TARGETING THE EGFR AND PI3K PATHWAYS AS A THERAPEUTIC STRATEGY FOR PROSTATE CANCER

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part been submitted to any University for a degree.

Signature: ..... 1 December 2014 **Date: .....**

ABSTRACT

Targeted therapy for prostate cancer may offer potential improvement over current conventional therapies because of its specificity. Although conventional treatments are effective, they are not curative and have several limitations. In prostate cancer, activation of both the epidermal growth factor receptor (EGFR) and the phosphatidylinositol 3 – kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway have been implicated in tumorigenesis and resistance to both conventional and targeted anticancer therapies. Having a better understanding of the molecular mechanisms involved in PCa development, progression and resistance to therapy, could assist in the design of novel therapeutic strategies.

The objective of this study was to inhibit key molecular targets of the human epidermal growth factor receptor signalling pathway and expose prostate cell lines to doses of radiation, so as to establish potential therapeutic targets that may be amenable to combined modality therapy, and formulate a cocktail of inhibitors to evaluate its radiosensitising capability.

The EGFR/PI3K/mTOR pathway plays an important role in the radiosensitivity of the human prostate carcinoma cell line (DU145) and the normal cell line (1542N). In our study we have shown that AG-1478, an EGFR inhibitor, and BEZ-235, a dual inhibitor of the PI3K/mTOR pathway, singly or in combination, at low and relatively high radiation doses, resulted in radiosensitisation of DU145 cells. Radio-protection was achieved in 1542N cells. AG-1478 had no effect on radiosensitivity.

OPSOMMING

Geteikende terapies wens hul spesifisiteit teenoor konvensionele terapies vir prostaat kanker, mag potensieel verbetering offer. Konvensionele behandeling is wel effektief maar nie genesend nie wens ‘n aantal beperkings, sowel as die toksisiteit vir normale selle. In prostaat kanker is die aktivering van beide die epidermiese groei faktor reseptor (EGFR) en fosfatidielinositol 3-kinase/Akt/soogdier teiken vir rapamisien (mTOR) seingewing baan sterk betrek by tumor groeisel en weerstand teen konvensionele en geteikende anti-kanker terapies.

Beter begrip van die molekulêre meganismes betrokke by prostaat kanker ontwikkeling, bevordering en weerstand teen terapie, kan die ontwerp van nuwe terapeutiese strategies ondersteun.

Die doelwit van hierdie studie was om sleutel molekulêre teikens van die epidermiese groei faktor reseptor seingewing baan te inhibeer en om prostaat selle bloot te stel aan dosisse bestraling, om potensiële terapeutiese teikens te vestig wat vatbaar is vir gekombineerde modaliteit terapie, om ‘n mengsel van stremmiddels te formuleer, en om die straling gevoeligmaking bekwaamheid daarvan te evalueer.

Die EGFR/PI3K/mTOR seingewingbaan speel ‘n belangrike rol in the radiosensitiwiteit van die menslike prostaat kanker sellyn (DU145) en die normale prostaat sellyn (1542N). Die studie bevind dat AG-1478, ‘n EGFR stremmer, en BEZ-235, ‘n tweevoudige beperker van die fosforinositied 3-kinase (PI3K) en soogdier teiken vir rapamisien (mTOR) seingewingbaan, enkel of in kombinasie die DU145 selle radiosensitiseer vir straling dosisse van 2 en 6 Gy. Stralings beskerming was verkry met die 1542N sellyn. AG-1478 het geen effek getoon op radiosensitiwiteit nie.

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DEDICATION

Those who will always be loved and dearly missed

Mme Maria Mohapi

Mama Sebolelo Nyakale

Ausi Tono Mohapi

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LIST OF ABBREVIATIONS

ADT	Androgen Deprivation Therapy
AG 1478	AG 1478 hydrochloride
BSA	Bovine serum albumin
CO_2	Carbon Dioxide
DMSO	Dimethyl Sulfoxide
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor
IgG	Immunoglobulin G
RMPI-1640	Roswell Park Memorial Medium
MARK	Mitogen – Activated Protein Kinase
MEM	Minimum Essential Medium Eagle
FBS	Feotal Bovine Serum
HRPC	Hormone Refractory Prostate Cancer
HPV-16	Human papilloma virus serotype 16
Pen-Strep	Penicillin – Streptomycin
PBS	Phosphate Buffer Saline
PI3K	Phosphatidylinositol 3-kinase

mTOR	Mammalian target of rapamycin
RIPA	Radio Immuno Precipitation Assay
PCa	Prostate Cancer
PBS	Phosphate buffered saline
PE	Plating efficiency
SDS	Sodium dodecyl sulphate
SF2	Survival fraction at 2Gy
SF6	Survival fraction at 6Gy
SSD	Source-to-sample distance
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
PVDF	Polyvinylidine difluoride
TBST	Tris buffered saline-tween 20
Gy	Gray (1 Gy = 100 rad)
α	Linear coefficient of inactivation after ^{60} y-irradiation
β	Quadratic coefficient of inactivation after ^{60} y-irradiation
TEMEDN,N,N' ,N'	Tretramethylethylenediamine
TLD-chips	Thermo Luminescent dosimetry chips
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

CHAPTER 1

1.1. Introduction

Prostate cancer (PCa) is the second most common cancer in men, accounting for 903 500 new diagnoses and 258 400 deaths per year worldwide (Siegel et al., 2012). The incidence and mortality rates of prostate cancer are on the rise globally, especially in developed countries, also in parts of Africa, including South Africa (Rebbeck et al., 2013). Radiotherapy and surgical resection are the potential curative treatment protocols for patients with localized PCa (Di Lorenzo et al., 2002). Despite early screening, most men are diagnosed at an advanced stage, with 25-29% of patients with early stage disease relapsing within 5 years (Di Lorenzo et al., 2002; Siegel et al., 2012).

During the development of PCa, the first line treatment for patients who have relapsed, or with advanced PCa, is androgen deprivation therapy (ADT); this is because the majority of the tumours remain androgen-dependent (Carrión-Salip et al., 2012). However, in most cases, the tumour cells progress to a hormone refractory state, where androgen-independent tumours are generated (Carrión-Salip et al., 2012; Di Lorenzo et al., 2002). Once the first line therapy fails, therapeutic options are limited and survival is about 6-12 months for patients with androgen-independent hormone refractory prostate cancer (HRPC)(Di Lorenzo et al., 2002).

The major clinical challenge is the resistance prostate cancer cells develop against treatment, resulting in tumour recurrences. Approximately 30-40% of prostate cancer patients reveal failure after radiotherapy (Skvortsova et al., 2008). The epidermal growth factor receptor (EGFR) and phosphatidylinositol-3-Kinase (PI3K) pathways are implicated in disease progression and survival of prostate cancer cells after

radiation exposure, resulting in resistance to radiotherapy (Gao et al., 2003; Guérin et al., 2010; Sarker et al., 2009).

To address these challenges, there is an urgent need to develop novel therapeutic strategies targeting the affected pathways within the androgen-independent tumours for optimum treatment of prostate cancer. It is envisioned that using a cocktail of targeted therapeutic agents to inhibit survival signalling markers like EGFR, PI3K and mammalian target of rapamycin (mTOR), in combination with radiotherapy, could be more effective in treating prostate cancer.

1.2. Literature Review

1.2.1. The Prostate

The prostate is a gland about the size of a walnut. It makes and stores the liquid that carries sperm. The prostate is located near the bladder and rectum, just below the bladder, and surrounds the urethra. The glandular epithelium of the normal prostate is composed of three cell types. The two major cell types, basal and secretory luminal cells, express several specific markers by which they can be distinguished (Abate-Shen and Shen, 2000; Shen and Abate-Shen 2010).

1.2.2 Prostate Cancer – Clinical Picture

Prostate cancer, like other cancers, arises in differentiated epithelial cells in which embryonic pathways are reactivated through the activation of oncogenes and the loss of tumour suppressor genes, leading to growth and survival advantage (Taichman et al., 2007). The initiation and progression of prostate cancer is

illustrated in Figure 1. The disease presents as heterogeneous and multi-stage, both at the clinical and histological level. This makes it difficult to understand the factors involved in the onset and progression of the disease (Van Bokhoven et al, 2003).

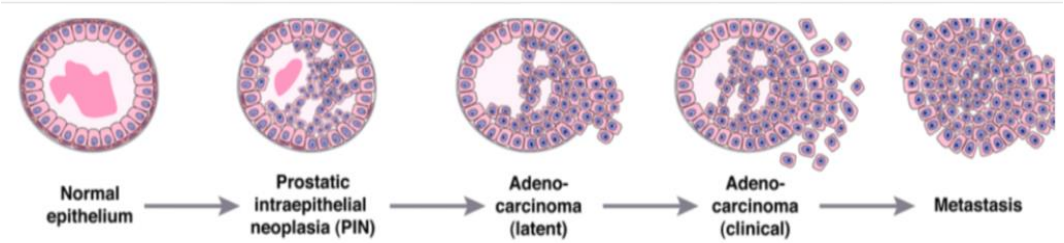

Figure1. Initiation and progression of human prostate cancer.

1.2.3. Epidemiology

Prostate cancer is of significant human health concern because it is the most diagnosed form of cancer in men. It remains one of the most common causes of cancer deaths amongst men worldwide, accounting for about 14% of total new cases and 6% mortality rates (Ghosh et al., 2005; Rajasekhar et al., 2011). The incidence of this disease is on the rise, especially in developed countries, due to a high quality of living and an ageing population (Bolus et al, 2001; Heyns et al. 2011; Sherwood, 2008). Similar trends have been documented in developing countries, and especially in South Africa (Heyns et al., 2011). The incidence and mortality rates of prostate cancer are, respectively, about 1.75 and 2.50 times higher among African-American men than in men of other ethnic groups (Heyns et al., 2011), and increasing mortality rates remain problematic because of the lack of curative treatment in androgen-resistant metastatic disease (Li et al., 2005). With the abovementioned, it is clear that prostate cancer remains one of the most common causes of cancer deaths amongst men.

