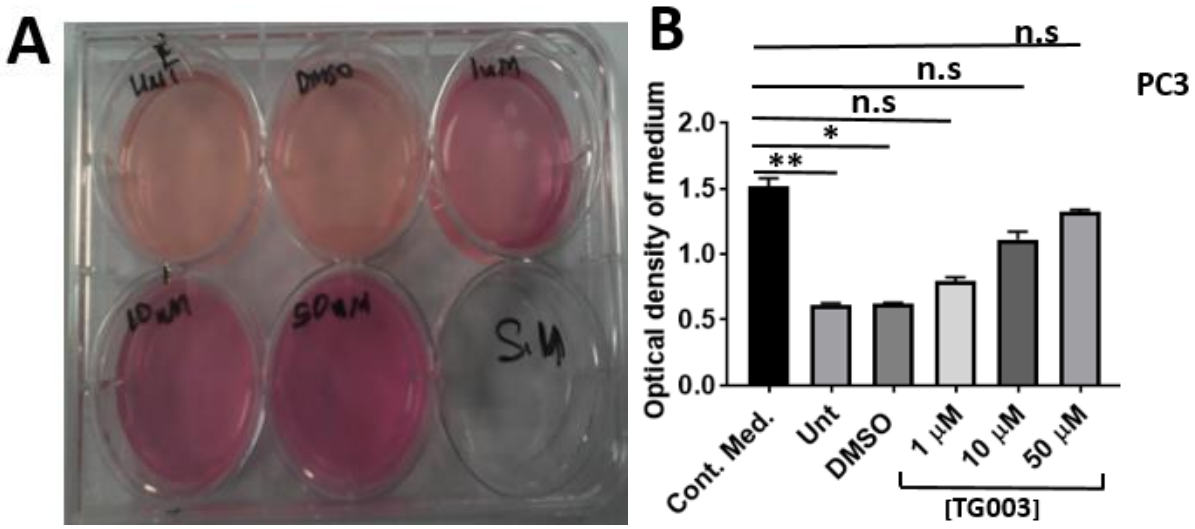


Figure (4.4): Spectrophotometric measurement of colour changes and media depletion following TG003 treatment. (A) Spectrophotometric measurement of media colours for the untreated, DMSO, 1 µM, 10 µM and 50 µM of TG003 was taken at 540 nm after 96 h incubation at 37^{o}C. Untreated and DMSO are controls. (B) Graphical representation **of colour changes and media depletion following TG003 treatment.** N=3 repeats. One-way ANOVA, in all experiments n.s depicts not significant, *=p≤0.05, **=p≤0.01.Error bars are indication of SE.

To confirm the above results, and to be sure that CLK1 inhibition with TG003 causes reduction of prostate cancer cell proliferation, Ki67 analysis was performed. Ki67 is an antibody that binds only to the nucleus of proliferating cells. Eight hours serum starved $3.0X10^5$ PCa cells were treated with increasing doses of TG003 (1 μM to 50 μM) for 48 h. 4% paraformaldehyde (PFA) was used to fix the treated cells for 10 minutes after being washed in warm PBS. Cells were permeabilized in 0.25% triton-PBS for 10 minutes and incubated overnight with 1:1000 anti-KI67 antibody at +4ºC. Goat anti-rabbit antibody (Alexa fluor 488) 1:750 was used as secondary antibody incubated at room temperature for 1 h. Cells were counterstained with DAPI for 10 minutes and matched images of both Ki67 and DAPI were obtained using image pro plus. Cell proliferation levels were measured using imageJ. Figure 4.5 shows that the proliferation levels of PCa cells were impaired following TG003 treatment especially at 10 μM and 50 μM concentrations.

Having examined and confirmed a decline in proliferation of prostate cancer cell lines (PC3 and DU145) following TG003 treatment, we also examined the proliferation of the normal prostate epithelium cell line (PNT2) subjected in the same conditions. In terms of proliferation PNT2 appeared to be less sensitive to TG003 (figure 4.5C).

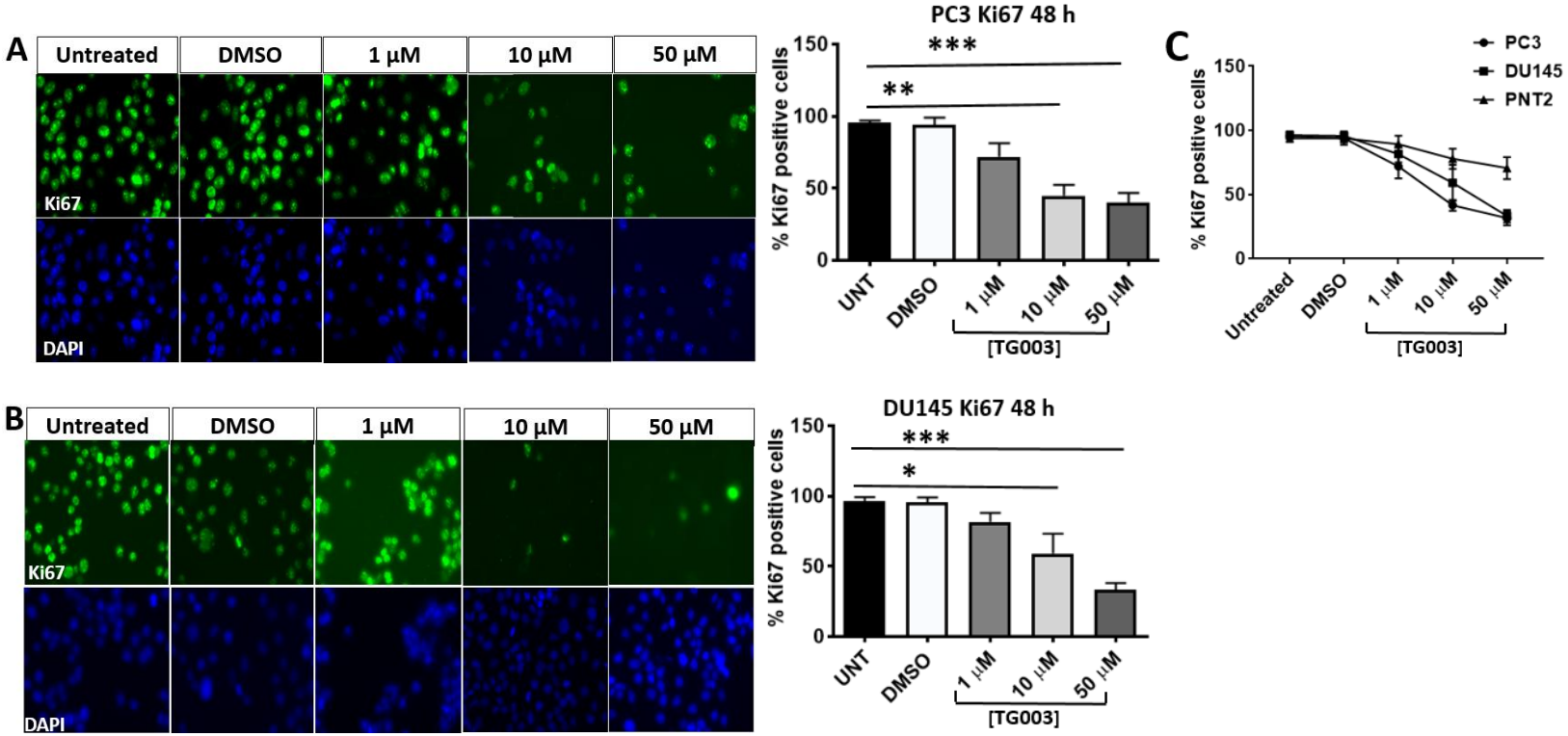


Figure (4.5) TG003 Treatment induces cell proliferation reduction): 8 h serum starved PCa cells were treated with increasing doses of TG003 (1 µM to 50 µM) for 48 h. Treated cells were fixed in 4% paraformaldehyde (PFA) and incubated overnight with Ki67 antibody at +4ºC and subsequently anti- rabbit antibody (Alexa fluor 488) . Cells were counterstained with DAPI for 10 minutes and washed three times in PBS. Images of both Ki67 and DAPI were

obtained using image pro-plus and measured using imageJ. Graphical and representative images are shown above. A and B are representative images of prostate cancer cell lines (PC3 and DU145 respectively) treated with TG003 concentrations and stained with Ki67 antibody. DAPI images are controls. Their respective statistical analyses are indicated by the sides. N=3 (One-way ANOVA). In all experiments, *=p≤0.05, **=p≤0.01, ***=p≤0.001. (C) is the graphical representation of the proliferation analysis of prostate cancer cell lines (PC3 and DU145) compared with the normal prostate epithelium cell line (PNT2). N=3 repeats (Two-ways ANOVA). Shown are the means of groups of data +/- 95% confidence interval. PC3 vs DU145 not significant, PC3 vs PNT2 p≤0.001, DU145 vs PNT2 p≤0.05.

In summary, in both PC3 and DU145 cells, there was an evidence of cell growth and viability suppression in a dose (especially 10 µM and 50 µM) and time dependent manner of TG003 treatment. Significant decreased cell proliferation was also observed in both cell lines following TG003 treatment. However, a greater percentage Ki67 positive cells were recorded in normal prostate epithelium cell line (PNT2) than the prostate cancer cell lines (DU145 and PC3) suggesting the reduced sensitivity of normal prostate cells to TG003.

4.4 Effect of CLK1 inhibition on apoptosis

The term apoptosis otherwise known as programmed cell death describes the process of deliberately eliminating damaged or unwanted cells (Formigli *et al*., 2000; Sperandio *et al*., 2000; Debnath *et al*., 2005). It is a normal homeostatic mechanism involved in the aging and developmental processes to sustain tissues' cell populations. Apoptosis is a highly organised process regulated by many genes (Lowe and Lin 2000). Alterations of the alternative splicing of several regulatory genes results in deregulation of apoptosis leading to metastatic development and progression (Mercatante and Kole 2001).

The *Bclx* apoptotic regulator is alternatively spliced into anti-apoptotic (*BclxL*) and proapoptotic (*Bclxs*) variants. *BclxL* is overexpressed in many cancers including prostate cancer and has been linked to chemoresistance and a vital target for cancer therapy. Increased prostate cancer metastasis is associated with over expression of *BclxL* (Mercatante *et al*., 2001) Apoptotic induction and increased chemosensitivity has been found in both breast and prostate cancer cells through redirection of *BclxL*

to *Bclxs* pre-mRNA splicing by blockade of the alternative 5' splice site with an antisense oligonucleotide in Bclx intron 2 (Mercatante *et al*., 2001). *Bclxs* is a potential antagonist of *BclxL* and inhibits the pro-survival properties of *BclxL* to induce apoptosis. High levels of apoptotic *Bclxs* variant have been found in patients with stomach and colon cancers and in nude mice with breast cancer (Gryco *et al*., 2012). Many studies suggest that *BclxL* overexpression in cancer cells is a clear indication of oncogenic anti-apoptotic stimuli while the overexpression of the isoform *Bclxs* is an evidence of pro-apoptotic stimuli (Finch *et al*., 2006; Coluccia *et al*., 2004). Increased levels of *BclxL* are also found in cancer cells that tends to resist the chemotherapeutic effect of some agents (Shultz *et al*., 2012). Both transcriptional and post-transcriptional processes are needed for the regulation of *BclxL* expression. In the later, the Bclx gene through alternative splicing of exon 2 5' splice site selection, produces either upstream 5'ss selection (proapoptotic) or downstream 5'ss selection (antiapoptotic) isoforms. However, in contrast to *BclxL*, many studies have suggested that the *Bclxs* isoform triggers apoptosis (Minn *et al*., 1996; Ban *et al*., 1998).

PCa (PC3 and DU145) cells were treated for 48 h with TG003 and stained with 12% acridine orange in phosphate buffer solution. Slides were analysed microscopically using a fluorescence microscope with BG-12 excitation filter. Results suggest progressively increasing number of apoptotic cells in a TG003 dose dependent manner (figure 4.6). This suggests that chemical treatment of cells with TG003 induces apoptosis in prostate cancer cell lines.

