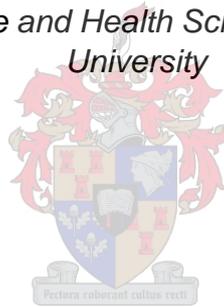


**RADIOSENSITISATION OF ANDROGEN-DEPENDENT AND INDEPENDENT
TUMOUR CELLS AS A THERAPEUTIC STRATEGY IN PROSTATE CANCER**

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*Thesis presented in partial fulfilment of the requirements for
the degree of Doctor of Philosophy in Radiobiology in the
Faculty of Medicine and Health Sciences at Stellenbosch
University*



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December 2019

Declaration

I, Sechaba Maleka 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.

Sechaba Maleka

December 2019

Signature

Date

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Abstract

Metastatic prostate cancer continues to be a leading cause of cancer-related death in men. Increased incidences and mortality have been reported globally, although treatment of locally confined prostate cancer has been shown to be successful. However, aggressive and incurable castration-resistant prostate cancer (CRPC) is a major clinical concern.

The combination of radiotherapy and androgen deprivation therapy (ADT) is the current standard-of-care treatment strategy for prostate cancer (PCa). The androgen-dependent stage of tumour is successfully managed, until the cancer switches to androgen-independence when resistance to treatment severe. The mechanism underlying this switch is still not clear and poorly understood. However, the implicated survival pathways of PI3K/mTOR, EGFR and AR might help explain remissions of PCa after treatment. Thus, the rationale for this study was to target these pathways with respective inhibitors, namely, MDV3100 (for AR), AG-1478 (for EGFR), and NVP-BEZ235 (for PI3K and mTOR).

The “traditional” prostate cancer cell lines, (DU145 and LNCaP), which are derived from metastatic regions, and (1542N and BPH-1) from normal tissue and a primary benign tumour, respectively, served as biological models in this research. The following were investigated: (1) androgen sensitivity of cell lines, (2) the intrinsic cellular radiosensitivity, (3) the cytotoxicity of specific inhibitors of AR, EGFR, PI3K and mTOR, (4) interaction of the respective inhibitors, and (5) the radiomodulatory effects of inhibitors, either singly or in combination.

The “classical” androgen-independent cell lines were found to switch into androgen-dependence when treated with high concentrations of 5 α -DHT.

Very strong synergistic interactions of inhibitors were demonstrated in all cell lines, except the LNCaP cell line in which inhibitors were antagonistic. Concomitant use of these inhibitors in intrinsically androgen-dependent prostate cancers might not be beneficial. The use of inhibitor cocktails with radiation at low doses (2 Gy) is highly desirable as the normal cells were protected, especially with the dual inhibitor of PI3K/mTOR (NVP-BEZ235). However, at higher doses (6 Gy) the potential benefit is great in tumour cell lines, but very limited in the normal cell line. Therefore, at fractional doses of relevance to conventional radiotherapy, use of cocktails containing the PI3K/mTOR inhibitor as an adjuvant may be beneficial in the management of androgen-dependent cancer.

It is concluded that these findings might assist in the design of more effective treatment approaches for cancers that typically display resistance to radiotherapy and chemotherapy.

Opsomming

Metastatiese prostaatkanker (PCa) bly steeds een van die leidende oorsake van kankerverwante sterfte in mans. 'n Toename in die insidensie en mortaliteit is wêreldwyd gerapporteer, alhoewel behandeling van prostaatkanker wat lokaal beperk is, bewys is om suksesvol te wees. Daarenteen bly aggressiewe en ongeneeslike kastrasieweerstandige prostaatkanker (KWPK) 'n ernstige kliniese probleem.