1.2.4. Treatment for Prostate Cancer

There are numerous treatment options for prostate cancer, each with its own limitations. An effective treatment regimen, ideally, must result in less morbidity; decreased recurrence of tumours, and be specific for destroying cancer cells without damaging normal cells (Spitzweg et al., 2000). Effective therapies have been developed to address the heterogeneity of PCa, but no curative therapeutic strategies exist. Most patients with organ-confined disease undergo radical prostatectomy, radiotherapy or androgen deprivation therapy (ADT) (Lee et al., 2008).

The function of the prostate is initially controlled by androgens which are important in maintaining and regulating the expression of specific genes (Carrión-Salip et al., 2012). EGFR and their respective receptors mediate proliferation of the androgen-independent PCa and may interact with androgen receptors in the absence of androgen ligand binding, constituting an essential signalling pathway for tumour growth, invasiveness and metastasis (Eichhorn et al., 2008)

The majority of prostate tumours are dependent on androgens for growth (Ghosh et al., 2005) and advanced prostate cancers are generally treated with ADT (Jain et al., 2012). However, the majority of prostate cancers have been known to develop resistance against ADT (Jain et al., 2012). Another factor known to increase tumour cell resistance to treatment is the presence of overexpressed or activated oncogenes, or the loss of function in tumour suppressor proteins (Cully et al., 2006).

1.2.5. Conventional Therapy

The primary treatment for localised prostate cancer is ionising radiation, or complete removal of the prostate (radical prostatectomy) (Lee et al., 2008). Due to resistance to therapy many patients relapse, resulting in re-growth of the cancer. The next step to follow is ADT; cancerous cells die due to androgen blockade. Most tumours develop resistance, eventually growing in the absence of testosterone. This is known as hormone relapse, and patients in this stage are defined as hormone-refractory (Lee et al., 2008), and are also known as presenting with castration-resistant prostate cancer (CRPCa) (Jain et al, 2012).

After hormone relapse, therapeutic options are limited. Chemotherapy is also utilised in the clinical setting but has been found to be ineffective in prostate cancer. It leads to toxicity with severe side effects to patients (Lee et al., 2008).

Below is a list of conventional therapies and their limitations**:**

- Radiotherapy: the problem is toxicity and the lack of specificity to the site of the tumour. Normal cells are also killed during treatment (Lee et al., 2008).
- Surgery is invasive and can only be performed on localised prostate cancer. In addition to complications like incisional hernia and rectal injury, surgery has a high morbidity rate (Bolus et al. 2001).
- Long-term hormonal therapy, also known as ADT, reduces the quality of life of an individual resulting in a loss of stamina, atrophy, premature osteoporosis and increased fatigue (Spitzweg et al., 2000). Most prostate cancers develop resistance against ADT (Jain et al., 2012).
- Chemotherapy involves very toxic drugs that kill fast growing cells, as well as, normal cells. For example, blood cells are destroyed during chemotherapy,

and immunological function is suppressed and compromised. This results in infections and poor immune response (Bolus et al. 2001).

1.2.6. Personalised/Targeted Therapy

The development of monoclonal antibody therapy targeted at tumour antigens for treatment of cancer has had some stunning success. Examples include the use of trastuzumab for HER-2-positive breast cancer, rituximab for CD20-positive non-Hodgkins lymphomas, cetuximab for EGFR-positive colorectal lymphomas, and gemtuzumab for CD33-positive malignancies (Fleuren et al., 2014; Harris, 2004; Stern and Herrmann, 2005).

The potential for EGFR-targeted therapy in the treatment of cancers has shown great success, especially in breast cancer therapy (Liang et al., 2003). ErbB2, also known as HER-2, has been shown to be overexpressed in about 30% of patients with breast cancer (Liang et al., 2003). Therapeutic strategies using inhibitory agents directed at the growth receptor, and other components of the intracellular signalling pathway, have shown great success in the treatment of breast cancer with trastuzmab (Herceptin), which is a monoclonal antibody binding to the HER-2 receptor, thus providing evidence to validate this type of approach (Liang et al., 2003).

The hormone-resistant state observed in breast cancer is similar to that in prostate cancer. Therefore, the management of prostate cancer through targeted therapy may be developed by translation of the successful treatment approach for breast cancer (Liang et al., 2003).

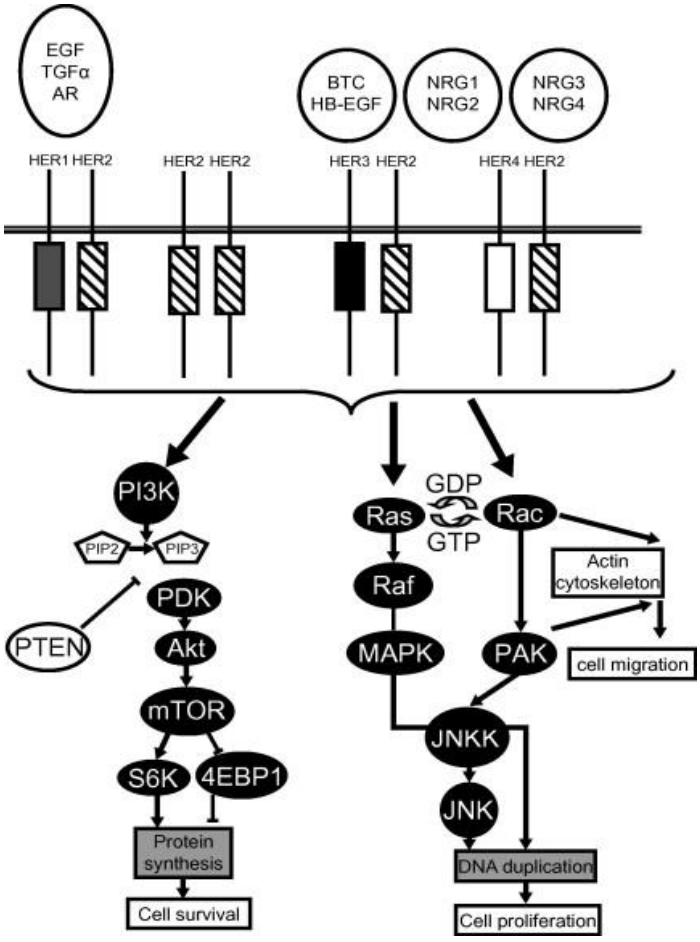

Figure 2. Human epidermal growth factor receptor family signal transduction pathways. Homodimerisation and heterodimerisation of HER-2 leads to tyrosine kinase activation and downstream signalling via the PI3K/Akt/mammalian target of rapamycin pathway, and the Ras/Raf/mitogen-activated protein kinase pathway to stimulate processes involved in cell survival and proliferation (Hurvitz et al., 2013).

1.2.7. The PI3K/Akt/mTOR Signalling Pathway

Cells exposed to stress, such as ionising radiation, activate multiple intracellular signalling pathways. Activating these pathways plays a critical role in controlling cell survival and proliferation rates (Dent et al., 2003). The PI3K/Akt/mTOR pathway, as depicted in Figure 2, plays a central role in the development and progression of

cancer (Liu et al., 2009) and has a diverse array of functions, including differentiation, growth, metabolism, migration and regulation of cell survival (Bitting and Armstrong, 2013). Studies indicate that the PI3K/Akt/mTOR pathway is central in the development and progression of specific human malignancies (Bitting and Armstrong, 2013; Carracedo and Pandolfi, 2008; Liu et al., 2009; Morgan et al., 2009). There are three classes of PI3K and the class most implicated in cancer is Class 1, specifically Class 1A. Class 1A PI3K consists of two subunits**:** a regulatory subunit, p85, and a catalytic subunit, p110 (Courtney et al., 2010). Initial activation of the pathway occurs at the cell membrane, where the signal is translated through Class 1A (LoPiccolo et al., 2008).

Activation of the pathway can occur through a tyrosine kinase growth factor receptor, such as epidermal growth factor receptor (EGFR) (Liu et al., 2009; LoPiccolo et al., 2008). The process leads to the phosphorylation of phosphatidylinositol 4, 5-bisphosphate ($PI(4,5)P_2$) to phosphatidylinositol 3, 4, 5-trisphosphate ($PI(3,4,5)P_3$), followed by the recruitment and activation of 'Akt' to the plasma membrane (Courtney et al., 2010; Liu et al., 2009). The activation of Akt leads to the phosphorylation and regulation of a wide spectrum of substrates involved in multiple cellular processes, including cell survival, cell growth, cell differentiation, cell cycle progression and cell proliferation (Bitting and Armstrong, 2013; Liu et al., 2009).

After activation of Akt, numerous downstream proteins, including mTOR, are phosphorylated thereby regulating a range of cellular processes (Bitting and Armstrong, 2013). mTOR is a master regulator of protein transcription, playing an important role in protein synthesis and cell survival.

1.2.8. The PI3K/Akt/mTOR Signalling Pathway in Prostate Cancer

Prostate cancer cells utilise multiple molecular pathways to proliferate, survive and invade tissue during the course of tumour progression (Shukla et al., 2007). The two main pathways are; PI3K/Akt/mTOR that leads to cell survival, and mitogen-activated protein kinase (MAPK) that leads to cell proliferation. Among several prostate signalling pathways, up-regulation of PI3K/Akt/mTOR signalling through mutations in the PTEN gene and activation of growth factor receptors are important for the identification of therapeutic targets (Lee et al., 2008; Shukla et al., 2007).

In prostate cancer, alterations of the PI3K/Akt/mTOR pathway have been reported in 42% of primary prostate tumours and 100% of metastatic tumours (Bitting and Armstrong, 2013). These include mutations and altered expression in the proteins within the pathway, leading to increased PIK3/Akt/mTOR signalling activity. The detection of altered PI3K/Akt/mTOR has been found in prostate tissue. This suggests the importance of the pathway in the development and progression of PCa. Up-regulation of the components of this pathway occurs in about 30-50% of PCa, and the signalling of the molecules in this pathway has also been detected in PCa cell lines and xenografts (Morgan et al., 2009).

The PI3K/PTEN/Akt/mTOR pathway is the most accepted pathway to study the survival of prostate cancer cells compared to that of MAPK (Li et al, 2005). Within the prostate gland, the population of cells that undergoes active proliferation is 1-3% (Lee et al., 2008). This observation explains why diagnosis of the disease only takes place in the later stages of life in men between 40-60 years. Pathways that mediate survival rather than proliferation may be more attractive targets for reducing tumour growth in prostate cancer patients (Lee et al., 2008; Li et al, 2005).