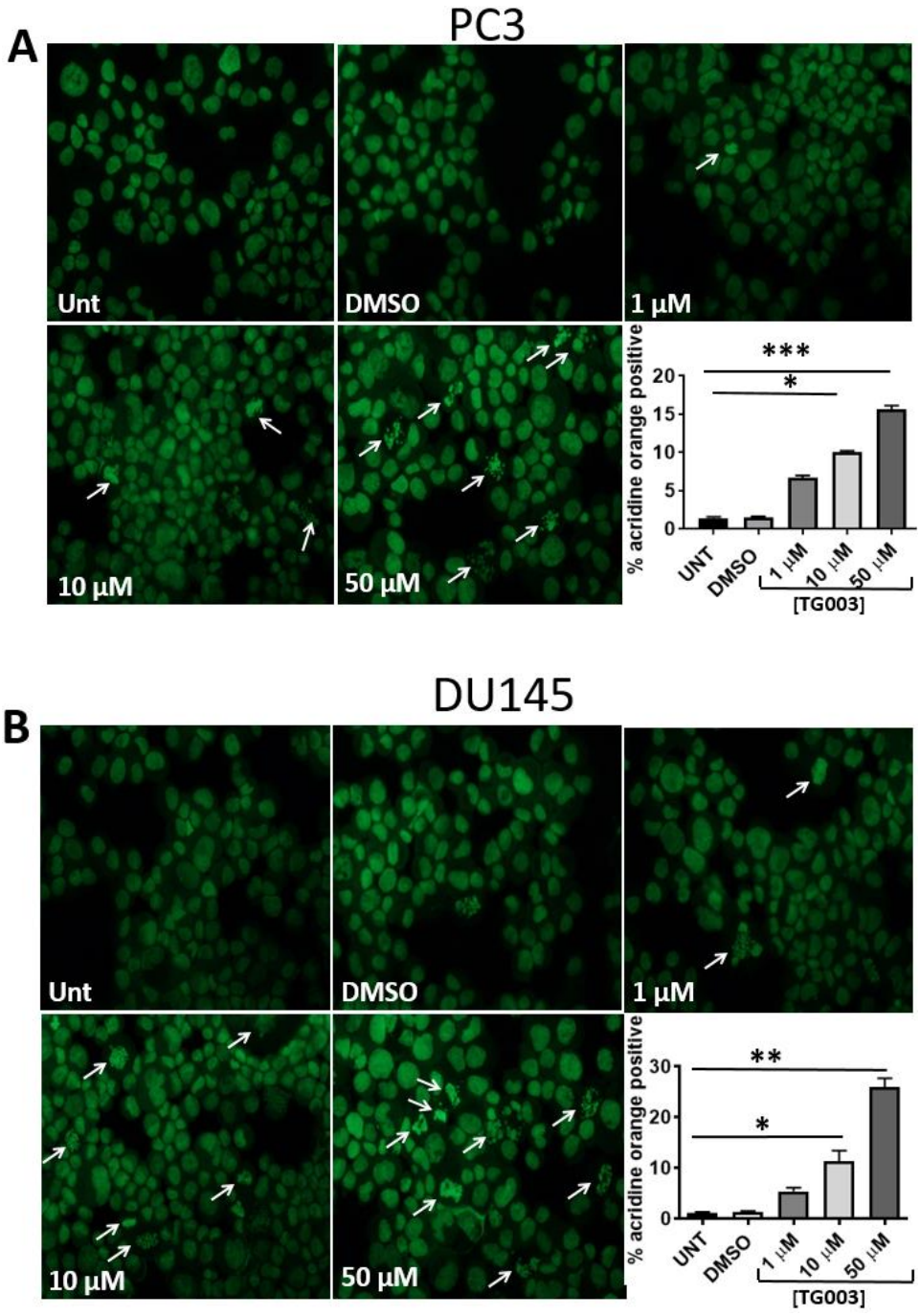


Figure (4.6). TG003 induces apoptosis. PCa (DU145 and PC3) Cells were treated with TG003 concentrations (1 µM to 50 µM) for 48 h. Treated cells were further stained with 12% acridine orange. 2000 cells were scored per treatment, percentage apoptotic cells in the mix of other cells were also scored and recorded. The untreated and DMSO controls received no TG003 treatment. Representative sections are shown while, the arrows indicate representative apoptotic cells. N=3 repeats (One-way ANOVA). Shown are the means of groups of data +/- 95% confidence interval. In all experiments, *=p≤0.05, **=p≤0.01, ***=p≤0.001.

RNA was extracted from 48 h TG003 treated prostate cancer cells. RNA quality was ascertained using nanodrop technics before cDNA synthesis. cDNA from the treated and untreated (controls) cells were run in 2% agarose gel at 400A and 95V for one hour. PCR analysis were performed with apoptotic genes such as *Bclx*, *caspase 9, Mcl1* and *survivin* (figure 4.7). There was increased expression of the pro-apoptotic variants (isoforms) following higher concentrations of TG003 which strongly correlate the results that *CLK1* inhibition with TG003 induces apoptosis.

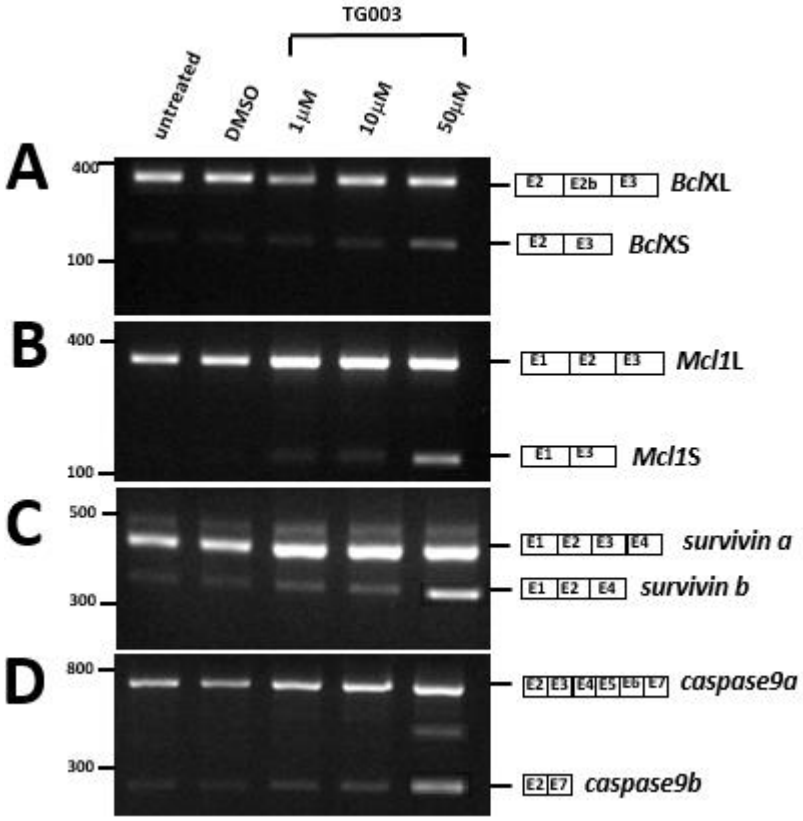


Figure 4.7. Study of *CLK1* inhibition in apoptotic genes (*BclX, Mcl1, Survivin and Caspase 9*): A, B, C and D are *BclX, Mcl1, Surviving and Caspase 9* genes respectively. BclXL and BclXS are anti and pro-apoptotic isoforms of BclX respectively, Mcl1L and Mcl1S represent anti and pro-apoptotic isoforms of Mcl respectively, Survivin a and Survivin b represent anti and pro-apoptotic isoforms of Survivin respectively, Caspase 9a and Caspase 9b represent anti and pro-apoptotic isoforms of Caspase 9 respectively. Figure adapted from Uzor *et al*., (2018).

To obtain further evidence that TG003 treatment induces apoptosis, prostate cancer cell lines (PC3 and DU145) compared with normal prostate epithelia cell lines (PNT2) treated for 48 h with TG003 were stained with an apoptotic marker (caspase 3/7). A significant increase in apoptosis was recorded in a dose dependent manner, especially in high doses of TG003 treatment (figures 4.8A, B, C). Statistical analyses of the three cell lines suggest that prostate cancer cell lines (DU145 and PC3) displayed consistent induction of apoptosis in a dose dependent manner. The normal prostate epithelia cell lines (PNT2) displayed a much reduced induction of apoptosis (figure 4.8C).

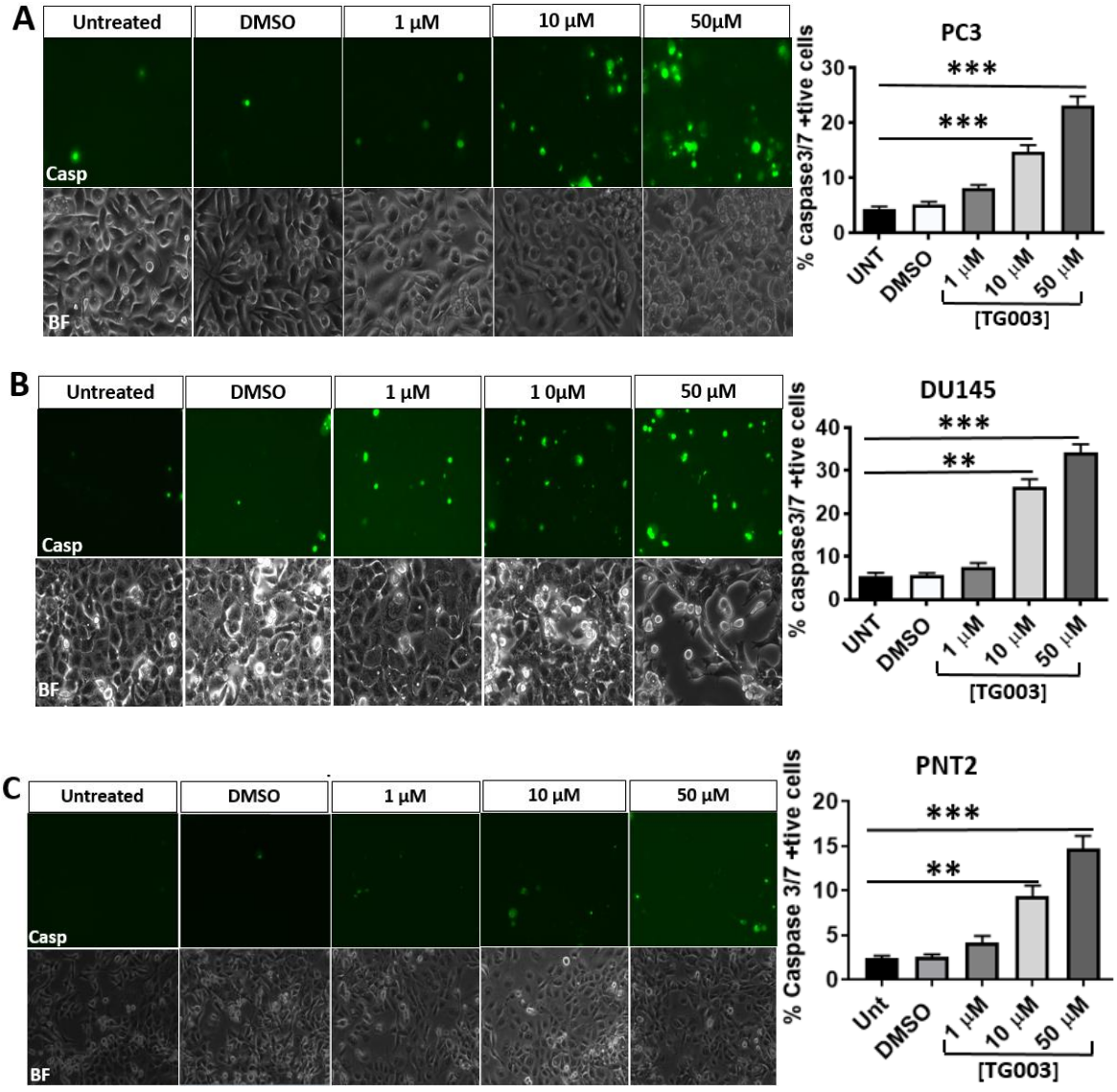


Figure 4.8. TG003 treatment induces apoptosis. Apoptosis was meaured via caspase 3/7. TG003 treated (48 h) PCa cell lines were stained with caspase 3/7 (green channel) for 45 minutes, matched images (green channel and bright field) of each treatment were obtained using fluorescent phase contrast microscope (image pro plus). Untreated and DMSO (solvent) are controls. TG003

concentrations for each treatment are indicated. A, B and C are representative images of PC3, DU145 and PNT2 cell lines respectively stained with caspase 3/7 immunofluorescence antibody. Their respective statistical analyses are indicated by the sides. For each experiment, N=3 repeats (Two-way ANOVA using Tukey's multiple comparisons test). Shown are the means of groups of data +/- 95% confidence interval. In summary, at 10 µM TG003, DU145 vs PC3 $p \leq 0.0001$, DU145 vs PNT2 $p \leq 0.0001$, PC3 vs PNT2 $p \leq 0.01$. At 50 µM TG003, DU145 vs PC3 $p \leq 0.0001$, DU145 vs PNT2 $p \leq 0.0001$, PC3 vs PNT2 $p \leq 0.0001$.

4.5 Effect of *CLK1* inhibition on prostate cancer cell migration and invasion

It has been postulated that greater than 80% male mortality of prostate cancer is due to its invasiveness and rapid metastasis to the bone marrow (Coleman, 2006). The major target for prostate cancer metastasis is the bone; when this happens, poor prostate cancer prognosis is indicated and survival rate declines (Jin *et al*., 2013). The nature of the bone microenvironment and its interaction with malignant tumour cells makes it the most susceptible site of metastasis. In prostate cancer cases, about 68% incidence of prostate cancer bone metastasis has been reported (Suva *et al*., 2011). For prostate cancer cells to thrive in the bone marrow, it must interact with osteoclasts and osteoblasts which triggers and amplifies its overwhelming metastasis within the bone environment (Casimiro, *et al*., 2009). Furthermore, Wang *et al*., (2011) suggested that an essential therapeutic strategy for malignant prostate cancer that has spread to the bone is to inhibit cancer cell migration and osteoclast differentiation. Wang *et al* also found that lanthanides (Ln) and gadolinium (Gd) are examples of such compounds with the potential of inhibiting cell migration. They investigated the potency of $GdCl_3$ in cell migration inhibition and obtained a statistically significant result (Wang *et al*., 2011). Another compound of interest capable of inhibiting prostate cancer cell migration, proliferation and invasion as found within the context of this study could be TG003.

4.5.1 TG003 reduces cell migration and invasion (transwell assay).

To determine cell migration using a transwell insert, half a million ($0.5x10^6$) PC3 cells were seeded in starving media for 8 h in a twenty-four well plate which was allowed under sterile conditions to warm up to room temperature. After eight hours starvation at 37^oC, the starving media was replaced with a complete DMEM containing TG003

and re-incubated at 37^{o}C for 48 h. The cells were washed twice with warm PBS, trypsinized, spun down and the supernatant removed and replaced with DMEM without FBS. Exactly 500 µL of DMEM containing 10% FBS (complete DMEM) was added to the lower chamber while 300 µl containing 1.0x10^{5}cells/ml in a starving DMEM was added to the transwell inserted over the 24 well plate and incubated for 24 h at 37^{o}C. Media from the transwell was carefully aspirated and the insert washed twice with PBS for 5 minutes. The cells were fixed in a clean well containing 225 µL of 4%(w/v) paraformaldehyde (PFA) for 10 minutes. Non migrated cells were scraped off from the insert with cotton swab. The insert was washed twice with PBS for 5 minutes each and permeabilized with absolute methanol for 20 minutes at room temperature. Using a tweezer, the membrane was carefully removed and turned upside down on a clean slide and incubated for 1 h at room temperature with a drop of Hoechst under a coverslip. Cells were counted from random sight using a fluorescence microscope.