Die kombinasie van radioterapie en androgeenontnemingsterapie (AOT) is die huidige standaardbehandelingstrategie vir PCa. Die androgeenafhanklike stadium van die tumor word suksesvol beheer, totdat die kanker oorgaan na androgeenonafhanklikheid, wat ernstige weerstand teen behandeling beteken. Die onderliggende meganisme, wat hierdie omskakeling bewerkstellig, is steeds nog onduidelik en onverklaarbaar. Die paaie van PI3K/mTOR, EGFR en AR mag van waarde wees om remissies van PCa na behandeling te verklaar. Teen hierdie agtergrond is die doelwit van hierdie studie om hierdie paaie te teken met die onderskeie inhibitoare, naamlik; MDV3100 (vir AR), AG-1478 (vir EGFR) en NVP-BEZ235 (vir PI3K/mTOR).

Die tradisionele prostaatkankersellyne (DU145 en LNCaP), verkry vanaf metastatiese streke, asook (1542N en BPH-1), verkry vanaf normale weefsels en 'n primêre benigne tumor respektiewelik, het gedien as biologiese modelle in hierdie navorsing. Die volgende aspekte is ondersoek: (1) Androgeensensitiwiteit van sellyne; (2) Die intrinsieke radiosensitiwiteit van die selle; (3) Die sitotoksiteit van spesifieke

inhibitore van AR, EGFR, PI3K en mTOR; (4) Interaksie van die onderskeie inhibitore;
(5) Die radiomodulerende uitwerking van inhibitore, afsonderlik of gekombineerd.

Dit is gevind dat die “klassieke” androgeen- onafhanklike sellyne omgeskakel het na androgeenafhankheid wanneer dit behandel is met hoë konsentrasies van 5 α -dihidrottestosteron (5 α -DHT).

Merkwaardige sinergistiese interaksies tussen inhibitore is gedemonstreer in alle sellyne, behalwe die LNCaP-sellyn waar die inhibitore antagonisties was. Gekombineerde gebruik van hierdie inhibitore in intrinsieke androgeenafhanklike prostaatkankers mag nie voordelig wees nie. Die gebruik van inhibitormengsels saam met bestraling teen 'n lae dosis (2Gy) is hoogs wenslik, omdat die normale selle beskerm is, veral met die dubbele inhibitor van PI3K/mTOR (NVP-BEZ235). Daarenteen is die potensiël voordeel van 'n hoë dosis (6Gy) groot in tumorsellyne, maar baie beperk teen die normale sellyne. Gevolglik, teen fraksionele dosisse van betekenis by konvensionele radioterapie, mag die aanwending van 'n kombinasie van die PI3K/mTOR – inhibitor as adjuvant voordelig wees by die behandeling van androgeenafhanklike kanker.

Die gevogtrekking word gemaak dat hierdie bevindinge kan bydra tot die ontwerp van meer doeltreffende behandelingstrategieë teen kankers wat tipies weerstandig is teen radioterapie en chemoterapie.

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Opinions expressed and the conclusions arrived at in this dissertation are those of the author, and are not necessarily to be attributed to the Faculty of Medicine and Health Sciences (Stellenbosch University), the NRF or the Harry Crossley Foundation.

Dedication

Being the 1st Ph.D in the Maleka and Mohapi Family – this is for you,

To inspire the current and generations to come

By leaving a roadmap to academia.

*To the Journey of becoming a Clinician –Scientist. In answering my calling as I
prepare for the journey ahead. Medical Science has been a great journey, now am
heading to my calling – Medicine.*

Sechaba-Thabiso Maleka - Mohapi

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LIST OF ABBREVIATIONS

5 α -DHT:	5 α -dihydrotestosterone
α :	linear coefficient of cell inactivation
ADT:	androgen deprivation therapy
Akt:	serine-threonine protein kinase
AR:	androgen receptor
β :	quadratic coefficient of cell inactivation
BSA:	bovine serum albumin
CO ₂ :	carbon dioxide
CI:	combination index
CRPC:	castration-resistant prostate cancer
csFBS:	charcoal-stripped foetal bovine serum
\bar{D} :	mean inactivation dose
DMSO:	dimethyl sulfoxide
EGFR:	epidermal growth factor receptor
EC ₅₀ :	inhibitor concentration for 50% inhibition
EBRT:	external-beam radiation therapy
FBS:	foetal bovine serum
Gy:	gray
HRPC:	hormone refractory prostate cancer
HPV-16:	human papillomavirus serotype 16
MAPK:	mitogen-activated protein kinase
MEM:	minimum essential medium
MF:	modifying factor
mTOR:	mammalian target of rapamycin
OD:	optical density
PCa:	prostate cancer