PTEN, a lipid phosphatase, is a well-known negative regulator of the PI3K/Akt/mTOR signal transduction cascade (Lee et al., 2008). The loss of PTEN function and up-regulation of PI3K leads to the inhibition of apoptosis, and activation of the survival pathway (Cully et al., 2006; Lee et al., 2008). The majority of prostate cancer cells that have been evaluated show inactivation of the PTEN tumour suppressor gene (Li et al, 2005). Loss of PTEN function in advanced prostate cancer takes place in 50-80% of patients (Lee et al., 2008). The mutated PTEN protein initiates the activation of the cascade which presents the cell with a number of cancer-like properties (Cully et al, 2006).

The PI3K/Akt/mTOR pathway is fundamental to the metastatic potential of PCa and provides a strong rationale for targeting this pathway in prostate disease.

1.2.9. Inhibition of the PI3K/ Akt /mTOR Signalling Pathway

Given the importance of the PI3K/Akt/mTOR pathway in cell survival signalling and the high prevalence of activation of the pathway in PCa, its inhibition has great potential for improved clinical outcome in men with advanced prostate cancer (Bitting and Armstrong, 2013).

The catalytic domain of the p110 subunits and mTOR belong to the phosphatidylinositol kinase-related family of kinases, and are, therefore, structurally similar. Recent developments in the inhibition of the PI3K/Akt/mTOR pathway, termed dual-inhibition, means that two sites in the PI3K/Akt/mTOR pathway are targeted, resulting in optimum blockage of the pathway. Compared to single targeting of specific components of the pathway, a dual inhibitor has the potential of

inhibiting all the PI3K catalytic isoforms, thus effectively switching off the pathway completely and overcoming feedback inhibition, normally observed with mTORC1 inhibitors (Bitting and Armstrong, 2013; Courtney et al., 2010).

There are a number of agents currently being studied and utilised in the clinic today that target the PI3K/Akt/mTOR pathway. One well-known agent is LY294002, a dual PI3K-mTOR inhibitor, which has been studied extensively and used in preclinical studies, but this agent is not suitable for patients (Bitting and Armstrong, 2013; Courtney et al., 2010). Other dual inhibitors, such as the NVP BEZ235 and NVP – BGT226 have entered phase 1 clinical trials. NVP BEZ235 has been extensively evaluated clinically, with positive results. It has been shown to be well-tolerated, with no significant changes in the body weight of mice, and slowed the growth of PTEN-deficient human cancer cells (Courtney et al., 2010). In addition, HER-2 amplification in breast cancer cell lines appeared sensitive to NVP BEZ235 (Brachmann et al., 2009; Serra et al., 2008), and was shown to induce apoptosis in oestrogen-deprived oestrogen-positive breast cancer cells (Brachmann et al., 2009; Serra et al., 2008). In the clinic, BEZ235 has been well-tolerated in patients with solid tumours (Choi et al, 2010). A study using genetically engineered mice also demonstrated that BEZ235 was highly effective at shrinking murine lung tumours (Roper et al., 2011).

1.2.10. Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR) family, consists of four members, namely; ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4 (Hynes and MacDonald, 2009). They are composed of 3 functional domains, namely, an extracellular ligand binding

domain, a hydrophobic trans-membrane, and an intracellular domain that has catalytic tyrosine kinase activity (Hynes and MacDonald, 2009).

Activation of the EGFR signalling pathway takes place when the EGFR family of peptides binds to their receptive extracellular domains (Hynes and MacDonald, 2009). Dimerisation of the receptor takes place with another member of the ErbB family or an EGFR monomer (Baselga, 2002). This is followed by the induction of intrinsic protein tyrosine kinase activity, and tyrosine autophosphorylation, leading to the recruitment of several intracellular substrates resulting in mutagenic signalling and other cellular activities (Baselga, 2002). The most important and well-studied signalling route of the EGFR is the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol 3-kinase (PI3K)(Baselga, 2002).

1.2.11. EGFR and Prostate Cancer

The ErbB receptors are expressed in a variety of epithelial tissues where they play an important role in development, proliferation and differentiation (Guerin et al, 2010). High levels of EGFR have been observed in a variety of tumours, including prostate, breast, colorectal and ovarian (Baselga, 2002; Di Lorenzo et al., 2002). Bladder cancer and renal cell cancer, too, are reported to express EGFR (Baselga, 2002). ErbB2 and EGFR have been implicated in the development of numerous cancers, including PCa (Guérin et al., 2010; Hynes and MacDonald, 2009). Genetic changes detected in the cancers lead to the deregulation of receptors, resulting in the overexpression of EGFR proteins. Activation of EGFR has been shown to enhance tumour growth and tumour progression (Baselga, 2002; Scaltriti and Baselga, 2006). Poor therapy response and disease progression has been

correlated with EGFR overexpression in tumours. This leads to the development of resistance to cytotoxic agents and radiation therapy (Baselga, 2002; Scaltriti and Baselga, 2006).

1.2.12. Inhibition of the EGFR

The majority of new therapeutic approaches for PCa currently in the clinic, are directed against the growth factor signalling pathway involving the EGFR family of receptors and the downstream components that transduce signals to the cell nucleus (Guérin et al., 2010). Targeted therapies in oncology, to date, include two main categories of molecules**:** monoclonal antibodies and tyrosine kinase inhibitors (TKI) (Guérin et al., 2010).

Preclinical studies have demonstrated that EGFR-targeting agents have the potential to be used in combination with cytotoxic chemotherapy and radiotherapy. IMC-C225 (cetuximab) has been shown to enhance the effects of cytotoxic agents and radiotherapy (Baselga, 2002; Fleuren et al., 2014; Harris, 2004; Stern and Herrmann, 2005). This is achieved by inhibiting cell growth and survival both *in vitro* and *in vivo*.

1.2.13. Radiosensitisation of Prostate Cancer

The radiosensitisation of prostate cancer is achieved by inhibiting proliferating and survival pathways, leading to the suppression of prostate cancer cells (Valerie et al, 2007). Tumour cell growth is reduced and the sensitivity of the prostate cancer cells

to physiological stress, such as ionising radiation, will be increased (Dent et al., 2003).

Conventional external beam irradiation is the preferred non-invasive treatment option for many prostate cancer patients (Dent et al., 2003). Radio-recurrence and resistance of the disease are mostly due to the fact that the conventional radiation dose does not irradiate the tumour completely (Lee et al., 2008). The radiation dose is usually increased to overcome the aforementioned problem, but dose escalation is directly associated with high normal tissue toxicity (Lee et al., 2008). This option is, therefore, not clinically relevant because of the severe acute effects suffered by patients. Curative radiation therapy aims to prevent tumour regrowth by inducing tumour cell death and loss of reproductive integrity.

After radiation therapy, more than 99% of the malignant cells typically are killed. The surviving fraction, which amounts to more than a million cells per gram of tumour, has the potential to actively contribute to radio-recurrence of prostate cancer and is the major target for radiosensitisation (Lee et al., 2008). The surviving tumour subpopulation can be targeted by monoclonal antibodies and agents that inhibit the EGFR and PI3K pathways (Dent et al., 2003; Lee et al., 2008), in combination with radiation. This approach proves that the concept of combination therapy results in a better treatment outcome than therapy with a single modality.

1.3. Hypothesis

It is hypothesised that low doses of ionising radiation in combination with BEZ235 and Tyrphostin AG-1478 (inhibitors) as a cocktail can significantly sensitise prostate cancer cells to radiotherapy.

1.4. Thesis Objectives

The current study aims to understand the molecular mechanisms involved in the evolution of prostate cancer cells surviving ionising radiation treatment, by identifying and validating the expression of potential therapeutic targets responsible for the survival pathways**:** the PI3K/Akt/mTOR and EGFR signalling pathways. The idea is to sensitise prostate cancer cells to radiation therapy. It is hoped that an improved outcome to radiotherapy will be achieved by concomitant treatment of prostate cells with low doses of ionising radiation and BEZ235 (dual inhibitor of PI3K and mTOR) and Tyrphostin AG-1478 (inhibitor of EGFR), either singly or as a cocktail.

These novel therapies will improve the outcome of radiotherapy by targeting the molecular elements that drive the radiation resistance. It is expected that this approach will make a significant contribution towards the formulation of patient-specific therapeutic cocktails that will revolutionise the treatment of prostate cancer.

The main objectives of this study were to:

1. Determine the intrinsic radiosensitivity of two prostate cell lines**:** DU145 (prostate cancer cells) and 1542N (normal prostate cells).

2. Identify and validate the expression of EGFR and PI3K proteins.
3. Sensitise the prostate cell lines with BEZ235 and AG-1478, individually and in combination, to ideally enhance the effectiveness of radiotherapy.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Lines

1542-NPTX

The benign 1542-NPTX (1542N) human prostate epithelial cell culture was derived from a primary adenocarcinoma of the prostate, and immortalised with E6 and E7 genes of the human papilloma virus 16 (Bright et al, 1997). The cells were grown in Roswell Park Memorial Institute medium (RPMI-1640) (Sigma-Aldrich, Germany) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (HyClone, UK), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Lonza, Belgium). The cell line was provided by Professor JRW Masters (Prostate Cancer Research Centre, University College London, UK).

DU145

DU145 is a human prostate cell line which is derived from a metastatic lesion of the central nervous system (Stone et al, 1978). DU145 cells which have an epithelial-like morphology, were grown in Minimum Essential Medium (MEM) (Sigma-Aldrich, Germany) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (HyClone, UK), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Lonza, Belgium). The cell line was obtained from Professor P Bouic (Synexa Life Sciences, Montague Gardens, South Africa).

2.2. Cell Culture Maintenance

The cells were routinely maintained at 37ºC in a humidified atmosphere (95% air and 5% CO_2) in SHEL LAB incubators (Sheldon Manufacturing Inc, USA) and all cell culture procedures were carried out in vertical laminar flow cabinets using aseptic techniques. Cells were routinely grown in 75 cm^2 flasks, and were passaged when culture confluency was between 80-90%. For cryopreservation, cells were trypsinised, pelleted by centrifugation (4000 RPM for 5 minutes), resuspended in a mixture of 0.9 ml foetal bovine serum and 0.1 ml of dimethyl sulfoxide (DMSO), stored at -80°C overnight, and then transferred into liquid nitrogen for use at a later stage.