The procedure to determine the effect of TG003 treatment on PC3 cell line invasion was same as that of cell migration except that the transwell chambers, 24 well plates, serum free DMEM, micropipettes and pipette tips were kept refrigerated overnight prior to the assay. Stock extra cellular matrix (ECM) matrigel was defrosted in 4^{o}C refrigerator overnight and diluted with serum free DMEM. It was statistically confirmed that TG003 treatment slowed down prostate cancer (PC3) cell migration and invasion in a concentration-dependent manner especially at ≥ 10 µM TG003 concentrations (figure 4.9).

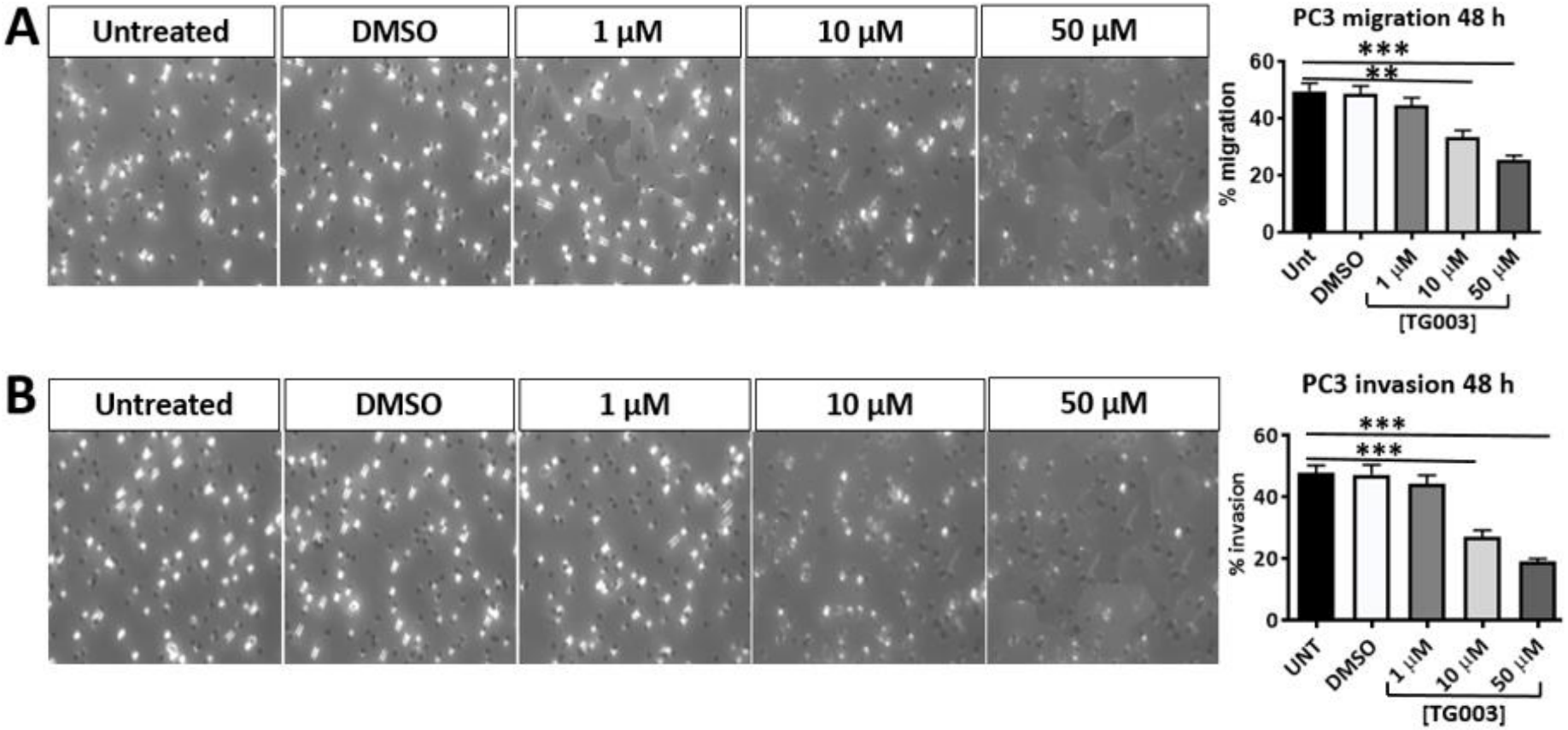


Figure 4.9. PCa (PC3) cell migration (A) and invasion (B). 48 h PC3 cells were subjected to a migration and invasion assay using a transwell insert. Untreated and DMSO are the controls; TG003 was used at 1 µM, 10 µM, and 50 µM. N=3 repeats (Kruskal-Wallis). Shown are the means of groups of data +/- 95% confidence interval. In all experiments, **=p≤0.01, ***=p≤0.001.

4.5.2 Effect of TG003 treatment on cell migration (scratch assay)

Scratch assays were performed to further study the effect of TG003 on cell migration. A camera (lumascope) was inserted in a CO_2 incubator to record the migration of PCa cell lines (PC3 and DU145 independently) over a scratch made with 200µL yellow pipette tip on fully confluent cells in six-well plates. Results show that the scratch closed completely on the controls after 72 h incubation in CO_2 incubator at 37^{o}C while the TG003 treated cells remain unclosed at 72 h incubation. TG003 treatment inhibited scratch closure in a concentration and time dependent manner in PCa PC3 (figure 4.10) and DU145 (figure 4.11).

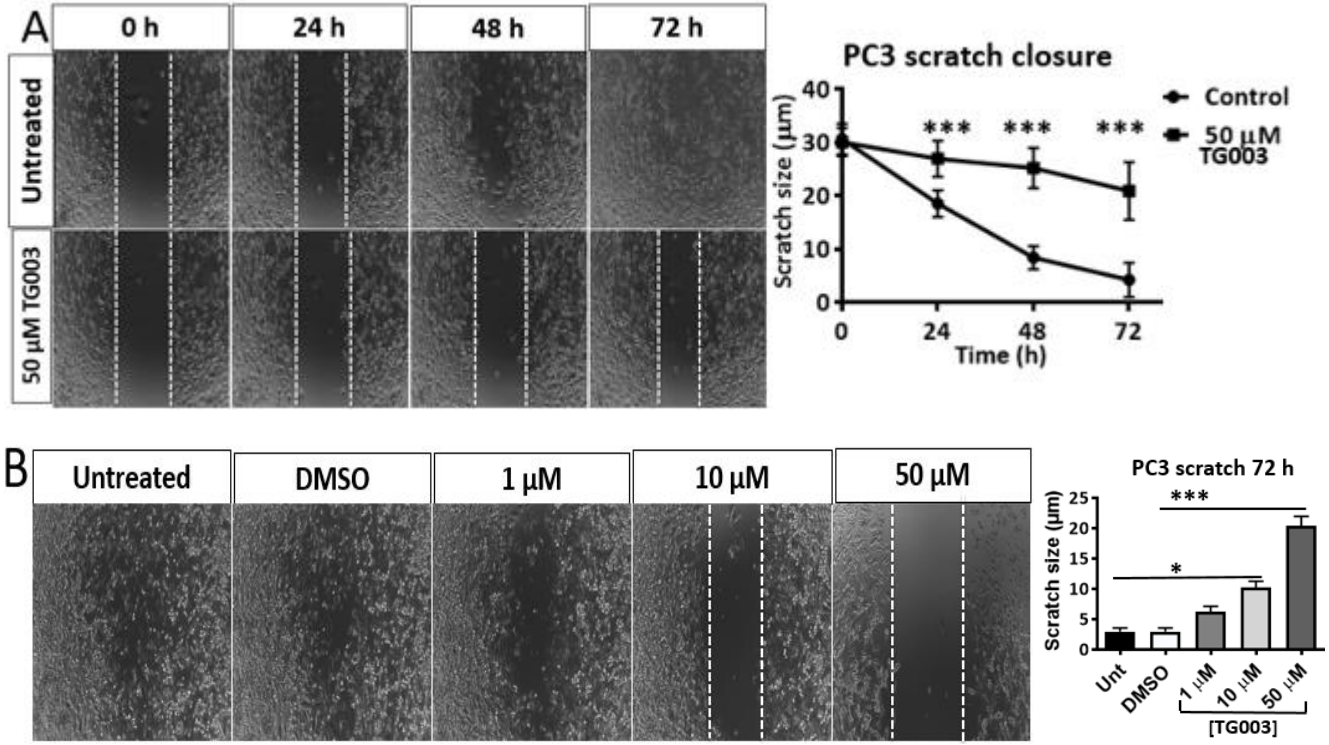


Figure 4.10. TG003 treatment inhibits scratch closure in prostate cancer PC3 cell line. (A) and (B) are scratch assays of PCa PC3 cell line. (A) is treated with 50 µM TG003 concentration and incubated in CO_2 incubator at 37°C under lumascope from 0 h to 72 h. (B) is treated with various concentrations of TG003 as indicated and incubated for 72 h at 37°C under lumascope. Untreated and DMSO are controls. Their respective statistical analyses are indicated beside each image. N=3 repeats (Kruskal- Wallis). Error bars are indication of SE. In all experiments *=p≤0.05, ***=p≤0.001.

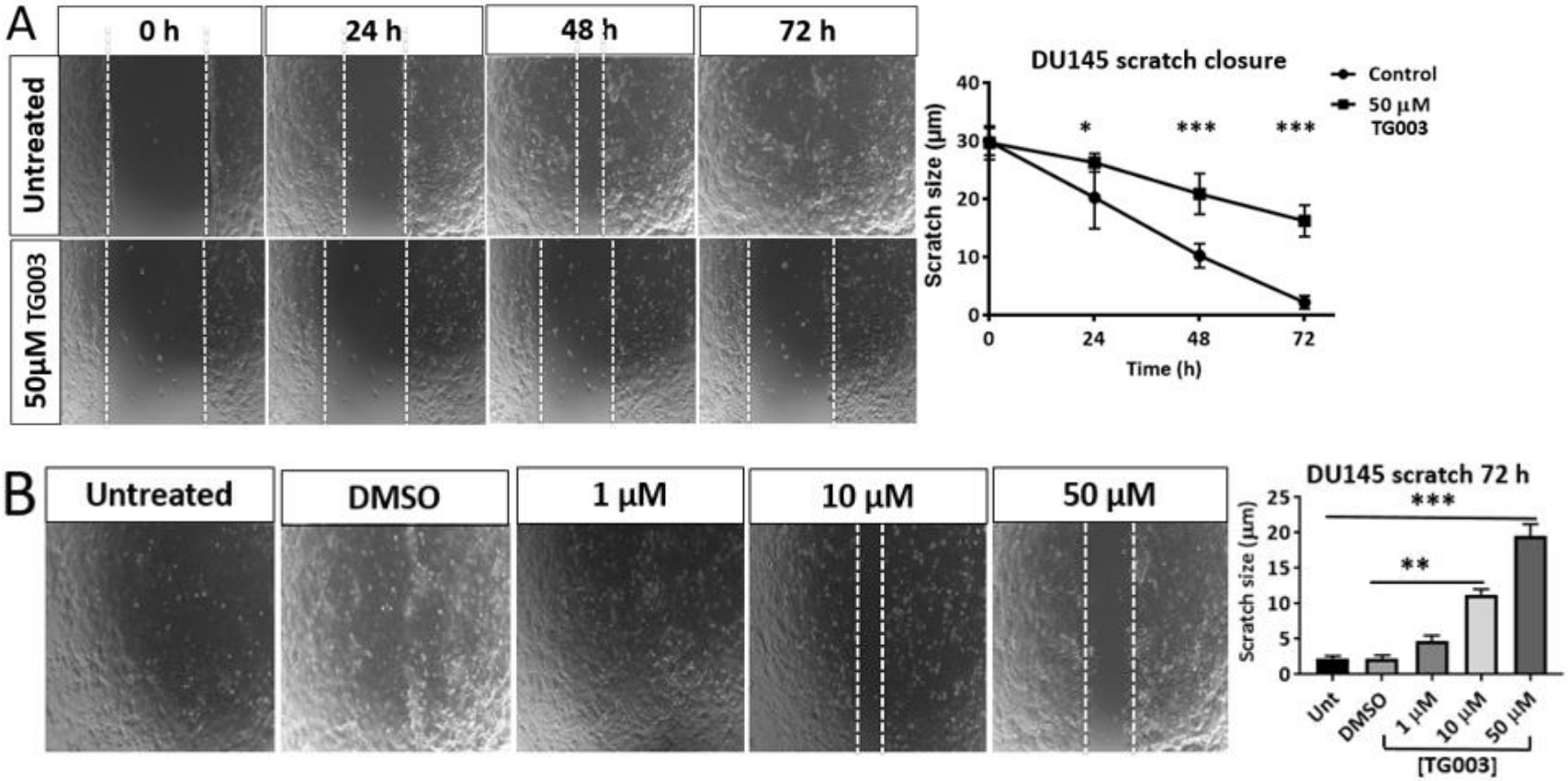


Figure 4.11. TG003 treatment inhibits scratch closure in prostate cancer DU145 cell line. (A) and (B) are scratch assays of PCa DU145 cell line. (A) is treated with 50 µM TG003 concentration and incubated in CO_2 incubator at 37°C under lumascope from 0 h to 72 h. (B) is treated with various concentrations of TG003 as indicated and incubated for 72 h at 37°C under lumascope. Untreated and DMSO are controls. Their respective statistical analyses are indicated beside each image. N=3 repeats (Kruskal- Wallis). Error bars are indication of SE. In all experiments *=p≤0.05, **=p≤0.01 ***=p≤0.001.