PBS:	phosphate buffered saline
PI3K:	phosphatidylinositol 3-kinase
RPMI:	Roswell Park Memorial Institute
SF ₂ :	surviving fraction at 2 Gy
SF ₆ :	surviving fraction at 6 Gy

CHAPTER 1: INTRODUCTION

1.1. Introduction

Prostate cancer (PCa) remains one of the commonly diagnosed cancers, especially in men over the age of 40 years, with an increasing incidence and mortality globally (Taitt, 2018; Bray et al., 2018). Several treatment options are available for localised and early stage PCa, however, ongoing research is needed to improve the clinical outcome of metastasised and resistant PCa (Heidenreich et al., 2011; Gómez-Millán et al., 2015; Litwin and Tan, 2017). Understanding the biology, genetic basis, and risk stratification of the prostate, holistically, before treatment, is very advantageous in the management of PCa.

Radical prostatectomy, external-beam radiation therapy (EBRT) and brachytherapy are the gold standards in the treatment of localised PCa and have proven to be beneficial for post-operative local control. However, radioresistance remains the main clinical challenge, which finally leads to tumour recurrences (An et al., 2007; Heidenreich et al., 2011; Heidenreich et al., 2014; Chang et al., 2015; Ost et al., 2016). As the disease progresses and the genotype changes, treatment strategies need to be altered to address the problem. Chemotherapeutics, such as enzalutamide, abiraterone and prednisone have yielded positive outcomes but for the toxicity to normal tissue (An et al., 2007; Kuban et al., 2008; Wei et al., 2017; Taneja, 2018). However, normal tissue can be spared while therapy is administered by dose alterations, and by employing radiation and chemotherapy combination strategies (Blagosklonny et al., 2002; Maleka et al., 2015). This has shown great results in addressing the side effects of high doses and toxicity of chemotherapy to the patients (Akudugu et al., 2008; Hamunyela et al., 2017; Wei et al., 2017).

Maleka and colleagues demonstrated that NVP-BEZ235 results in better cancer control than traditional radiotherapy (Maleka et al., 2019). Advanced research has resulted in a wealth of knowledge to address the limitations of conventional treatment, and said limitations can be addressed with non-conventional therapy, such as immunotherapy and targeted therapy.

Once the biology of PCa is appreciated and fully understood, treatment can be planned with the goal being (1) to protect normal tissue and organs surrounding the prostate, such as bladder, rectum, testicles, and (2) destroying the cancerous cells within the prostate gland, in the case of localised disease. Knowledge of the mechanics of disease targeted-therapy can be combined with conventional treatment to achieve long term patient survival.

Targeted therapy looks at the critical pathways involved in the initiation and progression of PCa (LoPiccolo et al., 2008; Hurvitz et al., 2013; Kratochwil et al., 2016). The androgen receptor (AR), at the epicentre of PCa research, paradoxically remains neglected and is involved in crosstalk between pathways (Sadar, 1999; Migliaccio et al., 2006). Since the disease is driven by hormones, it should be controlled by hormones, for example, androgen deprivation therapy (ADT), but resistance limits therapeutic advantage (Lai et al., 2013; Caffo et al., 2015). Capitalising on the crosstalk between pathways by targeting the PI3K/mTOR/EGFR pathway might assist in solving the problem of PCa resistance to hormonal therapy (Di Lorenzo et al., 2003; Wang et al., 2008; Carracedo and Pandolfi, 2008; Carracedo et al., 2008; Hurvitz et al., 2013). The dual inhibitor of PI3K and mTOR, NVP-BEZ235, has been studied in breast, head and neck, and colon cancers

(Brachmann et al., 2009; Fokas et al., 2012; Chen et al., 2018). Our previous investigations have shown that effects of NVP-BEZ235 may be novel in prostate tumours (Maleka et al., 2015).