2.3. Target Inhibitors

BEZ235 is a dual inhibitor of phosphoinositide-3-kinase (PI3K) and mammalian target of rapamycin (mTOR), with an inhibitory concentration at 50% (IC_{50}) of 7.5 nM for PI3K and 5 nM for mTOR in highly metastatic human prostate tumour cells (Maira et al., 2008; Potiron et al., 2013). BEZ235 has a molecular weight of 469.55 and chemical formula $C_{30}H_{23}N_5O$ (Santa Cruz Biotechnology, Texas, USA), and is soluble in dimethyl sulfoxide (DMSO).

AG-1478 is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor with an IC_{50} of 3 nM in non-small cell lung cancer (Levitzki and Gazit, 1995; Puri and Salgia, 2008). AG-1478 has a molecular weight of 315.8 and chemical formula $C_{16}H_{14}ClN_3O_2$ (Tocris Bioscience, UK), and is soluble in DMSO.

2.4. Irradiation

Cell lines grown in 25 cm^2 tissue culture flasks were irradiated using the ^{60}Co γ-irradiation source at Tygerberg Academic Hospital. Dosimetry was by thermoluminescent dosimetry (TLD-chips). The beam configuration was vertical with a source-to-sample distance (SSD) of 66.5 cm measured to the base of the experimental cell culture flasks. The field size was 30 x 30 cm^2. Build-up consisted of 10 ml of medium in the 25 cm^2 culture flasks, and a 0.5 cm perspex sheet positioned on top of the culture flasks. The backscatter radiation was absorbed by a 5 cm thick perspex sheet and an 8.5 cm thick foamalite slab.

For all assays cell cultures were irradiated at room temperature (22°C) over a dose range of 0-10 Gy at an average dose rate of 0.827 Gy/min (range**:** 0.782 - 0.873 Gy/min).

2.5. Clonogenic Survival Assay

Near-confluent stock cultures were washed with sterile phosphate buffered saline (PBS), trypsinised and counted using a haemocytometer. Cells were seeded in triplicate per experiment in 25 cm^2 culture flasks at varying density from 300 to 100 000 cells per flask, depending on cell type and on the radiation absorbed dose that cells received, and left to settle for 4 – 5 hours. The cell cultures were then exposed to graded doses ranging from 0 - 10 Gy. After an appropriate incubation period (usually 10 - 14 days), the colonies were fixed by decanting the medium in the flask, and replacing it with 10 ml of fixative, consisting of 100 ml glacial acetic acid, 100 ml methanol and 800 ml distilled water, for 10 minutes. The fixative was then decanted

and replaced with 10 ml of Amido Black stain, consisting of 10 ml of 0.01% amido black in 1 litre of fixative. The colonies were left to stain for 10 minutes. The stain was then decanted, and the flasks left to dry. The colonies were counted using a dissection microscope, and corresponding surviving fractions calculated. The means (± SD) of the surviving fractions for the three experiments were plotted against the irradiation dose, and cell survival curves were obtained by fitting the data to the linear-quadratic equation**:**

$$Survival(S) = e^{-(\alpha D + \beta D^2)}$$

where *S* is the surviving fraction, α and β are the linear and quadratic cell inactivation constants, respectively, and *D* is the dose in Gy.

2.6. Radiosensitisation Effect of Inhibitors

Radiosensitisation induced by inhibitors, BEZ235 and AG-1478, added 30 minutes prior to irradiation, was assessed using the clonogenic assay in the DU145 and 1542N cell lines. Cells were seeded in 25 cm^2 tissue culture flasks with an appropriate number of cells (300 to 50 000) per flask depending on radiation dose. After 4 hours of cell attachment, cells were treated with either 18 nM of BEZ235, 15 nM of AG-1478 or a combination of the two inhibitors as a cocktail, before radiation. The flasks were then irradiated at 2 and 6 Gy with ^{60}Co γ-irradiation. After an incubation period of 10 - 14 days, colonies were fixed, stained and scored. Unirradiated cell cultures with and without inhibitor served as controls. Radiosensitisation by each inhibitor was expressed as a radiation enhancement

factor (EF), given by the ratio of surviving fractions with and without inhibitor as follows:

$$REF = \frac{SF(2\ Gy)}{SF\ (2\ Gy + inhibitor)}$$

or

$$REF = \frac{SF(6\ Gy)}{SF\ (6\ Gy + inhibitor)}$$

The criteria for inhibition, no effect, and enhancement of radiosensitivity by inhibitors were *REF* < 1.0, *REF* ≈ 1.0 and *REF* > 1.0, respectively.

2.7. Protein Extraction

Total protein extraction from cells was performed on ice (4°C). Cells were harvested at 0, 6 and 24 hours after treatment with inhibitors and radiation. The medium was decanted, and the treated flasks rinsed with cold PBS. The cells were mechanically harvested by gentle scraping with a cell scraper. Each cell suspension was then transferred to a tube containing growth medium. The cells were pelleted by centrifugation (4000 RPM for 5 minutes), washed and resuspended in cold PBS, followed by a second centrifugation. The pellet was then resuspended in 200 µl TBS/1%Triton X-100 extraction buffer and placed on a tube roller mixer overnight at 4°C. Lysates were clarified by centrifugation (14000 RPM for 15 minutes) to remove

insoluble cellular debris, and the supernatant was collected and stored at -80°C for use at a later stage.

2.8. Determination of Protein Concentration

Protein determination was performed on ice (4°C) by means of the bicinchoninic acid colorimetric assay kit, manufactured by Pierce (Rockford, IL, USA). Briefly, samples were diluted in TBS/1%Triton X-100 buffer (1:2 and 1:5), and 10 µl of the diluted protein placed in a 96-well multiwell plate. Two hundred microliters of working reagent (Reagent A and Reagent B mixed in a 51:1 ratio, to produce a clear light green solution) was added to each well and the plate incubated at 37°C for 30 minutes. A purple colour developed. The reaction was stopped by incubating the multiwell plate at 4°C for 10 minutes before measuring the absorbance values with a Labtech L-4000 microplate reader (Sussex, UK) at a wavelength of 570 nm.

Serial dilutions were made from a 2 mg/ml bovine serum albumin (BSA) stock vial and a standard curve was produced by plotting the average blank-corrected 570 nm measurement for each BSA standard against its concentration in µg/ml. The standard curve was then used to determine the protein concentration of each unknown sample. The working range of the assay was 125 - 2000 µg/ml.

2.9. Preparation of Acrylamide Gels

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine changes in the expression of PI3K, mTOR and EGFR total proteins, including the phosphorylated proteins. This method separates proteins by the

relative distance they migrate across a polyacrylamide gel matrix, based on their molecular weights.

A 10% separating gel and a 5% stacking gel were used for optimum resolution of the PI3K, mTOR and EGFR proteins. Briefly, the separating gel was prepared by mixing volumes of the following components**:** 5.9 ml of distilled water, 5 ml of 30% acrylamide/Bis solution (29**:**1; 3.3%), 3.8 ml of 1.5 M Tris (pH 8.8) and 150 µl of 10% SDS. One hundred and fifty microlitres of freshly prepared 10% ammonium persulfate and 9 µl of tetramethylethylenediamine (TEMED) were added to initiate polymerisation of the gel. The stacking gel was prepared as follows**:** 3.4 ml of distilled water, 83 µl of 30% acrylamide/Bis solution 29**:**1 (3.3%), 63 µl of 1.0 M Tris (pH 6.8) and 50 µl of 10% SDS. Fifty microlitres of freshly prepared 10% ammonium persulfate and 5 µl of TEMED were added to initiate polymerisation.

Samples were boiled at 95°C for 5 minutes in Laemmli sample buffer (Bio-Rad, USA) before loading onto the gel. Equivalent amounts of protein (20 µg) were loaded per lane. Ten microlitres of a precision pre-stained 10 - 250 kDa molecular weight marker (Bio-Rad, USA) was loaded in the first lane to assist with the orientation and size determination of separated proteins.

Proteins were fractioned by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a Mini-PROTEAN® Tetra cell vertical electrophoresis system (Bio-Rad, USA) at a constant voltage of 100 V (AA Hoefer Power Pac PS300-B, USA) until the migration front reached the bottom of the gel.

2.10. Western Blotting and Immunoblot

The cells were assayed for levels of protein expression before and after exposure to ionising radiation, by Western blotting with antibodies specific for EGFR, PI3K and mTOR.

The resolved proteins were electro-transferred onto a polyvinylidene difluoride membrane (PVDF Immun-Blot™) (Bio-Rad, USA) for 2½ hours on ice at a current of 200 mA, using a vertical wet transfer system (Mini- PROTEAN® Tetra cell) (Bio-Rad, USA).

The proteins were transferred from the gel to the membrane in transfer buffer containing glycine (192 mM), Tris (25 mM) and methanol (20%).

2.11. Ponceau S Staining of PVDF Membranes

PVDF membranes were stained with Ponceau S (Sigma, Germany) to expose the protein bands and allow for an assessment of sample loading. Following protein transfer by electroblotting, membranes were soaked in Ponceau S for 60 seconds and rinsed in distilled water, before blocking with Tris buffered saline with Tween 20 (TBST) containing 5% non-fat milk or 5% BSA.

2.12. Immuno-detection

The membranes were blocked in Tris buffered saline with Tween 20 (TBST) containing 5% non-fat milk for 25 – 30 minutes at room temperature, followed by three washes in Tris buffered saline (TBS)/Tween 20 solution, before the primary

antibodies were added and membranes left rotating overnight at 4°C. Incubation in primary antibodies against anti-PI3 kinase p110 beta (PI3K) mouse monoclonal antibody to PI3 kinase p110 beta (Abcam, UK), EGFR (mouse monoclonal antibody) (Santa Cruz Biotechnology, USA), and mammalian target of rapamycin (mTOR) (7C10) rabbit monoclonal antibody (Cell Signaling Inc., USA) was followed by several washes in TBS/Tween 20 solution, before the addition of a secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG horseradish peroxidase antibody) (Santa Cruz Biotechnology, USA), and incubated for 1 hour at room temperature. The membranes were then washed several times in TBS/Tween 20 solution, before protein detection.