4.5.3 Effect of *CLK1* inhibition (TG003 treatment) on EMT marker expression.

During metastatic prostate cell progression, prostate tumour cells become invasive and degrade the basement membrane of the extracellular matrix (ECM) through a process known as epithelial-to-mesenchymal transition (EMT). EMT is involved in wound healing, neural crest formation and mesoderm development. However, in cancer, EMT is associated with increased migration and invasiveness, cell invasion is the migration of cells through tissue after overcoming the extracellular matrix barrier (Tiwari *et al*., 2012). Invasive prostate cancer cells are characterised by a loss of expression of crucial epithelial biomarkers including cell to cell tight junction and basal epical polarity (Kalluri and Weinberg, 2009). Apart from tumour progression, EMT also occurs during normal physiological development including wound healing. EMT prostate cell migration can be studied by investigating the extent of E-cadherin and vimentin expressions (Schaeffer *et al*., 2014)

Cadherins are a group of transmembrane proteins with calcium ions dependent function and are important adhesion cell molecule that help cells interact with each other. Extracellular cadherin regulates cell-cell adhesion while the cadherin adhesome comprisese the cytoplasmic intracellular tail. E- Cadherin is a cell adhesion molecule highly dependent on calcium ion that determines embryonic epithelial development and adult homeostatic epithelium differentiation. Cadherins suppress tumour invasion. Aberrant cadherin expression has also been linked with aggressive and invasive form of prostate cancer with high Gleason scores (Bengaroll *et al*., 2016; Putzke *et al*., 2011).

We continued to explore the effect of TG003 treatment on cell biology examining the EMT markers E-cadherin and vimentin. Prostate cancer cell lines (PC3 and DU145) were grown on cover slips in six well plates for 48 h with 4 ml of complete DMEM and TG003 treatments. Cells were washed twice with warm PBS and fixed with 4% (w/v) PFA for 10 minutes. PFA fixed cells on cover slips in 6-well plates were washed three times with PBS and permeabilized in 0.25% (v/v) triton at RT for 10 minutes. Permeabilized cells were blocked with 10% FBS in PBS for 1 h, after which E-cadherin and vimentin primary antibodies (from rabbit and mouse respectively) were incubated in the cells overnight in cold room. Cells were washed and counter stained with their respective secondary antibodies for one and half hour. The cover slips were mounted on slides with mounting media and images were viewed and obtained with image pro-plus and analysed with image J.

It was found that the untreated (PC3 and DU145 cells) continued to proliferate, migrate and invade the extra cellular matrix (ECM) matrigel with low E-cadherin (figure 4.12A and B) with a stronger vimentin signal (figure 4.12C and D). However, TG003 treated cells displayed higher E-cadherin positivity and lower vimentin (figure 4.12) in a dose dependent manner.

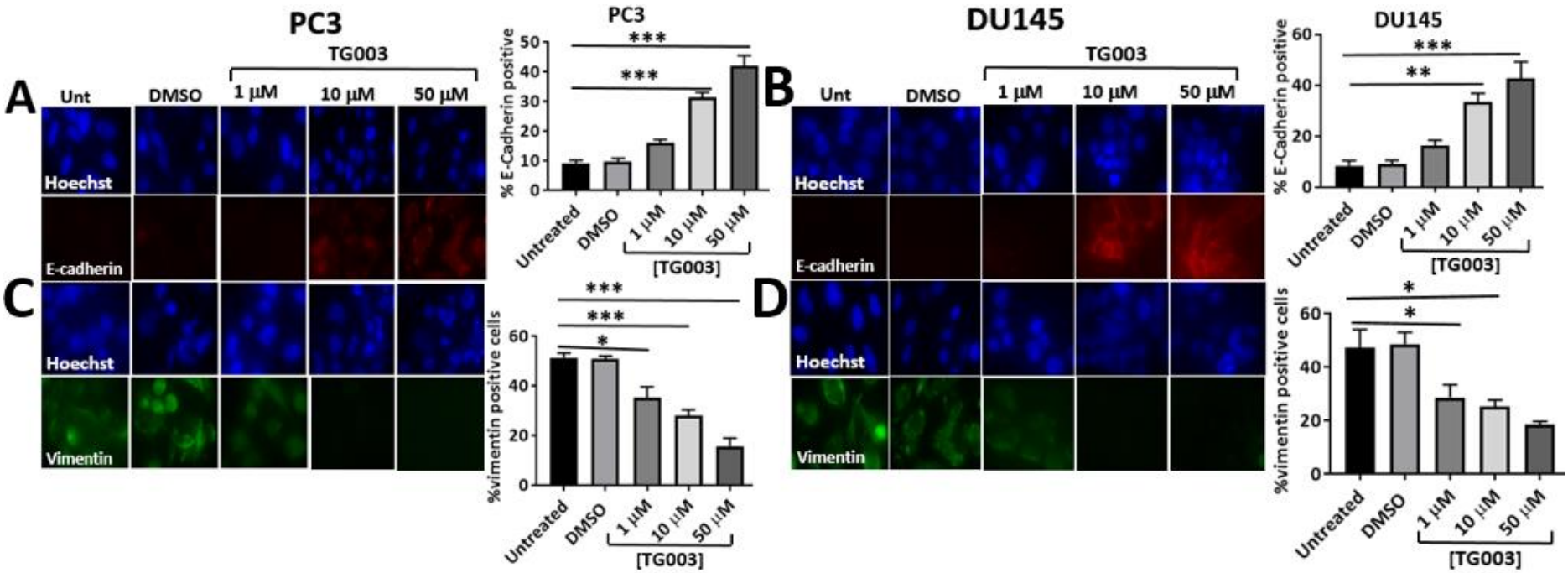


Figure 4.12. Epithelial – mesenchymal transition (EMT) marker expression in prostate cancer cell lines: A and B are representative images of 48 h TG003 (1 µM-50 µM) treated PC3 and DU145 cell lines respectively incubated with anti-E-cadherin antibody. C and D are representative images of PC3 and DU145 cell lines under same condition as in A and B above but incubated with anti-Vimentin antibody. Hoechst staining highlights the position of cells. TG003 concentrations are indicated. Untreated and DMSO are also shown. The percentage E-cadherin and vimentin for each cell lines were statistically analysed and are indicated graphically. Shown are the means of groups of data +/- the 95% confidence interval around the means in each case. N=3 repeats (One-way ANOVA). In all experiments, *=p≤0.05, **=p≤0.01, ***=p≤0.001.

4.6 Summary

As observed in (figure 4.1), the phosphorylation level of regulatory splice factors specifically SRSF4, SRSF6 and SRSF5 decrease in a concentration dependent of TG003 treatment. Interestingly, cell growth and proliferation is suppressed in both prostate cancer cell lines (PC3 and DU145) following TG003 treatment as found in trypan blue cell count (figure 4.2), cell viability assay (figure 4.3), indirect cell proliferation and ki67 assays (figures 4.4 and 4.5) respectively. Furthermore, there was increased apoptosis in both PCa (PC3 and DU145) cell lines in a dose dependent manner of TG003 treatment as observed in acridine orange, apoptotic gene polymerase chain reaction (PCR) and caspase 3/7 immunofluorescence staining (figures 4.6, 4.7 and 4.8) respectively. In this study, TG003 also reduces prostate cancer (PC3) cell migration and invasion in a concentration dependent manner (figures 4.9). TG003 effect was also tested on scratch closure of both PC3 and DU145 prostate cancer cell lines. It is obvious that the scratch closure on both cell lines are inhibited in a dose and time dependent manner of TG003 treatment (figures 4.10 and 4.11).

Lastly, as observed in figure 4.12, there is a significant effect of TG003 on EMT markers expression. Here, there is increased E-cadherin and decreased vimentin expressions in dose dependent manner of TG003 treatment. However, it is interesting to note that the normal prostate epithelial cells are less sensitive to apoptosis and ki67 proliferation markers(figures 4.8 and 4.5C respectively) as compared with PCa (DU145 and PC3) cell lines.

Chapter 5

Effect of CLK1 inhibition on tumour growth (xenografts)

5.1 Aim

Having observed that TG003 has a potent effect on prostate cancer cell line growth, proliferation, migration, invasion, scratch closure and apoptosis, we next examined its effect on xenograft growth in vivo. Before that we first performed an siRNA-mediated knockdown of CLK1 in prostate cancer PC3 cells knockdown to confirm that the effects observed with TG003 treatments are likely due to a reduction in CLK1 activity.

5.2 CLK1 knock down recapitulates the effects of TG003

To confirm that the effects of TG003 are due to CLK1 inhibition, and not because of the effect of TG003 on other protein kinases, we decided to knockdown CLK1 using standard siRNAs (figure 5.1A). The knockdown recapitulated the results observed with TG003. For instance, there was increased apoptosis with caspase 3/7 immunofluorescence green detection (figure 5.1B), decreased cell proliferation with Ki67 (figure 5.2A) and decreased cell migration (scratch closure) (figure 5.2B) following CLK1 siRNA knockdown compared with controls.

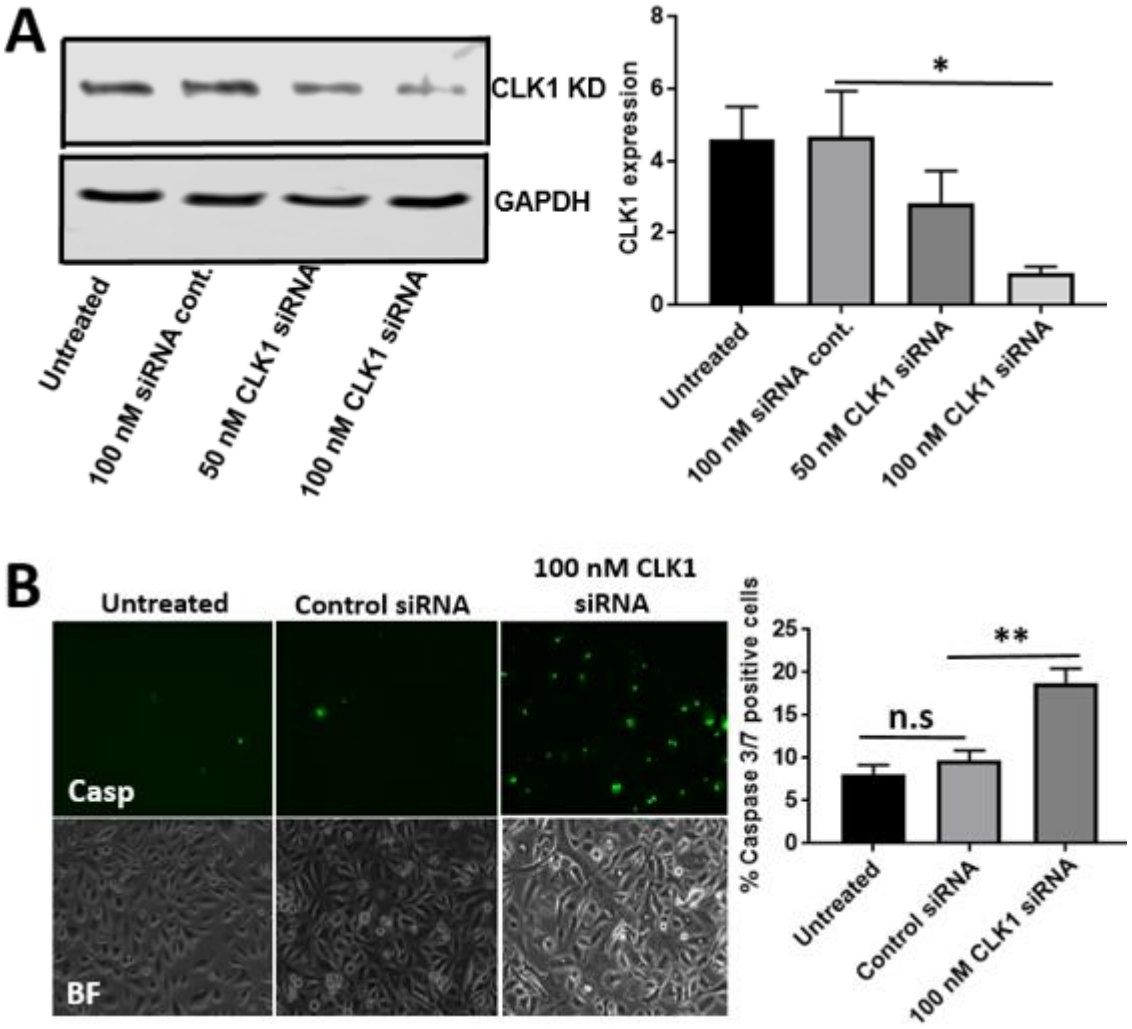


Figure 5.1. CLK1 siRNA knock down induces apoptosis: (A) Western blot of CLK1 siRNA knock down with the quantitation indicated by the side. N=3 repeats (B) caspase 3/7 antibody staining (apoptotic cells) with the quantitation graphically indicated by the side. N = 3 repeats. Shown are the means of groups of data +/- the 95% confidence interval around the means in each case (One-way ANOVA). In all experiments, n.s depicts not significant, *=p≤0.05, **=p≤0.01.