Cellular exposure to ionising radiation has been proven to activate the epidermal growth factor receptor, EGFR, and induce phosphoinositide 3-kinase (PI3K), serine-threonine protein kinase (Akt) and mammalian target of rapamycin (mTOR) activity downstream of the EGFR signalling pathway (Li et al., 2005; Valerie et al., 2007; Lee et al., 2008; Sarker et al., 2009; Palacios et al., 2013). Cell survival is promoted by this pathway which can also lead to treatment resistance.

In designing novel therapeutic interventions for the treatment or management of PCa, molecular mechanisms that are responsible for the development and progression of the disease need to be understood. Specifically, the transition of prostate tumour cells from androgen-dependent to androgen-independent is crucial in developing new treatment approaches (Shen and Abate-Shen, 2010).

The problems that are faced due to acquired resistance to therapy and tumour recurrences thus warrant a continuous search for better and alternative treatment options for PCa.

1.2. Rationale and Problem Statement

PCa management is faced with numerous clinical challenges though conventional therapy has proved to be successful in the management of localised and early

diagnostic cases. Clinicians are still battling to address the cellular resistance with late diagnostic cases that have metastasised. Resistance can be attributed to mutations, over-treatment, and mistreatment, resulting in tumour recurrences (LoPiccolo et al., 2008; Eichhorn et al., 2008). Resistance of PCa cells to radiotherapy and ADT limits treatment response (Shen and Abate-Shen, 2010; Palacios et al., 2013). Cells survive through activation of molecular signalling routes such as the EGFR and PI3K/Akt/mTOR pathways (Lee, et al., 2004; Skvortsova et al., 2008; Farooqi and Sarkar, 2015). Crosstalk between signalling pathways has also been cited as the main reason for resistances and recurrences (Bektas et al., 2005; Migliaccio et al., 2006; Courtney et al., 2010; Bitting and Armstrong, 2013). Furthermore, inhibition of the androgen receptor signalling pathway in ADT initially reduces androgen-dependent prostate tumour growth (Pienta and Bradley, 2006; Ryan and Tindall, 2011; Carrión-Salip et al., 2012). This is often followed by a relapse with the disease presenting as androgen-independent. Development of androgen-independence may be due to an EGFR-mediated activation of the PI3K/Akt/mTOR pathway, leading to enhanced tumour cell survival (Traish and Morgentaler, 2009; Bitting and Armstrong, 2013; Taneja, 2018; Tomasello et al., 2018). Development of new treatment strategies are needed to address these therapeutic dilemmas.

1.3. Hypothesis

It is hypothesised that targeting the EGFR family proteins, PI3K and mTOR, and the androgen receptor pathway, with specific inhibitors, singly or in combination, can

significantly sensitise androgen dependent and androgen independent prostate cancer cells to low therapeutic doses of ionising radiation.

1.4. Aims and Objectives

In search of alternative treatment approaches this current study aims to understand the molecular mechanisms involved in the evolution of prostate cancer, specifically the cross-talk that takes place during survival pathway inhibition and cells surviving ionising radiation treatment, by identifying and validating the expression of potential therapeutic targets responsible for the survival pathways: the PI3K/Akt/mTOR, EGFR and AR 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 NVP-BEZ235 (dual inhibitor of PI3K and mTOR), Tyrphostin AG-1478 (inhibitor of EGFR) and MDV3100 (inhibitor of AR), 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 overarching aim of the study was to sensitise androgen dependent and independent prostate cancer cells by inhibiting potential therapeutic targets with their respective inhibitors. For this, the following objectives were pursued:

- Determine the intrinsic radiosensitivity of a panel of benign and malignant prostate cancer cell lines following exposure to X-rays.
- Determine the androgen sensitivity of each cell line.
- Determine the cytotoxicity of specific inhibitors of AR, EGFR, PI3K and mTOR in each cell line.
- Evaluate the roles of AR, EGFR, PI3K and mTOR in androgen dependent and independent cell lines with respect to radiosensitivity.
- Determine the radiomodulatory effects of the abovementioned inhibitors, singly or in combination, in each cell line.