2.13. Western Blotting Detection System

Following Western blot transfer, the immobilised proteins were detected using the tetramethylbenzidine membrane peroxidase substrate system (KPL Inc., USA). This is a sensitive colorimetric detection method for the presence of peroxidase conjugates, permitting easy visualisation of blue bands.

2.14. Data Evaluation

Data are presented as the mean ± standard error of the mean (SEM) of three independent experiments as indicated by error bars. Statistical analysis and data fitting were performed by means of GraphPad Prism (GraphPad Software, San Diego, USA). A two-sided Student's t-test was used to compare the means between sample groups, p-values < 0.05 were declared significant.

Chapter 3

Results

3.1. Radiosensitivity

The cellular radiosensitivity was determined using the clonogenic cell survival assay. Cell survival data for the human prostate carcinoma cell line were fitted to the linear-quadratic model and are presented in Figure 3.

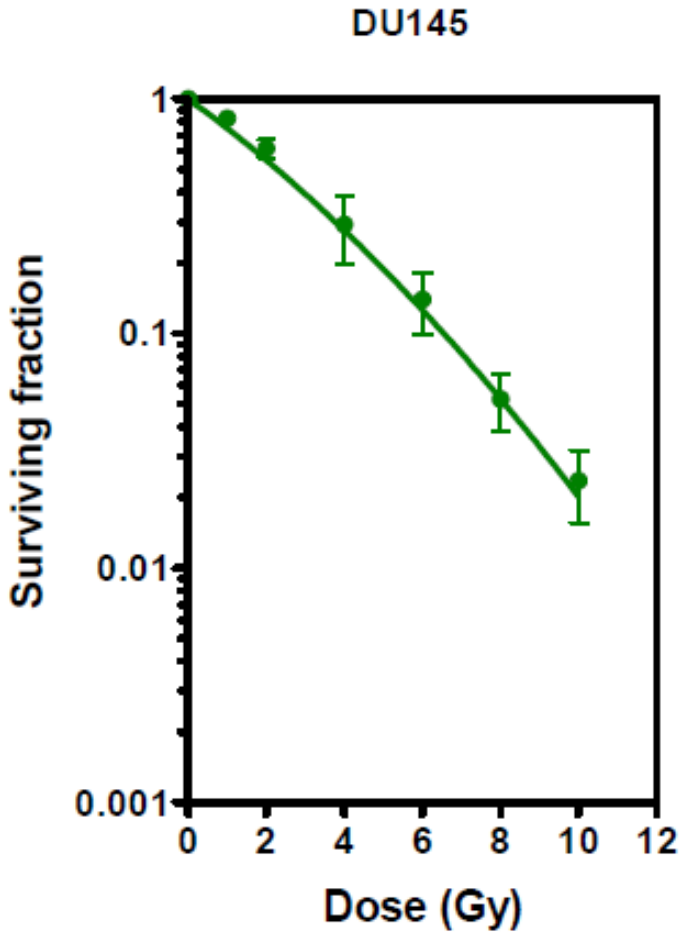

Figure 3: Clonogenic survival curve for the prostate carcinoma cell line (DU145) after ^{60}Co γ-irradiation. Symbols represent mean (± SD) surviving fractions from three independent experiments. The survival curve was obtained by fitting experimental data to the linear-quadratic model.

Similarly, cell survival data for the normal prostate cell line are shown in Figure 4.

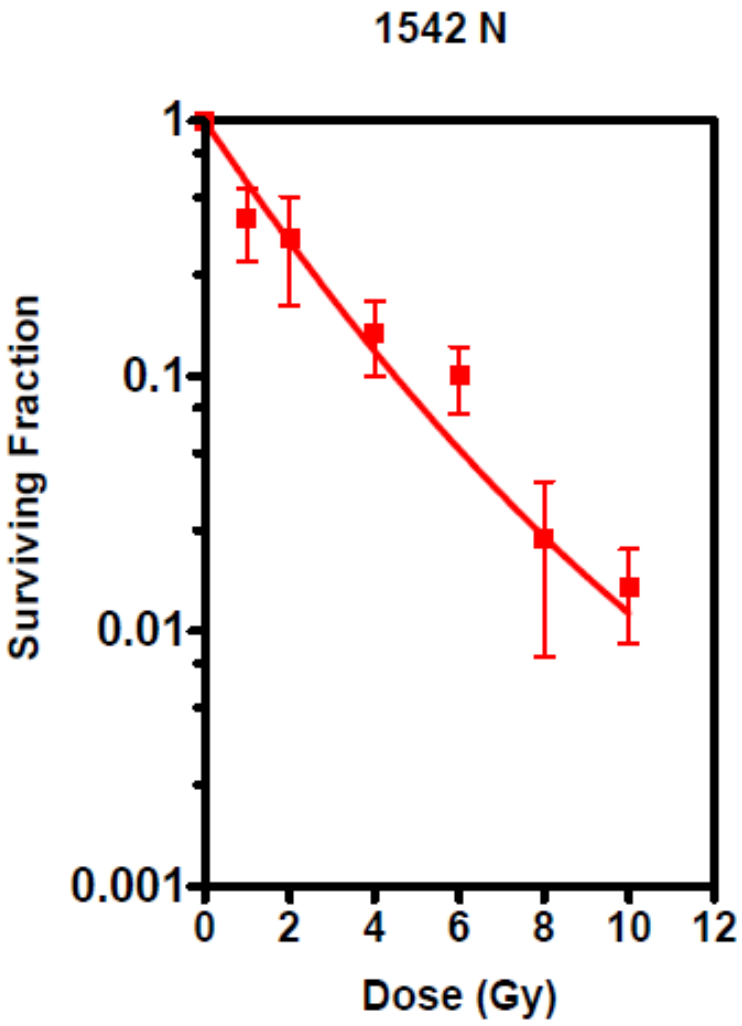

Figure 4: Clonogenic survival curve for the normal prostate cell line (1542N) after ^{60}Co γ-irradiation. Symbols represent mean (± SD) surviving fractions from three independent experiments. The survival curve was obtained by fitting experimental data to the linear-quadratic model.

From the dose-response curves presented in Figures 3 and 4, it is apparent that the normal cell line is more radiosensitive than its malignant counterpart. The survival

curve for 1542N is relatively steeper than that for DU145. The α- and β-coefficients for the two cell lines were found to be 0.57 ± 0.11 Gy^{-1} and 0.01 ± 0.01 Gy^{-2} and 0.28 ± 0.06 Gy^{-1} and 0.01 ± 0.01 Gy^{-2}, respectively. For comparison, intrinsic cellular radiosensitivity was expressed in terms of the surviving fraction at 2 Gy (SF_2). Indeed, the DU145 cell line was found to be more radioresistant than the 1542N cell line. The SF_2-values emerged as 0.53 ± 0.07 and 0.36 ± 0.09 for DU145 and 1542N, respectively, and were not statistically different (*P* = 0.1894).

3.2. Cytotoxicity of Inhibitors

To assess the cytotoxicity of the PI3K, mTOR and EGFR inhibitors, plating efficiencies (PE) of cell cultures were determined in the presence of the inhibitors, either singly or in combination, and compared with those obtained for cells cultured without inhibitors. The PE-values for the DU145 cell line were found to be 0.0229 ± 0.0075 and 0.1210 ± 0.0996 in the absence and presence of BEZ235, respectively, and were not significantly different (*P* = 0.3819). On average, treatment of these cells with 17.5 nM of BEZ235 appeared to improve plating efficiency. The PE-value for AG-1478 emerged as 0.0203 ± 0.0084 and was essentially the same as the PE value without inhibitor (*P* = 0.8292), corresponding to no cell kill. The plating efficiency in DU145 cell cultures treated with a cocktail of the two inhibitors was 0.1130 ± 0.0826, and was not statistically different from that obtained for untreated cell cultures (*P* = 0.9537). For the 1542N cell line, the plating efficiencies were 0.0240 ± 0.0010 and 0.0110 ± 0.0006 in the absence and presence of BEZ235, respectively, and were significantly different (*P* = 0.0004). BEZ235 induced a 54%

cell kill in this cell line. The plating efficiency in AG-1478 treated 1542N cell cultures was 0.0350 ± 0.0020 and was significantly higher than that obtained for untreated cell cultures (*P* = 0.0114). AG-1478 appears to enhance plating efficiency in the normal prostate cell line. The PE-value for 1542N cells treated with a cocktail of two inhibitors was found to be 0.0073 ± 0.0009 and was also significantly lower than the PE value for untreated cell cultures (*P* = 0.0007), giving a 70% cell kill.

3.3. Modulation of Radiosensitivity of DU145 and 1542N at 2 Gy

To assess whether blocking the activities of PI3K, mTOR and EGFR, with specific inhibitors resulted in changes in cellular radiosensitivity, cell cultures were treated with BEZ235 (against PI3K and mTOR) and AG-1478 (against EGFR), and subsequently irradiated with 2 Gy. The cell survival data for DU145 are shown in Figure 5. Treatment with BEZ235 alone sensitised the cells, reducing SF_2 from 0.58 ± 0.12 to 0.39 ± 0.08, although the sensitisation was not statistically significant (*P* = 0.2432). A higher radiosensitisation was observed when cells were pre-treated with AG-1478, with SF_2 decreasing from 0.58 ± 0.12 to 0.32 ± 0.07 (*P* = 0.1274). The levels of radiosensitisation that was individually induced by the two agents were not significantly different (*P* = 0.5522). When the cells were treated with a cocktail of the two inhibitors BEZ235 and AG-1478, the surviving fraction at 2 Gy emerged as 0.35 ± 0.05 and was not significantly different from those obtained for single agent treatment (*P* = 0.1449 and *P* = 0.7342, respectively).