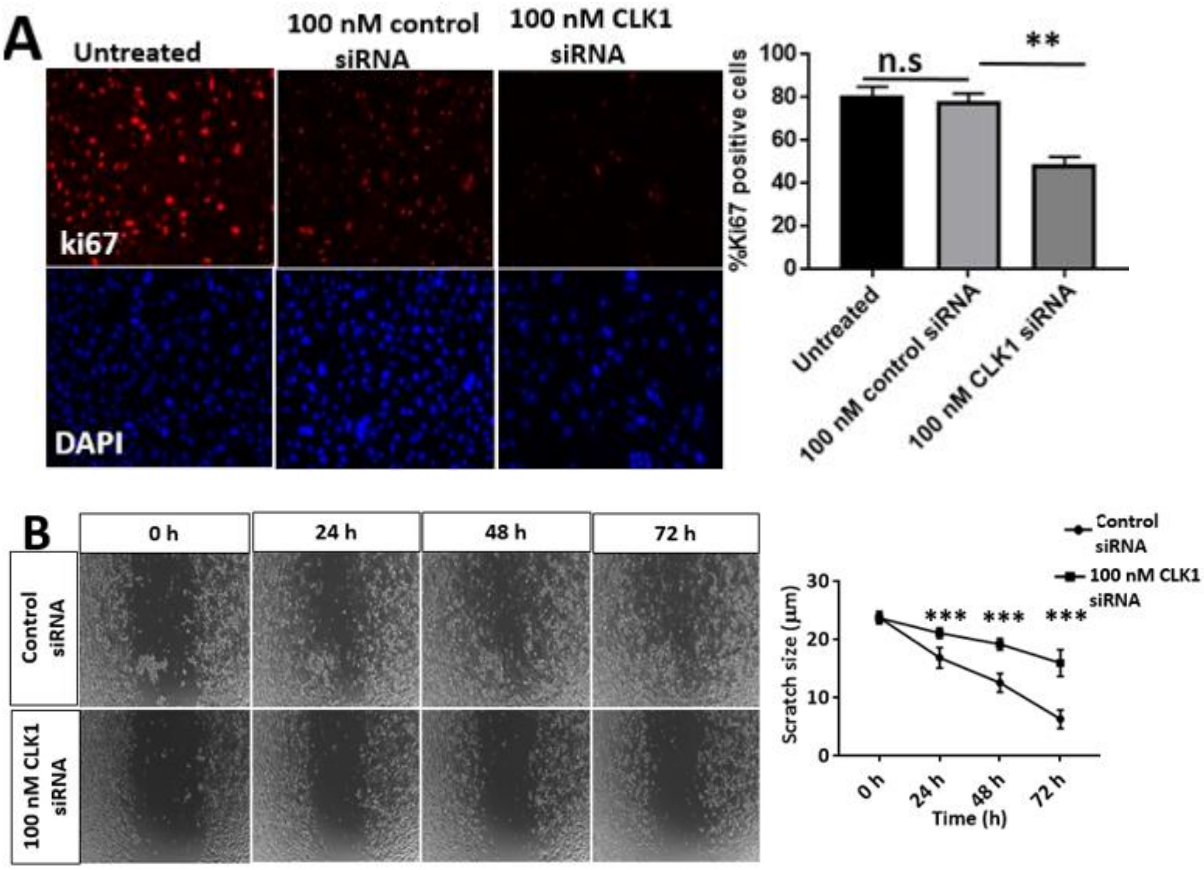


Figure 5.2. CLK1 siRNA knock down inhibits prostate cancer cell proliferation and scratch closure: (A) ki67 staining (proliferation assay) following CLK1 siRNA knock down in PC3 cells. Ki67 positive cells are stained in red while the control DAPI images are stained blue. N=3 repeats (One-way ANOVA). (B) Scratch assay following CLK1 knock down. Images were taken with lumascope inserted in CO_2 incubator at 37^{o}C every 2 h till 72 h. Their respective quantitation and statistical analyses are indicated by their sides. N=3 repeats (One-way ANOVA, Kruskal-Wallis). In both experiments (A and B), error bars are indication of SE, n.s depicts not significant, **=p≤0.01, ***=p≤0.001.

5.3 TG003 treatment reduces xenograft growth

Through a collaboration with Exeter University (Dr. Lin Ling and Dr. Sebastian Oltean), we looked at the impact of TG003 (a specific *CLK1* inhibitor) on the growth of PC3 xenografts in standard nude mice. PC3 human prostate cancer cells were detached from culture flasks and diluted in cold PBS to a concentration of $1x10^{7}$/ml and placed on ice. 100 µl of cell suspension, containing $1x10^{6}$ cells was

subcutaneously injected into each right flank of CD1- nude mice (Charles River). Tumour sizes were measured by caliper twice per week. When the tumour sizes reached 3mm x 3mm, 50µM TG003 or DMSO-containing vehicle were injected intraperitoneally two times a week, 6 mice per treatment group. Mice were culled by cervical dislocation (following Schedule 1 procedures) when the tumour size reached the maximum allowed (12mm x 12mm) and the tumours were extracted. Images of each tumours were taken, and the tumours weighed. Tumour volumes were calculated using formula volume=[(length+width)/2] *length*width. Quantitations of the tumour volumes were analysed by a two-way ANOVA using Prism software.

There are several published studies on the effect of in vivo protein kinase inhibition, but no xenograft in vivo work has been reported specifically with TG003. For instance, in 2015 Mavrou *et al* reported that SRPK1 inhibition with the compound SPHINX in prostate cancer cell line xenografts drastically reduced tumour volume in vivo. In 2012, Oltean *et al* claimed that SRPK1 inhibition is a potential manipulator of pro and anti-angiogenic VEGF-A alternative splicing was altered in xenografts towards the anti-angiogenic isoforms, thereby preventing abnormal blood vessels formation that could fuel cancer growth including prostate cancer. In mouse models, SRPK1 inhibition has also been performed to treat retinopathy by diminishing abnormal in vivo neovascularisation formation (Gammons *et al*., 2013; Gammons *et al*., 2013).

The xenograft experiments with TG003 show tumour volume reduction in both 10 µM and 50 µM TG003 treatments (figure 5.3 A and C) with clear statistical difference seen in 50 µM TG003 treatments (figures 5.3B, D; $p<0.0001$). The excised tumours from both 50µM TG003 treatments and DMSO control were weighed (figure 5.3 E). It is

important to note that the mice appeared otherwise normal throughout the experimental process until the tumours were excised. It is also worthy of note that DMSO had no effect on xenograft growth.

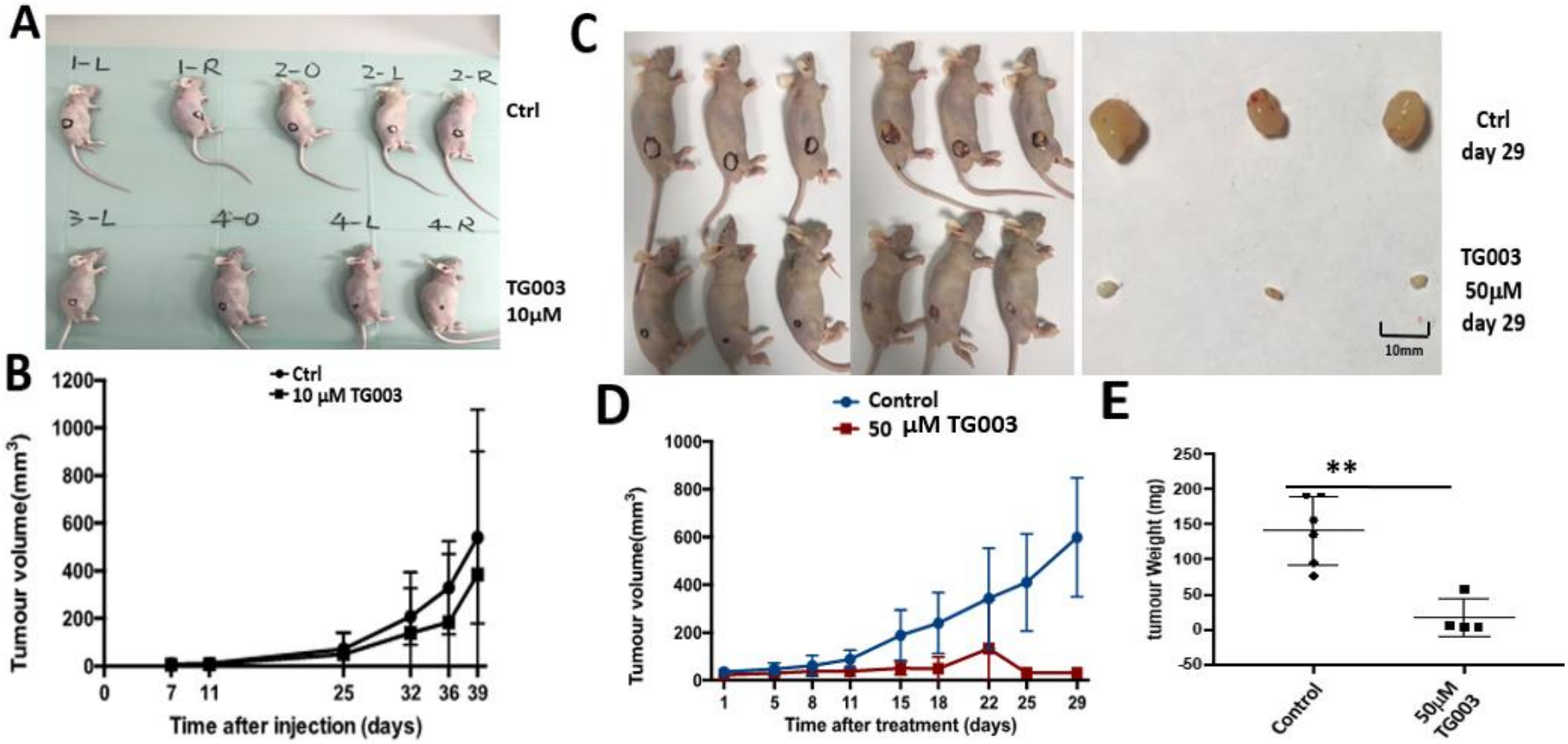


Figure (5.3). TG003 reduces tumour volume in vivo (In vivo xenograft of TG003 treated mice): (A) Mice treated with 10 µM TG003 after tumour growth. The controls are also indicated. (B) and (D) are quantitation of the tumour volume of 10 µM and 50 µM TG003 treatment respectively compared with their controls. (C) Mice treated with 50 µM TG003, the excised tumours from the 50 µM TG003 treatment and their controls are also indicated. (E) Quantitation of

the tumour weight of both 50 μM TG003 treatment and DMSO control. Shown are the means of groups of data +/- the 95% confidence interval around the means in each case. N=6 repeats (Two-Ways ANOVA). $p \leq 0.0001$, but for tumour weight $p \leq 0.01$.

5.4 Summary

It was observed that CLK1 knockdown on PCa PC3 cell line recapitulated the effect of TG003 on same cell line in terms of increased apoptosis, decreased proliferation and scratch closure (figures 5.1 and 5.2) in a dose dependent manner of TG003. This is an indication that the effect of TG003 is as a result of CLK1 inhibition. Figure 5.3 shows that TG003 reduces tumour volume in an in vivo xenograft.

Chapter 6

Discussion

6.1 Ongoing research on CLK1 function and the consequences of its inhibition

Other groups are exploring the biological roles and the consequences of targeting CLK protein kinases; in agreement with this growing body of literature, the work presented in this thesis confirms that CLK1 inhibition results in reproducible and potent effects on cell biology and proliferation. For example, Liu *et al*., (2013) demonstrated that in vitro protein kinase phosphorylation of SR proteins regulates cell adhesion and proliferation; and that CLK1 regulates not just the activity but also the expression of key splice factors through alternative splicing mechanisms. Whereas CLK1-dependent phosphorylation of the splice factor SPF45 promotes its expression, CLK1 inhibition with TG003 lowered SPF45 expression by inducing exon 6 exclusion (Liu *et al*., 2013). It has been shown that overexpression of SPF45 promotes cell invasion and migration depending on the regulatory function of the CLK1 (Liu *et al*., 2013; Araki *et al*., 2015). Muraki *et al*., (2004) reported that TG003 treatment of prostate cancer cell line potentiated SR protein phosphorylation suppression and nuclear speckles dissociation. In 2008, Yomoda and colleagues discovered the impact of active CLK1 in the phosphorylation of SR proteins. They reported that dephosphorylation of SR proteins occur one hour after administration of TG003 to cultured cells and rephosphorylation occurred immediately after removal of TG003.

Yun *et al* (1994) suggest that CLK family plays a crucial role during embryonic development, this is evidenced in the observed mutation in the *Drosophila melanogaster* homologue of CLK which resulted in differentiation, segmentation and general neuronal development defects. This is also supported by the finding of Myers

et al., (1994) that CLK1 expression in PC12 cells is required for neuronal differentiation. In *Xenopus leavis*, excess CLK1 activation causes neuronal defects and is rescued by the administration of a specific CLK1 inhibitor - TG003 (Zhoa *et al*., 2012).

Araki *et al*., 2015 reported that TG003 treatment of S6K cells caused significant reduction of SR protein phosphorylation and increased numbers of nuclear speckles in the cells. Similarly, we found that treatment of PC3 cells with TG003 diminished SR protein phosphorylation of three SR splice factors (SRSF4, SRSF5 and SRSF6) (figure 4.1). It is important to note as demonstrated by Araki and his colleagues that inhibition of CLK1 suppressed cell growth and induced apoptosis through splicing alterations in genes involved in cell growth and survival (Araki *et al*., 2015). Cell growth inhibition in TG003 concentration dependent manner was also observed in our results (figures 4.2 and 4.3).

6.2 Alternative splicing of human CLK1

We have confirmed alternative splicing of CLK1 previously reported in mouse cells, also in human prostate cancer cell lines (PC3, DU145 and VCaP), prostate epithelium cell line (PNT2) and leukemic cell lines (K562, TK6, MOLT4 and HL60) using intron and exon 4 primers. It is clear that both prostate and leukemic cell lines show various degrees of intron 4 retention and exon 4 skipping (figure 3.2A and B). There is more intron 4 retention in prostate cancer cell lines than leukemic cell lines. PC3 tends to possess less intron 4 retention than the rest of the prostate cancer cell lines. In summary there is evidence of differential rates of CLK1 exon 4 inclusion and intron 4 retention in a wide panel of human cell lines.