1.5. Significance of the Study

This research work demonstrates the following:

- High concentration of 5α -dihydrotestosterone (5α -DHT) switches androgen independent cells to androgen dependent.
- NVP-BEZ235 has the ability to protect normal cells, at the same time giving a therapeutic benefit to cancer cells.
- NVP-BEZ235 has a therapeutic advantage at EC_{50} over X-rays at D_{50} .
- A very strong synergism was demonstrated between NVP-BEZ235 and MVD3100.
- Pre-treatment of apparently normal and malignant human prostate cells with specific inhibitors of EGFR, PI3K, mTOR and AR, singly or in combination, sensitises PCa cells to low and high doses of ionising radiation.

1.6. Delineations

This study used inhibitors of AR, EGFR, PI3K and mTOR to sensitise four prostate cell lines (1542N, BPH-1, DU145 and LNCaP). These cell lines express the abovementioned targets, but the expression levels of said targets, reported in several studies, were not determined prior to inhibition.

The research variables determined in this study are (1) the intrinsic cellular radiosensitivity of the cell lines, (2) the cytotoxicity of specific inhibitors of AR, EGFR, PI3K and mTOR, (3) the radiomodulatory effects of inhibitors, either singly or in combination.

Clonogenic cell survival following ionising radiation was determined to compare the intrinsic cellular radiosensitivity of four cell lines. Cytotoxicity was determined, using the colony forming assay, by extracting the equivalent concentration for 50% cell kill (EC_{50}) for each cell line. Combination indices were determined from the cytotoxicity data in order to decide on the best inhibitor combination to be used in a cocktail, in which radiation modifying factors were derived to determine their impact on cellular radiosensitivity.

These research variables were deemed sufficient to prove the research hypothesis. Some of the data presented here have been published (Maleka et al., 2019; Appendix A) and presented at Conferences.

1.7. Background Literature

1.7.1. The Prostate Gland

The male reproduction system consists of the penis, testicles and the prostate, a walnut-shaped organ. The main and important function of the prostate is the production of the fluid that transports the sperm through the urethra. It is surrounded by the bladder and the rectum.

1.7.2. Incidences

Prostate cancer is a significant human health concern because it is the most diagnosed form of cancer in men (Rebbeck et al., 2013; Ferlay et al., 2019). Globally, and second to lung cancer, PCa remains one of the leading causes of cancer deaths amongst men. In developing countries in sub-Saharan Africa, including South Africa, the incidence of this disease is increasing rapidly (Rebbeck et al., 2013; Taitt, 2018) due to medical technology advancement, increased screening and awareness, a high quality of living, and an ageing population (Traish and Morgentaler, 2009; Rebbeck et al., 2013). Increasing mortality rates remain problematic because of the lack of curative treatment, especially in patients with androgen resistant metastatic disease (Scher et al., 2010; de Bono et al., 2010; Litwin and Tan, 2017).

1.7.3. Hallmarks of Cancer

Different mechanisms in the human body, such as apoptosis, necrosis and autophagy, regulate the growth of normal cells. When normal cells are not controlled due to a failure of these mechanisms, these cells evade the immune system and divide uncontrollably, resulting in a growth known as cancer. These abnormal cells become resistant and continue to spread, acquiring characteristics that aid their replication.