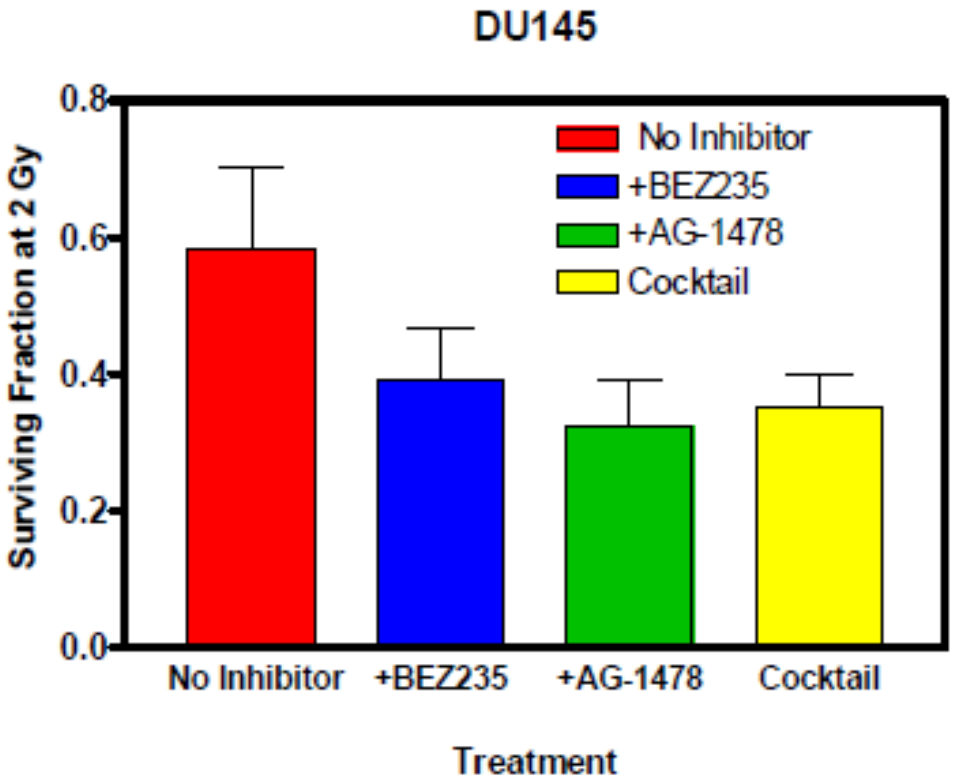

Figure 5: Clonogenic cell survival at 2 Gy of ^{60}Co γ-irradiation for a human prostate carcinoma cell line (DU145) following single or combined (cocktail) treatment with PI3K and mTOR inhibitor (BEZ235) and EGFR inhibitor (AG-1478).

The corresponding data for the normal cell line are presented in Figure 6. Interestingly, pre-treatment of 1542N cells with BEZ235 resulted in a high level of radioprotection, with the surviving fraction at 2 Gy increasing from 0.36 ± 0.06 to 0.69 ± 0.11 (P = 0.0509). On the other hand, when cells were treated with the EGFR inhibitor alone, SF_2 did not differ markedly from that for untreated cell cultures. The corresponding SF_2 was found to be 0.29 ± 0.06. Cellular treatment with a BEZ235/AG-1478 cocktail appeared to have no effect on the radiosensitivity of the 1542N cells, giving a surviving fraction at 2 Gy of 0.32 ± 0.03.

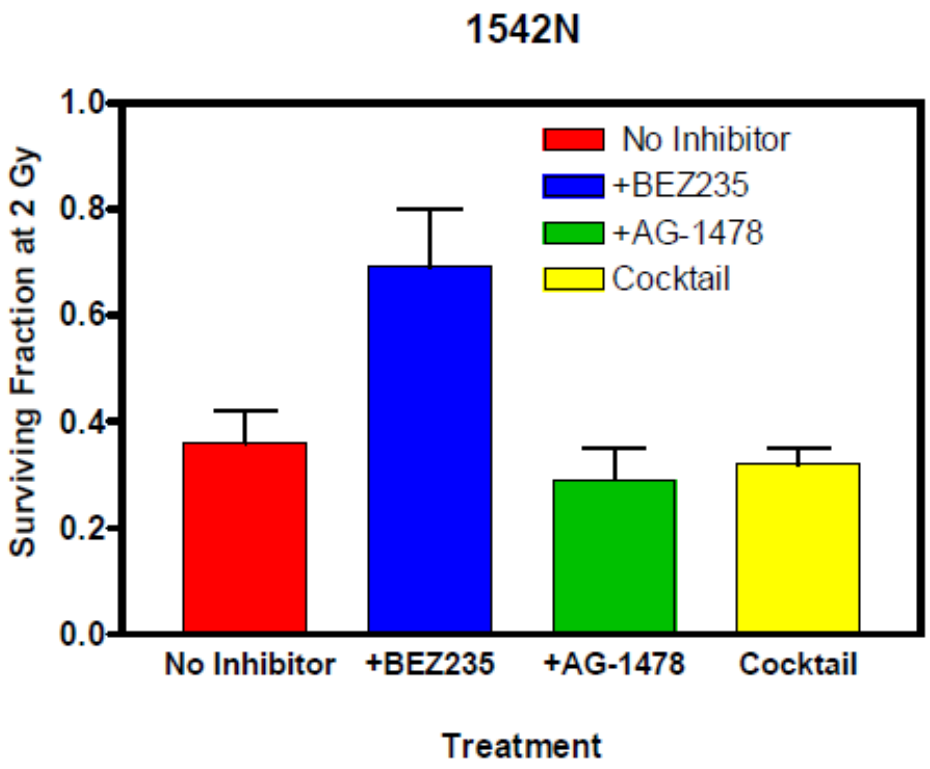

Figure 6: Clonogenic cell survival at 2 Gy of ^{60}Co γ-irradiation for a normal human prostate cell line (1542N) following single or combined (cocktail) treatment with PI3K and mTOR inhibitor (BEZ235) and EGFR inhibitor (AG-1478).

3.4. Modulation of Radiosensitivity of DU145 at 6 Gy

To investigate whether the apparent radiosensitisation seen in the prostate carcinoma cell line (DU145) exists at higher fractional doses as may be encountered in stereotactic radiotherapy, the effect of blocking the activities of PI3K, mTOR and EGFR and irradiating cell cultures to 6 Gy was assessed, and the cell survival data are presented in Figure 7. The reduction in cell survival due to pre-treatment with BEZ235, AG-1478, or the cocktail of the two inhibitors, was marginal, with the

surviving fraction at 6 Gy decreasing from 0.17 ± 0.09 to 0.10 ± 0.04 (*P* = 0.5509), 0.11 ± 0.02 (*P* = 0.5501), and 0.06 ± 0.02 (*P* = 0.3685), respectively.

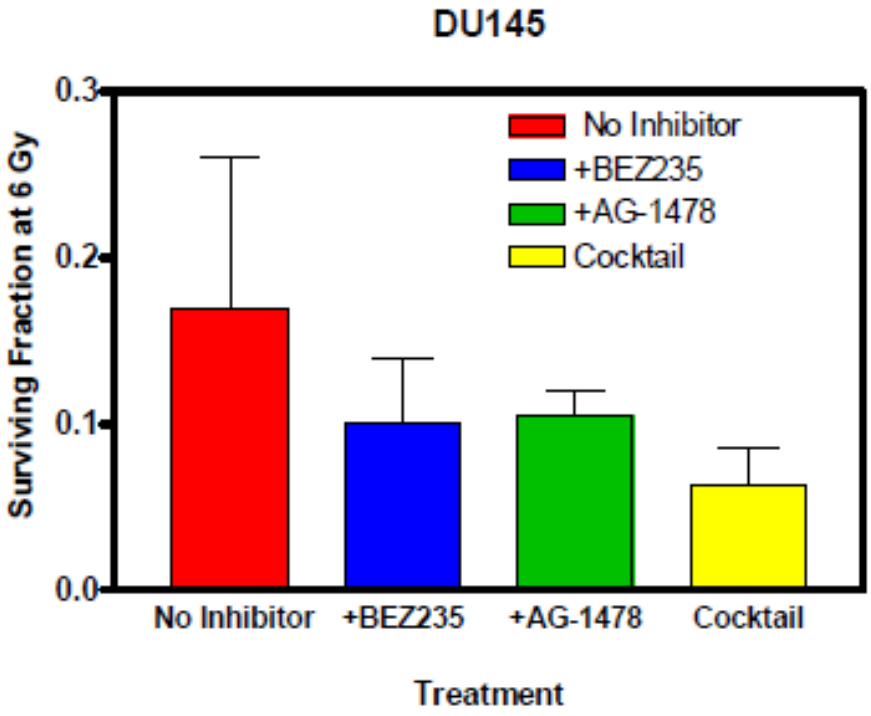

Figure 7: Clonogenic cell survival at 6 Gy of ^{60}Co γ-irradiation for a human prostate carcinoma cell line (DU145) following single or combined (cocktail) treatment with PI3K and mTOR inhibitor (BEZ235) and EGFR inhibitor (AG-1478).

3.5. Evaluation of Effect of Inhibitors on Radiotoxicity

To further evaluate the effect of inhibitors on radiation-induced cell kill, radiation enhancement factors were calculated for each cell line and treatment scenario from the data presented in Figures 5 - 7. The radiotoxicity enhancement data for the DU145 cell line are summarised in Table 1. On average, treatment with AG-1478

showed a higher level of radiation enhancement than BEZ235 at 2 Gy. The reverse was observed at 6 Gy where the effect of BEZ235 was higher. Treatment with both agents did not seem to yield an added effect at 2 Gy relative to single agent exposure. However, cocktail treatment at 6 Gy resulted in an over 2-fold radiosensitisation.

Table 1: Radiation enhancement data at 2 and 6 Gy for DU145 cells treated with BEZ235 and AG-1478.

Treatment	SF_2	REF*
2 Gy (no inhibitor)	0.58 ± 0.12	
2 Gy + BEZ235	0.39 ± 0.08	1.49 ± 0.43
2 Gy + AG-1478	0.32 ± 0.07	1.81 ± 0.55
2 Gy + Cocktail	0.35 ± 0.05	1.66 ± 0.42
6 Gy (no inhibitor)	0.17 ± 0.09	
6 Gy + BEZ235	0.10 ± 0.04	1.70 ± 1.13
6 Gy + AG-1478	0.11 ± 0.02	1.55 ± 0.88
6 Gy + Cocktail	0.06 ± 0.02	2.83 ± 1.77

*Errors calculated using an appropriate error propagation formula.