Knippschild *et al*., (2014) reported that CLK1 expression is highly dependent on the type of cell and some other factors such as treatment with inhibitors, viral transformation, stimulation with insulin and irradiation. It is now apparent that its alternative splicing is also highly variable. Here we show that administration of TG003 to cultured prostate cancer cells causes more efficient CLK1 splicing leading to more CLK1 protein (figure 3.4B) via reduction in exon 4 skipping and intron 4 retention (figure 3.4A and C). The same effect is seen on exposure of prostate cancer DU145 cells to adverse environmental stress such as osmotic shock, heat shock and alkaloid treatments (figures 3.3A, B and C). This is in agreement with the findings of Ninomiya *et al*., 2011 who reported that exposure of cells to a specific CLK1 inhibitor caused elevation of CLK1 mature mRNA by promoting splicing of the intron retaining RNAs. Ninomiya *et al*., (2011), Duncan *et al*., (1997), Duncan *et al*., (1995) also noted that intron 4 retaining CLK1 RNA is located in the nucleus. They were able to investigate the nucleus localization of the CLK1 intron retaining RNA by the application of *in situ hybridization*. Herein TG003 treatment of human cell lines (figure 3.4A and C) also reduced exon 4 skipping in a dose dependent manner (10 nM-100 µM). This finding is consistent with the result of (Nishida *et al*., 2011).

A study shows that CLK hyper-phosphorylate SR proteins and induce their release from the nuclear speckles (Naro and Sette, 2013). It is also important to note that dephosphorylation of splice factors (SR proteins) is required for efficient splicing processes. In this study, we examined the effect of CLK1 inhibition via TG003 on SR protein phosphorylation using pan phosphor-SR antibody (1H4). We observe progressive dephosphorylation of splice factors (SR proteins) in a dose-dependent

manner of TG003 (figure 4.1). Araki *et al*., 2015 also observed decreased phosphorylation of SR proteins with CLK1 inhibition detected using the same 1H4 phospho-SR antibody.

In summary we have confirmed that, as initially reported in mouse cells, human CLK1 inhibition also results in higher CLK1 protein expression presumably through a feedback mechanism that involves its alternative splicing. It will be interesting to determine how this feedback mechanism works (for example, which splice factors are specifically involved in regulating exon 4 skipping and intron 4 retention).

6.3 Effect of TG003 on PCa cell growth and proliferation

Having confirmed that TG003 causes reduced phosphorylation of SR proteins in prostate cancer cells we decided to test the effect on prostate cancer cell growth and proliferation using trypan blue and Ki67 assays. Ki67 is an established proliferation marker and has been currently recommended by researchers as a biomarker of choice for tumour proliferation. Ki67 was first discovered in the early 80s as nuclear histone proteins by Gerdes *et al*., 1984. For the purpose of evaluation as a tumour proliferation marker, Ki67 was found to be overexpressed in proliferating cells and absent in non-proliferating cells (Lopez *et al*., 1991). The precise function of Ki67 is not yet known but is thought to be involved in ribosomal RNA synthesis (Rahmanzadeh *et al*., 2007). Prognostic and predictive values of Ki67 has been shown in breast cancer cells (Viale, 2011; Romero *et al*., 2014

The Ki67 antigen is therefore implicated in cell proliferation and has proved to be a useful tool in predicting human tumour development. It has been reported that the Ki67 antigen is detectable in the nucleus during interphase, whereas majority of the antigen

translocates to the surface of chromosomes during mitosis. Ki67 antigen is absent in G0 phase and present in mitosis and all active stages of cell cycle (G1, S, G2), this quality has made Ki67 a potential excellent biomarker for determining the proliferating fraction of both normal and abnormal cells (Scholzen and Gerdes, 2000). As an excellent marker of cell growth and proliferation, Ki67 has been recommended for clinical use in monitoring and evaluation of cancer patients (Chen *et al*., 2018).

Berney *et al*., 2009 reported Ki67 a better promising biomarker in prostate cancer with more prognostic information than Gleason scare and serum PSA after 808 patients diagnosed with prostate cancer between 1990 and 1996 were treated and Ki67 was used to measure the rate of proliferation in response to treatment. Immunohistochemical staining demonstrates that Ki67 is a cell cycle regulatory protein (Jalava *et al*., 2006) and highly expressed in all proliferative phases of cell cycle (Jalava *et al*., 2006).

The heterogeneity of cancer is one of the limitations of biomarker integration into clinical practice. The heterogeneity of Ki67 as a marker has been described in other forms of cancers including breast and liver cancer but has not been sufficiently studied in prostate cancer (Dowsett *et al*., 2011). Mesko *et al*., 2013 studied Ki67 heterogeneity in prostate cancer. They analysed 77 prostate cancer cores and observed that both inter and intraprostatic Ki67 heterogeneity is significantly higher in high risk cancer patients, and is therefore recommended for clinical use.Desmeules *et al*., 2015 also investigated the quantitative consistency of prostate cancer Ki67 microscopic virtual scoring with that of digital image analysis. The emergence of Ki67 as a tool to distinguish aggressive from non-aggressive prostate cancer, will aid better evaluation of prognosis.

Here the administration of TG003 to cultured cells suppressed cells growth and proliferation in a dose dependent manner (figures 4.2, 4.3, and 4.4). This is consistent with the reports of Karlas *et al*., 2010 and Nishida *et al*., 2011 that administration of TG003 suppresses influenza virus proliferation and affects splicing both in vitro and in vivo. We confirm a significant decrease in prostate cancer cell proliferation in a dose dependent manner (figure 4.5). We also examined the effect of TG003 treatment on prostate cancer cell growth and viability (figure 4.3). Consistent growth was observed in untreated cells and in DMSO-treated controls while there was a significant decreased cell growth in a concentration dependent manner of TG003 treatment (figure 4.2) The same was observed in the percentage viability analysis of prostate cancer cell lines (PC3 and DU145) treated with TG003 (figure 4.3). There was about 50% reduction in cell survival in both prostate cancer cell lines (PC3 and DU145) at 50 µM TG003 concentrations. We also noticed that the growth media (DMEM) in PCa cell lines treated with TG003 changed colour after 72 h to 96 h incubation in a CO_2 incubator at 37^{o}C (figure 4.4). The colour change was measured spectrophotometrically, the result of the untreated controls and TG003 treated cells were matched with the control media. There was a significant difference between TG003 medium and the control media (figure 4.4), but there was no statistically significant difference between untreated controls including DMSO and the control medium. This suggest that there was medial depletion due to progressive growth and multiplication of prostate cancer cells in the untreated controls which was not same in the TG003 treated cells in the media. In summary, TG003 treatment decreases prostate cancer cell growth and proliferation in a concentration dependent manner.

We also considered the effect of TG003 on a normal prostate cell line (PNT2) compared with the prostate cancer cell lines (PC3 and DU145). Our results examining cell proliferation using Ki67 show that PNT2 (normal prostate epithelia cell line) is less sensitive to Ki67 than prostate cancer cell line (PC3 and DU145) (figure 4.5C).

6.4 Effect of TG003 on PCa cell apoptosis

Having observed that TG003 treatment inhibits prostate cancer cell growth, viability and proliferation, we examined the effect on apoptosis. Apoptosis (programmed cell death) occurs in response to severe cell damage (Norbury and Hickson, 2001). Apoptosis refers to a coordinated cascade of events and energy dependent processes leading to cysteine proteases (caspases) activation. Interestingly, cells respond and react differently to pathological and physiological apoptotic stimulating agents. For instance, corticosteroid hormones may trigger apoptosis in some cells such as thymocytes while other cells are unaffected. Cancer therapeutic drugs and irradiation results in DNA damage which can trigger apoptotic cell death in a TP53-dependent pathway. Apoptosis can also be triggered by TNF or Fas expressed by some cells. However, necrosis should not be mistaken for apoptosis as both processes result in cell death which may occur simultaneously, independently and sequentially depending on the degree, duration and type of stimulating agent (Zeiss, 2003). In most cases, it is the nature and the intensity of stimuli that determines whether a cell dies by necrosis or apoptosis. For instance, apoptosis may occur at low doses of heat shock, hypoxia, radiation and anticancer cytotoxic drugs; however, the same stimuli at increased doses can induce necrosis.

At the early stages of apoptosis, there is evidence of visible pyknosis and cell shrinkage. Pyknosis is due to chromatin condensation and is one of the major features

of apoptosis. Features of cell shrinkage include: smaller sized cells, dense cytoplasm and tightly packed organelles (Hacker, 2000; Kerr *et al*., 1972). Histologically, eosin and haematoxylin stains identify apoptotic cells as a purple nuclear fragment with a spherical dark eosinophilic cytoplasm.

Apoptosis involves two major pathways: (a) the death receptor pathway otherwise called the extrinsic pathway (b) the intrinsic or mitochondrial pathway. Research indicates that the two pathways are interlinked such that molecules from one pathway can influence the other. Another form of the intrinsic pathway is the granzyme/perforin which is a T-cell mediated cytotoxicity pathway (Igney and Krammer, 2002). The latter pathway can trigger apoptosis through granzyme A or B. The above pathways converge at the execution pathway initiated by caspase-3 and the whole process finally results to fragmentation of DNA, cytoskeletal and nuclear protein degeneration, protein cross linking apoptotic bodies formation, ligands expression for phagocytic cell receptors and uptake by phagocytic cells.

The Bclx apoptotic regulator is alternatively spliced into anti-apoptotic (BclxL) and proapoptotic (BclxS) variants. BclxL is overexpressed in many cancers including prostate cancer and has been linked to chemoresistance and a vital target for cancer therapy. Increased prostate cancer metastasis is associated with over expression of BclxL (Mercatante *et al*., 2001). Apoptotic induction and increased chemosensitivity has been found in both breast and prostate cancer cells through redirection of BclxL to BclxS pre-mRNA splicing by blockade of the alternative 5’ splice site with an antisense oligonucleotide in Bclx intron 2 (Mercatante *et al*., 2001). BclxS is a potential antagonist of BclxL and inhibits the pro-survival properties of BclxL to induce apoptosis. High level of apoptotic BclxSvariant has been found in patients with

resolving stomach and colon cancers and in nude mice with breast cancer (Gryco *et al*., 2012).

Using acridine orange staining, we investigated the effect of TG003 on apoptosis. We observed apoptotic bodies in cells treated with increasing concentrations of TG003 compared with the controls (figure 4.6). There was a statistically significant percentage apoptotic difference between control and TG003 treated prostate cancer cells especially at concentrations ≥10 µM of TG003. We proceeded to consolidate our results by staining both TG003 treated cells and untreated controls with caspase 3/7 antibody. There was a significant increase in apoptosis especially following higher doses of TG003 compared with the control (figure 4.8). We also tested for apoptosis using apoptotic genes such as *caspase 9, Bclx, Survivin* and *Mcl1* genes. In each of these genes, the pro-apoptotic isoforms were expressed in an increasing order of TG003 concentrations (figure 4.7). In summary, our findings suggest that chemical treatment with TG003 induces apoptosis as well as inhibiting cell proliferation.

Other groups have also reported similar findings. Marcel *et al*., (2014) reported increased apoptosis in TG003 treated cells. Araki *et al*., 2015 also reported that TG003 treatment increased apoptosis.

We also examined the effect of TG003 on apoptosis in PNT2, normal prostate epithelium cells. Caspase 3/7 apoptotic immunofluorescence staining suggested that less apoptosis was induced in PNT2 cells compared to PC3 and DU145 prostate cancer cell lines (figure 4.8).

6.5 Effect of TG003 treatment on prostate cancer cell biology

6.5.1. Effect of TG003 on cell migration and invasion

Having shown that CLK1 inhibition via TG003 induces apoptosis, suppresses growth and proliferation of prostate cancer cell lines, we then investigated the effect of CLK1 inhibition on cell biology such as cell migration and invasion, wound healing scratch assays of prostate cancer cell lines (figures 4.9, 4.10, respectively). Results show that TG003 inhibits prostate cancer cell (PC3) migration and invasion (figure 4.9 A and B) in transwell inserts in a dose dependent manner. There was a statistically significant difference between controls and 10- 50 µM TG003 treatments in scratch assays in a time and dose dependent manner particularly in PC3 cells (figure 4.10, 4.11).

6.5.2 Effect of TG003 on the expression of E-cadherin

In prostate cancer, E-cadherin has been implicated as a possible prognostic indicator because of its decreased expression in higher-grade tumours associated with poor outcome. Loss of E-cadherin is associated with the prostate cancer transition to the mesenchymal phenotype associated with cancer aggressiveness, migration and invasion (Putzke *et al*., 2011).

We therefore examined E-cadherin expression in treated cell lines (figure 4.12A and B). In untreated cells we could see clear vimentin expression and low E-cadherin (consistent with a mesenchymal phenotype) but this was reversed following TG003 treatment (figure 4.12C and D). E-cadherin expression is an important prostate cancer prognostic indicator (Umbas *et al*., 1992; Umbas *et al*., 1994). For instance, using monoclonal E-cadherin antibody and frozen snap prostate tissues, Umbas *et al*., reported high E-cadherin expression in a low Gleason score cancer from 4-7, while at the same time they observed low or almost complete absence of E-cadherin expression in metastatic advanced high grade prostate cancers with high Gleason

scores from 10 -13. In other words, they reported increased E-cadherin expression in a decreasing Gleason prostate tumour scores (Umbas *et al*., 1992; Wang *et al*., 2017). Furthermore, in 2017, Wang *et al*., reported that E-cadherin downregulation promotes prostate cancer chemoresistance (Wang *et al*., 2017). Fan et.al, 2012 proposed that prostate cancer metastasis is enhanced by loss of E-cadherin expression through *metastasis- associated-1- gene* (*MTA-1*) upregulation. Our findings therefore suggest that TG003 reverses the mesenchymal phenotype and this is consistent with the observed reduction in cell migration and invasion. This is also in agreement with the work of Deep *et al*., (2011) who also reported increased E-cadherin expression in the context of anti-invasion and anti-migration silibinin treatments of prostate cancer cells (Deep *et al*., 2011).

6.5.3 Effect of TG003 on the expression of vimentin

Increased vimentin expression is associated with prostate cancer metastasis, increased migration and invasion (Wei *et al*., 2008) consistent with the properties of PC3 cells, a highly proliferative and tumorigenic cell line that readily metastasizes. Other studies have also demonstrated high vimentin expression in metastatic and invasive prostate cancer cells (Craven *et al*., 2006; Singh *et al*., 2003; Vasko *et al*., 2007; Liu *et al*., 2003; Sateli and Li 2011; Vuoriluoto *et al*., 2010). It was suggested that vimentin is more expressed in motile or migratory and highly invasive prostate cancer cells, but down-regulated in non-motile or degreased migration and invasive prostate cancer cells (Lang *et al*., 2002). Based on this, it has been suggested that high vimentin expression is a poor prognostic marker in prostate cancer (Sateli and Li, 2011). Singh *et al*., (2003) also reported that upregulation of vimentin fuels the invasiveness of androgen independent prostate cancers. We noted a clear decrease in vimentin expression via immunofluorescence staining following TG003 exposure

(figure 4.12C, D). Additional experiments, however are needed to measure accurately the effect of TG003 on the expression of vimentin using more quantitative methods such as realtime PCR.

6.5.4 The importance of EMT in prostate cancer

The chemoresistance of prostate cancer (PCa) is invariably associated with the aggressiveness and metastasis of this disease. New emerging evidence indicates that the epithelial-to mesenchymal transition (EMT) may play pivotal roles in the development of chemoresistance and metastasis.

EMT is characterised by downregulation of an epithelial marker known as E- cadherin and upregulation of vimentin, N-cadherin and Snail which are known mesenchymal markers (Nakamura andTokura, 2011). E-cadherin expression suggests epithelial phenotype while loss of it suggests a complete EMT and is associated with metastasis (Bougen *et al*., 2013). Studies suggest that EMT is not only associated with aggravated prostate cancer invasion and metastasis but also a leading cause of prostate cancer chemoresistace (Duran *et al*., 2015; Li *et al*., 2014).

Rubin *et al*., (2001) systematically evaluated the role of decreased expression of E-cadherin on prostate cancer progression in a broad range of prostate tissue including clinically localised prostate cancer, benign prostate tumour and hormone-refractory metastatic prostate cancer. These prostate tissues were analysed using high-density tissue microarrays (TMA) under same condition. After analysing 1,220 prostate TMA samples, Rubin *et al* discovered that 87% E-cadherin expression in benign samples and clinically localised prostate cancers, while there was a statistically significant correlation between aberrant E-cadherin and higher Gleason score, large tumour size and PSA failure prostate cancer suggesting that loss of E-cadherin expression is

linked with aggressive, invasive and metastatic forms of prostate cancer. In general, aberrant E-cadherin is associated with advance form of cancers including prostate cancer. Furthermore, in several clinical studies, E-cadherin has been reported to be silenced, downregulated or aberrantly expressed in many cancer types (Sharma and Lichtenstein, 2009). Fan *et al*. (2012) showed the knockdown of E-cadherin results in increased expression of the MTA-1 gene. Overexpression of MTA-1, (metastatis-associated gene encoding a protein involved in chromatin remodelling) is associated with invasion, metastasis and high grade prostate cancer (Dannenmann *et al*., 2008; Ryu *et al*., 2008). Toh and Nicolson, (2009) reported that MTA-1 is capable of potentiating the conversion of cancer cells including prostate cancer into a devastating aggressive prostate cancer when factors such as hypoxia-inducible factor-1α (HIF-1α), oestrogen receptor α (ERα) and p53 protein are repressed. It is now very clear that MTA-1 and E-cadherin are deeply involved in the EMT process, however, it has not been investigated whether E-cadherin has any effect on MTA-1 expression (Radaelli *et al*., 2009).

Another EMT factor that play a role in the aetiology and pathogenesis of prostate cancer progression, migration, invasion, metastasis and aggressiveness is the vimentin. Vimentin is a 57KDa protein and a major component of intermediate filament (IF) widely expressed only in mesenchymal cells. One of the crucial roles of vimentin is to maintain cellular integrity and resists cellular stress (Satelli and Li., 2011). Overexpression of vimentin has been reported in prostate cancer epithelial as well as epithelial of other cancer types such as CNS tumour, GIT tumour, breast cancer, lung cancer, malignant melanoma and others. Overexpression of vimentin in prostate

cancer correlate with poor prognosis, rapid tumour growth, migration, invasion and prostate cancer aggressiveness.

Evaluation of vimentin expression in normal and malignant tissues is an essential tool in prostate and other cancers diagnosis and prognosis. During prostate cancer progression, vimentin expression is detectable in mobile cancers especially in bone metastasis and poorly differentiated prostate cancer (Zhao *et al*., 2008). Downregulation of vimentin in prostate cancer cells is associated with less tumour motility which also suggests decreased tumour migration and invasion. While the reverse is the case of vimentin overexpression. Many authors support the assertion that vimentin is upregulated in most aggressive prostate cancer and contributes to prostate cancer progression, migration and invasiveness (Sethi *et al*., 2010). Therefore, vimentin expression does not only serve the purpose of prostate cancer detection but also involved in the development and progression of prostate cancer. Vimentin is also expressed in other cancers such as breast, lung, gastrointestinal tract, renal and cervical cancers. Others include malignant melanoma, CNS tumour, thyroid and endometrial carcinomas (Satelli and Li., 2011; Coppola *et al*., 1998). Gilles *et al*., (2003) reported increased vimentin expression in many aggressive breast cancers which also correlate with increased invasion and migration of such cancers.

There is a growing interest by cell biologists on how to explore the therapeutic potentials of targeting vimentin E in the contest of human prostate cancer. This interest arose from the fact that vimentin E has been found to be overexpressed during malignant prostate cell transformation resulting in aggravated prostate cancer risk thereby presenting vimentin E as an excellent target for therapy (Klein *et al*., 2011).

In the current study, we have shown that TG003 (a potential and specific CLK1 inhibitor) exhibited a potential anti-migratory and anti-invasive properties in prostate cancer cells as evidenced by series of experiments such as migration, invasion and scratch assays. TG003 also caused a significant suppression of highly metastatic and invasive prostate cancer cells (PC3) (figure 4.9). We also demonstrated that TG003 exhibited an inhibitory function in both PC3 and DU145 prostate cancer cell motility which is closely related to cell migration (figure 4.10), this effect is more prominent in PC3 cell lines as demonstrated in scratch assays. However, the mechanism of the anti- migration and invasion of TG003 in metastatic prostate cancer cell lines is yet to be understood. The preliminary findings reported here, namely that TG003 reduces E-cadherin while increasing vimentin could be highly significant, given the importance of EMT in prostate cancer.

6.6 Effect of TG003 treatment on tumour volume in vivo

Drastic significant reduction of tumour volume was observed on administration of 50 µM of TG003 intraperitoneally in PC3 xenografts grown subcutaneously in nude mice in vivo (figure 5.3). In comparison, Moreira *et al*., (2018) have used the SRPK1 inhibitor SRPIN340 showing a significant decline of melanoma cell migration and invasion in vitro, and reduction of melanoma metastasis in vivo with no apparent sign of obvious toxicity to normal cells (Moreira *et al*., 2018). Gammons *et al*., (2014) also reported reduced growth of human melanoma in vivo by both SRPK1 knockdown and its pharmacological inhibition independently. Furthermore, in 2015, Mavrou *et al*., reported a decreased PC3 tumour growth in vivo on nude mice treated with SRPK1 inhibitor, and then proposed that SRPK1 inhibition could pose a future therapeutic strategy against prostate cancer. In regards to CLK inhibition, it is already known that

its inhibition could be useful in treatment of other pathologies such as viral infection.The in vivo inhibition of CLK1 which inhibits influenza viral replication as CLK1 is majorly involved in the alternative splicing of *M2* gene responsible for influenza viral proliferation and multiplication (Zu *et al*., 2015). In the context of cancer, interestingly, recent work has highlighted the potential utility of targeting CLK1 and CLK2 in triple negative breast cancer cell lines (Zhu *et al*, 2018). Work presented here further strengthens the idea that CLK inhibition, either as an alternative to or in combination with SRPK1 inhibition, could offer additional benefit in the development of therapies for treatment of prostate, breast and potentially many other types of cancer.

6.7 Summary of key findings

In conclusion, accumulated evidence suggests that chemical inhibition of CLK1 with TG003 treatment suppresses growth, proliferation, migration and invasion at increased concentrations and induced apoptosis in prostate cancer cell lines. Administration of TG003 to cultured prostate cancer cells causes CLK1 inhibition resulting to efficient CLK1 splicing leading to more CLK1 protein. We now know that alternative splicing of CLK1 itself regulates how much CLK1 protein is made. There is evidence of differently CLK1 splicing in a panel of human cell lines. TG003 treatment changes splicing and induced mutated exon 4 skipping in a dose dependent manner (10nM-100µM). There is evidence of feedback loop in which CLK1 actively regulates its own splicing. We also discovered that normal prostate cell line treated with TG003 are less sensitive to proliferation and apoptosis assays compared to its counterpart cancer cell lines.

In summary, TG003 treatment may be a valuable tool for alternative splicing modulation and potentially for the development of a novel anticancer therapy. However further work is necessary to determine the mechanism through which CLK1 inhibition modifies alternative splicing.

6.8 Concluding remarks

It is very clear that TG003 inhibits growth of prostate cancer cell lines, though the detailed underlying mechanism of the anti-cancer effect of TG003 is yet to be studied. In this report, TG003 treatment significantly inhibited prostate cancer cells proliferation in a time and dose dependent manner. TG003 also markedly suppressed prostate cancer cell migration and invasion, significantly induced apoptosis and reduced tumour volume in xenografts grown in nude mice. These investigations provide a novel insight into the molecular activities of TG003 and present it, and CLK inhibition more generally, as novel antitumour approach. Further studies are needed to investigate in more detail the therapeutic potential of CLK inhibition in the context of not only prostate but surely many other types of cancer.

6.9 Future work

6.9.1 siRNA knockdown in vivo

SiRNA-mediated RNA interference of CLK1 worked in in vitro assays confirming the effect of TG003 on cell proliferation, apoptosis and wound healing assays in PC3 cells. It would be useful to repeat CLK1 siRNA knockdowns in other prostate cancer cell lines, attempting additional assays including measurement of EMT markers, and transwell migration and invasion assays. We have also established that TG003 treatment reduces PC3 tumour xenograft growth in vivo. It would be of interest to establish an shRNA mediated stable or inducible knockdown of CLK1 in PC3 and other prostate cancer cell lines and to perform additional xenograft assays.

6.9.2 Determination of the effect of TG003 on normal tissues

In common with most drug treatments it is of course possible that TG003 cause widespread side effects. Within the scope of the experiments conducted so far, with 50μM TG003 injections there was no visible adverse effect on the mice. It would be useful to investigate this in more detail and examine the effect of TG003 on critical organs such as the liver and kidneys, sites of drug metabolism, for any toxic effects.

6.9.3 Testing novel CLK inhibitors in prostate and other cancer cell lines

This project was focused on CLK splice factor kinase inhibition, particularly CLK1, using a well-studied inhibitor, TG003. However, clearly more specific CLK inhibitors are needed, and we have begun a collaboration with the University of Strathclyde, Scotland, to test novel and ideally more specific CLK inhibitors. We will be repeating the assays describe herein examining alternative splicing, cell line proliferation, apoptosis, migration and invasion, and xenograft work. Similar experiments could also be conducted in other cell line models; there is a wide choice. One possible avenue,

in line with recent literature, could be to turn attention to triple negative breast cancer for which there are very limited treatment options.

6.9.4 Determination of the splice factors involved in the autoregulatory mechanism of CLK1 inhibition

We have confirmed that inhibition of human CLK1 results in increased CLK1 protein expression probably through an autoregulatory or feedback mechanism that involves its alternative splicing. It will be interesting to determine how this feedback mechanism works (for example, which splice factors are specifically involved in regulating exon 4 skipping and intron 4 retention). Presumably a specific set of splice factors bind to regulatory sequences within cassette exon 4, and surrounding intronic sequences, including intron 4. It could be speculated that in normal physiological conditions, for example, exon 4 inclusion is not maximised due to a trans-acting repressor of splicing, but that in cellular stress responses the repressor's activity is diminished. Conversely, an activator of exon 4 inclusion becomes more active in response to cellular stress. Future work will address these possibilities, identifying the splice factors involved. It will also be of interest to determine whether exon 4 inclusion and intron 4 retention are coordinately regulated, or if their regulation is through entirely different mechanisms.