The radiotoxicity enhancement data for the normal prostate cell line (1542N) are presented in Table 2. In this cell line, treatment with BEZ235 significantly protected cells against a radiation absorbed dose of 2 Gy. In contrast, AG-1478 treatment

yielded a radiation enhancement factor of 1.24. When cells were treated with a cocktail of the two inhibitors, no effect was apparent.

Table 2: Radiation enhancement data at 2 Gy for 1542N cells treated with BEZ235 and AG-1478.

Treatment	SF_2	REF*
2 Gy (no inhibitor)	0.36 ± 0.06	
2 Gy + BEZ235	0.69 ± 0.11	0.52 ± 0.12
2 Gy + AG-1478	0.29 ± 0.06	1.24 ± 0.33
2 Gy + Cocktail	0.32 ± 0.03	1.13 ± 0.22

*Errors calculated using an appropriate error propagation formula.

3.6. Western Blot Analysis

Ponceau Stain

Figure 8 shows a representative image of a PVDF membrane that has been Ponceau stained after it has been through protein transfer. The Ponceau stain is a general stain used to determine if the transfer of proteins has taken place. Bands representing the standard protein ladder and samples are illustrated with arrows to indicate the relative positions of the target proteins**:** EGFR, mTOR, and PI3K.

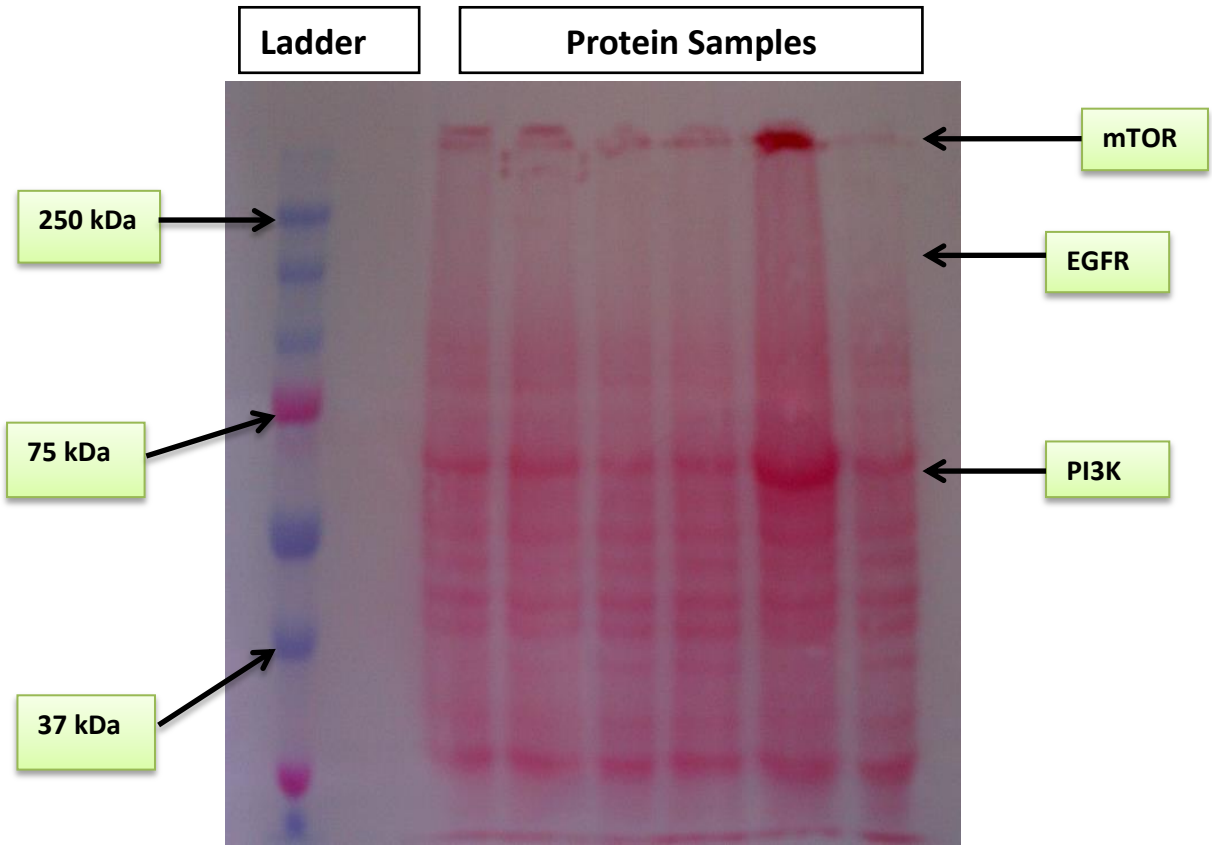

Figure 8: A representative image of a PVDF membrane that has been Ponceau stained after the transfer of proteins.

Immuno-Blots

Figure 9 is a representative image of a PVDF membrane developed from irradiated prostate cell cultures. Immuno-detection for total protein was used to identify EGFR, PI3K and mTOR. All proteins were expressed. Neither radiation dose nor incubation

time of the order of 6 hours had an impact on the expression levels of the target proteins.

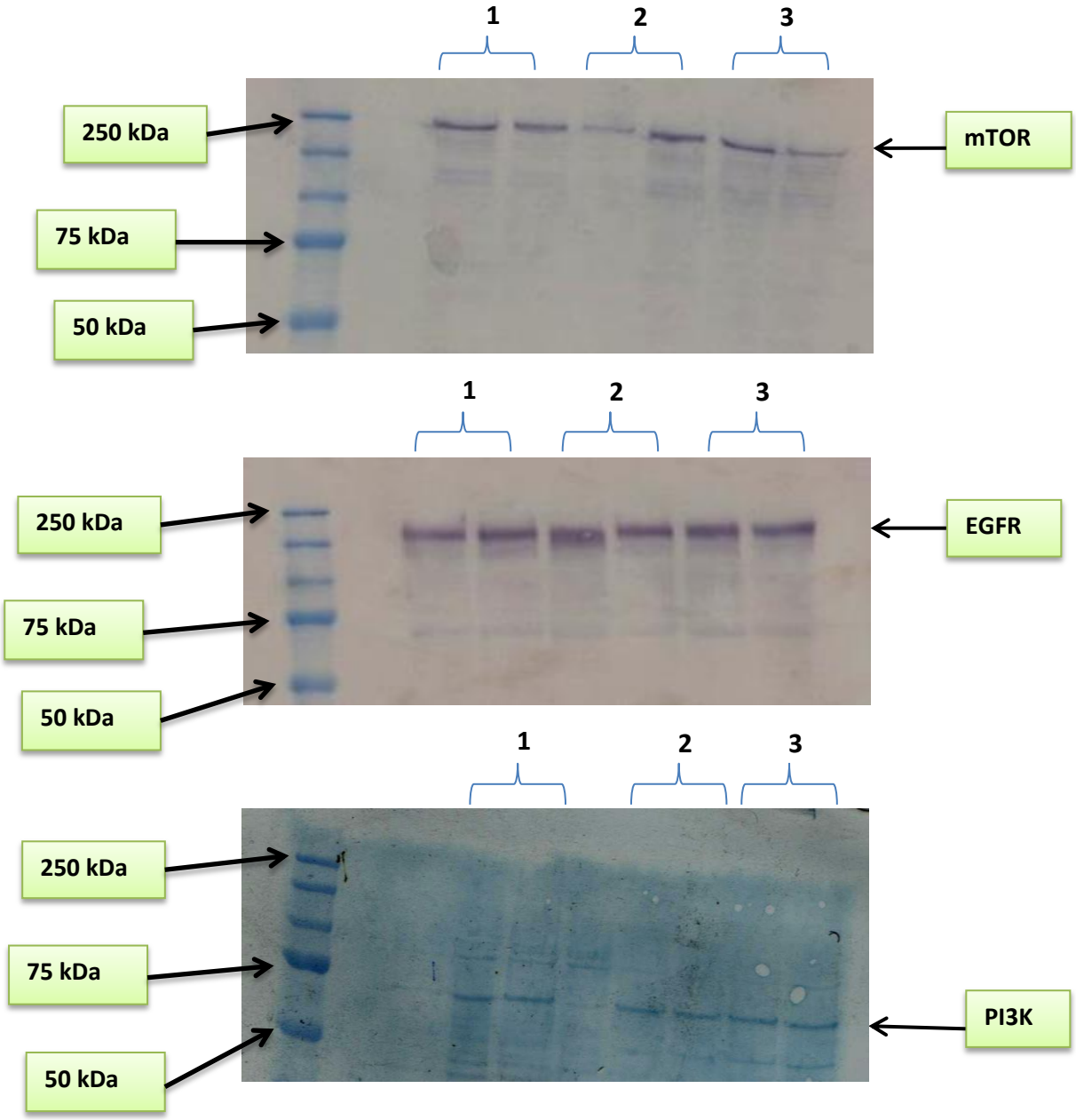

Figure 9: Representative immuno-blots of mTOR (*top panel*), EGFR (*middle panel*), and PI3K (*bottom panel*) expression. Protein samples were loaded in duplicate: Lanes 1 (unirradiated control); Lanes 2 (2 Gy with no incubation); and Lanes 3 (2 Gy with a 6-hour incubation).

CHAPTER 4

DISCUSSION

4.1. Cellular Resistance to Radiotherapy

Radiation therapy plays an important role in prostate cancer treatment (Skvortsova et al., 2008). However, the radioresistance of prostate cancer cells limits the outcome of radiotherapy (An et al., 2006). It is assumed that the surviving and repopulating cancer cells are capable of providing molecular protection against the cytotoxic effects of radiation therapy. This research aimed to investigate radio-enhancement for a better therapeutic outcome.

No therapy has yet demonstrated a clear superiority in terms of long-term survival (Lee et al., 2008; Kuban et al., 2008), the major clinical problem being the resistance observed in prostate cancer patients (Skvortsova et al., 2008). With the current standard protocol of radiation therapy, local and metastatic relapse remain frequent in high-risk patients. One well-recognised cause is the relative resistance of cells to radiation therapy (Potiron et al., 2013; Skvortsova et al., 2008). Knowledge of the radiosensitivity of prostate tumours could provide a rational basis for adjuvant radiotherapy approaches, and help optimise prostate cancer management. To address this need, a prostate cancer cell line (DU145) and a normal prostate cell line (1542N) were used in this study.