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APPENDICES

APPENDIX I: Alignment of human and mouse CLK1 mRNAs showing the position of designed primers

Note that R primers were designed as the antiparallel sequence. The position of exons is shown on the mouse: human alignment.

```
Clk1 exon 1FA        Unmodified DNA Oligo
                     5'-CTGGTAGAAGTGGAAGAAGTAG-3'
                     0.01

Clk1 exon 1FB        Unmodified DNA Oligo
                     5'-CAAGGATGTGAACCTGGACATCGC-3'
                     0.01

Clk1 exon 2FA        Unmodified DNA Oligo
                     5'-AGGAGTGTAGAGGATGATGAGG-3'
                     0.01

Clk1 exon 2FB        Unmodified DNA Oligo
                     5'-GGAGGGTCACCTGATCTGTCAG-3'
                     0.01

Clk1 exon 3RA        Unmodified DNA Oligo
                     5'-CTGCTACATGTCTACCTCCCGC-3'
                     0.01

Clk1 exon 3RB        Unmodified DNA Oligo
                     5'-CTCCTTCACCTAAAGTATCAAC-3'
                     0.01

VEGF 165b F          Unmodified DNA Oligo
```

	5'-GGCAGCTTGAGTTAAACGAACG-3'	
	0.01	
VEGF 165b R	Unmodified DNA Oligo	
	5'-ATGGATCCGTATCAGTCTTTCCTGG-3'	
	0.01	

```
MouseCLK1       GGTATAGGCCCCGCCCATC-----------------TACGTCACCAGTTTTTCCGAGAAG 43
HumanCLK1       -GTGCATGACCCGCCCCGCGGCGGAGACGCGCTCGCTGCGTCATCAGTGTTTTCGAGACG 59
                 **. * *.*******. *                 *.***** **** *** *****.*

MouseCLK1       AAGCTCG-CACTGCGTCTGTCCGCACCATTTTGTGTTGGGAGATCGTCGTAATCGTTTGC 102
HumanCLK1       AGTCTCGACGCAGCAGCTGTCAGCTCCATTTTGTTGTTGGTGCGCGACGCAGTCAGCTGC 119
                *. **** *.*:**. *****.**:*********  * **:*. **:** *.**.  ***
                      Exon1
MouseCLK1       AGACTTCTCGCCGTCGCCTTGTAAGCTTTGTCT---TCGCCTTGCAAGCTTTGTCTTCAG 159
HumanCLK1       GTGATTCCCGTGATTGCGTTACAAGCTTTGTCTCCTTCGACTTGGAGTCTTTGTC--CAG 177
                . ..*** **  .* ** **. ***********   ***.**** *. *******  ***
                          • Exon 2
MouseCLK1       GGTTGGAAAGATGAGACATTCAAAGAGAACTTACTGTCCTGACTGGGATGAAAGAGACTG 219
HumanCLK1       G------ACGATGAGACACTCAAAGAGAACTTACTGTCCTGATTGGGATGACAAGGATTG 231
                *      *.********* ********************** ********.*..** **

MouseCLK1       GGATTATGGAACATGGAGAAGCAGCAGCAGTCACAAAAGAAAGAAGAGATCACATAGCAG 279
HumanCLK1       GGATTATGGAAAATGGAGGAGCAGCAGCAGTCATAAAAGAAGGAAGAGATCACATAGCAG 291
                ***********.******.************** ********.*****************
                                                                •
MouseCLK1       CGCCCGTGAGCAAAAGCGCTGCAGGTACGATCACTCCAAAACGACAGACAGCTATTATCT 339
HumanCLK1       TGCCCAGGAGAACAAGCGCTGCAAATACAATCACTCTAAAATGTGTGATAGCCATTATTT 351
                 ****. ***.*.**********..***.******* **** *: :** *** ***** *
                    Exon 3
MouseCLK1       GGAAAGCAGATCCATAAATGAGAAAGCTTATCATAGTCGACGCTATGTTGATGAATACAG 399
HumanCLK1       GGAAAGCAGGTCTATAAATGAGAAAGATTATCATAGTCGACGCTACATTGATGAGTACAG 411
                *********.** *************.****************** .*******.*****

MouseCLK1       GAATGACTACAT---GGGCTACGAGCCAGGGCATCCCTATGGAGAACCTGGAAGCAGATA 456
HumanCLK1       AAATGACTACACTCAAGGATGTGAACCTGGACATCGCCAAAGAGACCATGAAAGCCGGTA 471
                             CAAGGATGTGAACCTGGACATCGC 1FB
                .**********    .**.*. **.**:**.**** * *:.****.*.**.****.*.**

MouseCLK1       CCAGATGCATAGTAGCAAGTCCTCTGGTAGGAGTGGAAGAAGCAGTTACAAAAGTAAACA 516
HumanCLK1       TCAGAACCATAGTAGCAAGTCTTCTGGTAGAAGTGGAAGAAGTAGTTATAAAAGCAAACA 531
                                    CTGGTAGAAGTGGAAGAAGTAG 1FA
                 ****: ************** ********.*********** ***** ***** *****

                                                          •       Exon 4
MouseCLK1       CAGGAGTCGCCACCACACTTCGCAGCACCATTCACACGGGAAGAGTCACCGAAGGAAAAG 576
HumanCLK1       CAGGATTCACCACAGTACTTCACATCGTCGTTCACATGGGAAGAGTCACCGAAGGAAAAG 591
                ***** **.****.. *****.** *. *.****** **********************

MouseCLK1       ATCGAGGAGTGTAGAGGATGATGAGGAGGGTCACCTGATCTGTCAGAGTGGAGACGTACT 636
HumanCLK1       AACCAGGAGTGTAGAGGATGATGAGGAGGGTCACCTGATCTGTCAGAGTGGAGACGTACT 651
                                  GGAGGGTCACCTGATCTGTCAG 2FB
                    AGGAGTGTAGAGGATGATGAGG 2FA
                *:* ******************************************************

                          •             Exon 5
MouseCLK1       AAGTGCAAGATATGAAATTGTTGATACTTTAGGTGAAGGTGCTTTCGGAAAAGTGGTGGA 696
HumanCLK1       AAGTGCAAGATATGAAATTGTTGATACTTTAGGTGAAGGAGCTTTTGGAAAAGTTGTGGA 711
                                 GTTGATACTTTAGGTGAAGGAG 3RB
                ****************************************:***** ******** *****

MouseCLK1       ATGCATCGATCATAAAGTGGGAGGTAGACGTGTAGCAGTAAAAATAGTTAAAAATGTGGA 756
HumanCLK1       GTGCATCGATCATAAAGCGGGAGGTAGACATGTAGCAGTAAAAATAGTTAAAAATGTGGA 771
                               GCGGGAGGTAGACATGTAGCAG 3RA
                .**************** ***********.******************************

MouseCLK1       TAGATACTGTGAAGCTGCTCAATCGGAAATACAAGTTTTGGAACACTTGAATACAACAGA 816
HumanCLK1       TAGATACTGTGAAGCTGCTCGCTCAGAAATACAAGTTCTGGAACATCTGAATACAACAGA 831
                ********************..**.************ *******  *************
                           •   Exon 6
MouseCLK1       CCCCCATAGTACTTTCCGTTGTGTCCAGATGTTGGAGTGGTTTGAGCATCGAGGTCACAT 876
HumanCLK1       CCCCAACAGTACTTTCCGCTGTGTCCAGATGTTGGAATGGTTTGAGCATCATGGTCACAT 891
                ****.* *********** *****************.*************.:*********
```

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MouseCLK1       TTGCATTGTGTTTGAACTTCTGGGGCTTAGTACTTATGATTTCATTAAGGAAAACAGTTT 936
HumanCLK1       TTGCATTGTTTTTGAACTATTGGGACTTAGTACTTACGACTTCATTAAAGAAAATGGTTT 951
                ********* ********: ****.*********** ** ********.***** .****

MouseCLK1       TCTGCCGTTTCGAATGGATCATATCAGGAAGATGGCATATCAAATATGCAAATCTGTAAA 996
HumanCLK1       TCTACCATTTCGACTGGATCATATCAGAAAGATGGCATATCAGATATGCAAGTCTGTGAA 1011
                ***.**.******.*************.**************.********.*****.**

                 •
MouseCLK1       CTTTTTGCATAGTAATAAATTGACTCATACAGACTTGAAGCCTGAAAACATCTTATTTGT 1056
HumanCLK1       TTTTTTGCACAGTAATAAGTTGACTCACACAGACTTAAAGCCTGAAAACATCTTATTTGT 1071
                 ******** ********.******** ********.**********************

MouseCLK1       GAAGTCTGACTACACAGAGGCTTATAATCCCAAAATGAAACGTGATGAACGTACTATAGT 1116
HumanCLK1       GCAGTCTGACTACACAGAGGCGTATAATCCCAAAATAAAACGTGATGAACGCACCTTAAT 1131
                *.****************** **************.************** ** :**.*

MouseCLK1       AAATCCAGATATTAAAGTGGTGGACTTTGGAAGTGCAACATATGATGATGAACACCACAG 1176
HumanCLK1       AAATCCAGATATTAAAGTTGTAGACTTTGGTAGTGCAACATATGATGACGAACATCACAG 1191
                ****************** **.********:****************** ***** *****

MouseCLK1       CACATTGGTATCTACAAGACATTATAGAGCACCGGAAGTTATTTTAGCCCTCGGGTGGTC 1236
HumanCLK1       TACATTGGTATCTACAAGACATTATAGAGCACCTGAAGTTATTTTAGCCCTAGGGTGGTC 1251
                 ******************************** *****************.********

MouseCLK1       ACAGCCATGTGATGTCTGGAGCATAGGATGTATTCTTATCGAGTATTATCTTGGATTTAC 1296
HumanCLK1       CCAACCATGTGATGTCTGGAGCATAGGATGCATTCTTATTGAATACTATCTTGGGTTTAC 1311
                .**.************************** ******** **.** ********.*****

MouseCLK1       AGTTTTTCCGACTCATGATAGCAGGGAACATTTAGCAATGATGGAAAGGATTCTTGGACC 1356
HumanCLK1       CGTATTTCCAACACACGATAGTAAGGAGCATTTAGCAATGATGGAAAGGATTCTTGGACC 1371
                .**:*****.**:** ***** *.***.********************************

MouseCLK1       ACTACCAAAGCACATGATACAGAAAACCAGGAAACGCAGATATTTCCATCATGATCGATT 1416
HumanCLK1       TCTACCAAAACATATGATACAGAAAACCAGGAAACGTAAATATTTTCACCACGATCGATT 1431
                :********.** ********************** *.****** ** ** ********

MouseCLK1       AGATTGGGATGAACACAGTTCTGCTGGCAGATATGTTTCTCGGCGCTGTAAACCTCTGAA 1476
HumanCLK1       AGACTGGGATGAACACAGTTCTGCCGGCAGATATGTTTCAAGACGCTGTAAACCTCTGAA 1491
                *** ******************** ***************:.*.*****************

MouseCLK1       GGAGTTTATGCTATCTCAGGATGCCGAACATGAGCTTCTCTTTGACCTCATTGGGAAAAT 1536
HumanCLK1       GGAATTTATGCTTTCTCAAGATGTTGAACATGAGCGTCTCTTTGACCTCATTCAGAAAAT 1551
                ***.********:*****.****  ********** ********************.******

MouseCLK1       GTTGGAGTATGATCCCGCCAAAAGAATTACTCTCAAAGAAGCCCTAAAGCATCCTTTCTT 1596
HumanCLK1       GTTGGAGTATGATCCAGCCAAAAGAATTACTCTCAGAGAAGCCTTAAAGCATCCTTTCTT 1611
                ***************.******************.******* ****************

MouseCLK1       TTACCCACTTAAAAAGCATACGTG-ATTTATAAAC--ACAGTGCTCTGAAAGGAATCTTA 1653
HumanCLK1       TGACCTTCTGAAGAAAAGTATATAGATCTGTAATTGGACAGCTCTCTCGAAGAGATCTTA 1671
                * *** :** **.**...** .*. ** *.***:   ****  **** .***..******

MouseCLK1       CAGACTGTATCAGTCTAGCTTTTAATT---AAGTTATTTTGTATAGCTT---AATTTGTA 1707
HumanCLK1       CAGACTGTATCAGTCTAATTTTTAAATTTTAAGTTATTTTGTACAGCTTTGTAAATTCTT 1731
                *****************. ******:*   ************* *****   **:** *:

MouseCLK1       AAACATTTTATGTT----------TTTT---------AGATGCTTTATTAAATACATGGC 1748
HumanCLK1       AACATTTTTATATTGCCATGTTTATTTTGTTTGGGTAATTTGGTTCATTAAGTACATAGC 1791
                **..:******.**          ****         * :** ** *****.*****.**

MouseCLK1       CAAACCAAATAACATCTTT--CAGTAATTATAGAATGATTTATTTGGAATAAAATTT--G 1804
HumanCLK1       TAAGGTAATGAACATCTTTTTCAGTAATTGTAAAGTGATTTATTCAGAATAAATTTTTTG 1851
                 **.  **: *********  ********.**.*.********* .*******:***  *

MouseCLK1       TGCTTATGAA----------------------TGTATAAAGGTCTATA------------ 1830
HumanCLK1       TGCTTATGAAGTTGATATGTATCTGAACAGTTTGTTCTAAGTACCATTTTTCTTCCTACT 1911
                **********                     ***: :*** :* **:
```

```
MouseCLK1       ----------------------
HumanCLK1       TCTATTAAAGAATGGACATAGA 1933
```

APPENDIX II: Copy of papers arising from research presented in this thesis. The first two are published; the third is submitted at the time of thesis submission.

1. Bowler, E., Porazinski, S., Uzor, S., Thibault, P., Durand, M., Lapointe, E., Rouschop, K.M.A., Hancock, J., Wilson, I., Ladomery, M. (2018) Hypoxia leads to significant changes in alternative splicing and elevated expression of CLK splice factor kinases in PC3 prostate cancer cells*. BMC Cancer*. **18**: pp. 1-11.
2. Uzor, S., Zorzou, P., Bowler, E., Porazinski, S., Wilson, I. D. and Ladomery, M. (2018) Autoregulation of the human splice factor kinase CLK1 through exon skipping and intron retention. *Gene*, **670**: pp. 46-54.
3. Uzor, S., Porazinski, S.R., Li, L., Ajiro, M., Iida, K., Hagiwara, M., Wilson, I.D., Oltean, S., Ladomery, M.R. (submitted for publication). CDC2-like (CLK) protein kinase inhibition as a novel targeted therapeutic strategy in prostate cancer. Submitted to *Scientific Reports*, reference SREP-19-06093