Figures 3 and 4 show survival curves for the DU145 and 1542N cell lines, respectively. The 1542N curve is relatively steeper than that of DU145, suggesting that the 1542N cell line is more radiosensitive than the malignant DU145 cell line. Similar results were previously reported for the 1542N cell line (Serafin et al., 2003).

4.2. Simultaneous Inhibition of EGFR and PI3K Pathways Enhance Radiosensitivity of Prostate Cancer Cells

Radiation therapy is an integral part of prostate cancer management, especially in combination with other cytotoxic agents (Galper et al., 2000; Mokbel, 2003). A significant reduction in incidences of local recurrence in prostate malignancies after therapy has been reported. However, subsets of patients are resistant to radiotherapy, due to mutations in the PI3K/mTOR survival pathway, and the overexpression of EGFR and other proteins involved cell survival pathways (Choi et al., 2010; Rikova et al., 2007). This severely limits the effects of radiotherapy. An appealing approach to overcoming this problem is combined molecular targeted therapy to sensitise tumours to radiotherapy. This approach has led to improved treatment outcomes in patients with advanced solid tumours, such as head and neck (Bonner et al., 2006).

This study explored whether the dual inhibitor of PI3K and mTOR, BEZ235, and AG-1478 which is an EGFR inhibitor, could sensitise human prostate cancer cells to ionising radiation. Furthermore, the study assessed whether a cocktail of the two inhibitors would yield a greater radiosensitisation in cells, in comparison to a single inhibitor. The effects of the inhibitors combined with radiation were assessed at a cellular level and clonogenic survival was measured. At the molecular level, expression of EGFR, PI3K and mTOR was evaluated using Western blot analysis.

Multiple features of tumour cells are involved in shaping their intrinsic and acquired radioresistance (Baumann et al., 2007). As mentioned earlier, the EGFR and PI3K

pathways have been implicated in disease progression and survival of prostate cancer cells after radiation exposure, resulting in resistance to radiotherapy. The pathways have also been shown to constitute one of many regulators of prostate cancer radiosensitivity (Bitting and Armstrong, 2013; Carracedo et al., 2008; Carracedo and Pandolfi, 2008; Liu et al., 2009).

The overexpression of EGFR activated by homodimer or heterodimer combinations, with other ErbB family members, was associated with poor prostate tumour response to radiotherapy (Baumann et al., 2007). Therefore, inhibition of EGFR signalling may enhance the radiosensitivity of cancer cells. It has been shown that inhibition of EGFR enhances the radiosensitivity of various tumours in preclinical and clinical studies (Magné et al., 2008). This is in agreement with the results presented here. Pre-treatment of DU145 cells with the EGFR inhibitor, AG-1478, reduced the surviving fractions at 2 and 6 Gy, giving average radiation enhancement factors of 1.81 and 1.55, respectively (Figures 5 and 7; Table 1). However, the resulting radiosensitisation did not reach statistical significance. The similarity in radiation enhancement at the two doses suggests that radiosensitisation of DU145 cells via inhibition of EGFR is likely independent of radiation dose, and that AG-1478 could potentially be used as a radiosensitiser in conventional, as well as, stereotactic fractionated radiotherapy. In contrast, it has been demonstrated elsewhere for head and neck, lung, and bladder tumour cell lines that BEZ235 exhibits greater enhancement at higher radiation doses (Konstantinidou et al., 2009; Fokas et al., 2012). The radiosensitisation observed in the 1542N cell line at 2 Gy is much less than that seen in the malignant cell line (Figure 6; Table 2), indicating that an

improved therapeutic benefit may be achieved with less extensive normal tissue toxicity when AG-1478 is used as an adjuvant to radiotherapy.

In addition, inhibition of the PI3K/Akt/mTOR pathway has been shown to enhance the radiosensitivity of various cancer cell types, including those from prostate origin (Toulany et al., 2006). In the current study, pre-treatment of DU145 cells with BEZ235, a dual inhibitor of PI3K and mTOR, was found to enhance the effect of radiation by levels similar to those produced by the EGFR inhibitor, but these were also not significantly different (Figures 5 and 7; Table 1). These data suggest that targeting the EGFR and PI3K/Akt/mTOR pathways singly has the same effect on the radiation response of the malignant cell line, regardless of radiation dose.

Interestingly, a significant radio-protective effect emerged when the 1542N normal prostate cell line was pre-treated with BEZ235 (Figure 6; Table 2). In fact, inhibiting PI3K and mTOR with BEZ235 almost doubled clonogenic survival in 1542N. Similar radio-protective properties of BEZ235 have been demonstrated in normal gut tissue (Potiron et al., 2013). The radiosensitisation seen in the DU145 may be attributable to the observation that BEZ235 inhibits double-strand-break repair in tumour cells and likely contributes to mitotic catastrophe (Wouters and Koritzinsky, 2008). This inhibition of DNA repair by BEZ235 should also enhance radiotoxicity in normal tissue, but no such enhancement was found in the gut (Potiron et al., 2013). Targeting the PI3K/mTOR pathway has anti-clonogenic effects in tumour cells (Potiron et al., 2013; Maira et al., 2008) and pro-clonogenic effects in normal cells (Martin et al., 2011), and should result in improved radiotherapy outcomes. The

radio-protection demonstrated here for BEZ235 in the normal prostate cell line, if validated, could have significant implications in prostate cancer management.

It is important to focus on the upstream target, EGFR, and the downstream targets, PI3K and mTOR, since inhibition of only one target upstream or downstream may not fully counteract the resistance of cells to irradiation. Co-targeting EGFR, PI3K and mTOR may, theoretically, maximise the radiosensitivity of cancer cells. The rationale or the desired expectation for this combination therapy approach is to target sub-populations of tumour cells that may be resistant to one or other cytotoxic agent, and, furthermore, to target all cells on a cell-by-cell basis at the same time, reducing normal tissue toxicity (Lee et al., 2008). Pre-treatment with a cocktail of AG-1478 and BEZ235 did not seem to be more superior at radiosensitising DU145 cells at 2 Gy than if cells were exposed to each agent individually (Figures 5 and 7; Table 1). However, at 6 Gy, the cocktail yielded a 3-fold radiosensitisation in these cells although no statistical significance was observed, suggesting that combined use of AG-1478 and BEZ235 in radiotherapy modalities that employ relative large fractional doses might be beneficial. More so, if it is demonstrated that the effect of the cocktail in normal cells is as marginal at higher radiation doses as found in the 1542N cell line at 2 Gy (Figure 6, Table 2).

It is worth noting that while AG-1478 and BEZ235 appeared to radiosensitise the malignant cell line, they seem to be non-toxic and growth promoting, respectively, when administered alone. These findings suggest that interference with the EGFR and PI3K/Akt/mTOR pathways without concomitant use of radiotherapy might not have a therapeutic benefit. In addition, the significant cytotoxicity seen in the normal

cell line for both pathway inhibitors in the absence of radiation requires further evaluation as it is highly desirable to keep normal tissue toxicity to the minimum.

4.3. Target Expression in Prostate Cells

To demonstrate capacity to detect expression of the pathway targets, Western blot analyses were performed on the cell lines. The representative immuno-blots for total protein in Figure 9 show that mTOR, EGFR, and PI3K are indeed expressed by DU145 and 1542N. Prostate carcinoma cells express EGFR, PI3K and mTOR under *in vitro* and *in vivo* conditions (Di Lorenzo et al., 2002, Baselga, 2002, Hynes and MacDonald, 2009). Expression of these proteins have been evaluated for their potential as prognostic indicators of disease progression (Di Lorenzo et al., 2002). The significant level of EGFR expression demonstrated in Figure 9 is consistent with those observed in patients treated with radical surgery, hormone therapy, as well as in patients with metastatic and hormone-refractory disease (Di Lorenzo et al., 2002). The finding that EGFR, PI3K and mTOR are expressed in both the malignant (DU145) and normal (1542N) cell lines, makes the cell lines suitable for evaluating how perturbation of the EGFR/PI3K/mTOR pathway may affect the radiation response of tumours and normal tissue. More extensive studies on the roles of these proteins in the radiation response of malignant and normal prostate cell lines could lead to more effective targeted therapeutic approaches.

4.4. Possible Future Avenues for Research

1. It would be of interest to validate the apparent radiosensitisation of the cancer cell line, using lower concentrations of the target inhibitors. This may provide useful information on the optimum inhibitor doses that can be used without inducing undue normal tissue toxicity;

2. Further molecular evaluation of the activity status of the targets by detection and measurement of the corresponding phosphorylated entities would shed more light on the signalling mechanisms underlying the radiation responses;

3. Studies seeking to understand why blocking PI3K and mTOR with BEZ235 leads to radiation protection in normal cells would have significant benefit not only in radiotherapy, but also in radiation protection.

4. The lack of statistical significance in the current results opens a door for a more extensive study to explore whether the findings are coincidental. This can be addressed by using varying concentrations of the inhibitors. If statistical significance is achieved, the finding might have a huge impact on the future management of prostate cancer.

CHAPTER 5

CONCLUSION

In the current study, it has been shown that treatment of a human prostate cancer cell line (DU145) with inhibitors of EGFR, PI3K, and mTOR, singly or in combination, at low and relatively high radiation doses resulted in a measurable but significant radiosensitisation. In the normal prostate cell line (1542N), blocking PI3K and mTOR had an appreciable level of radio-protection, while inhibition of EFGR had no effect on radiosensitivity. These data indicate that inhibition of the EGFR and PI3K/Akt/mTOR pathways may be a potential mode of reducing clonogenic cell survival in prostate cancer after radiation. In conclusion, PI3K and mTOR play an important role in EGFR/PI3K/mTOR-mediated radioresistance. This finding supports the molecular-target approach for sensitising prostate cancer cells to radiotherapy with novel inhibitors that are specific for cell survival pathways. These novel therapies will improve the outcome of radiotherapy by targeting the molecular elements that drive cellular resistance. It is expected that this approach will make a significant contribution towards the formulation of patient-specific therapeutic cocktails that will revolutionise the treatment of prostate cancer.

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