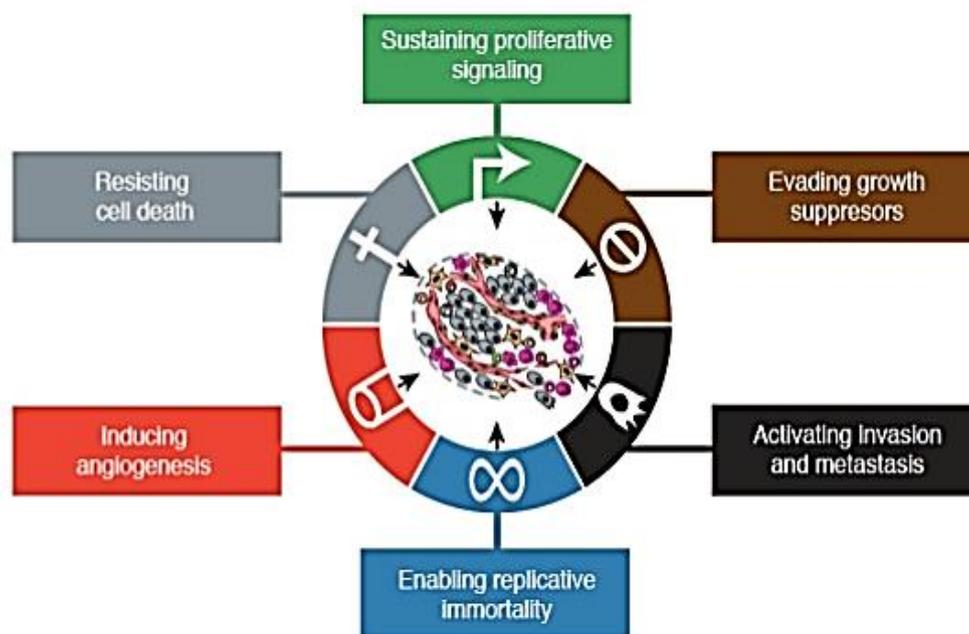


Figure 1.1: The Hallmarks of cancer as proposed by Hanahan and Weinberg (Hanahan and Weinberg, 2000).

Briefly, the six hallmarks depicted in Figure 1.1 include: (1) sustaining proliferative signalling for survival, (2) evading growth suppressors, (3) activating invasion and metastasis, (4) enabling replicative immortality, (5) inducing angiogenesis, which is the generation of blood vessels for tumour survival and growth, and (6) resisting cell

death by apoptosis, a mechanism that signals damaged cells to die (Hanahan and Weinberg, 2000; Pavlova and Thompson, 2016; Flavahan et al., 2017).

1.7.4. Management of PCa

The management and treatment planning of PCa has always been aligned with patient safety and survival (Heidenreich et al., 2011; Hannan et al., 2016; Wei et al., 2017). The goal of an effective treatment regimen (curative or palliative), ideally, must result in less morbidity, decreased recurrence of tumours, and be specific for destroying cancer cells without damaging normal cells (Potiron et al., 2013). Lastly, decisions are usually made as to the patient's preference (Heidenreich et al., 2014; Lowrance et al., 2018).

Through research and development, effective therapies have been developed to address the heterogeneity of PCa, but no curative therapeutic strategies exist (Sanchez et al., 2011). Most patients with organ-confined disease undergo radical prostatectomy, radiotherapy, or androgen deprivation therapy (Madsen et al., 2007; Rusthoven et al., 2016). Complications after treatment for prostate cancer are well documented (An et al., 2007; LoPiccolo et al., 2008; Hurvitz et al., 2013; Wei et al., 2017).

1.7.5. Conventional Therapies for PCa

In clinical settings, over 60% of solid tumours are treated with radiation, or a combination of radiation and other modalities. The primary treatment for localised

prostate cancer is ionising radiation, or complete removal of the prostate (radical prostatectomy). Due to resistance to therapy many patients relapse, resulting in re-growth of the cancer. The next step to follow is androgen deprivation therapy, because cancer cells die due to androgen blockade. Most tumours develop resistance, eventually growing in the absence of testosterone (Lai et al., 2013). This is known as hormone relapse, and patients in this stage are defined as hormone-refractory, also known as castration-resistant prostate cancer (CRPCa) (Guérin et al., 2010; Jain et al, 2012; Berkovic et al., 2013; Antonarakis et al., 2015).

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 (de Bono et al., 2010; Bitting and Armstrong, 2013). Prostate cancer is managed according to the stage of the cancer. In Stage I, the cancer is small and confined within the prostate and grows slowly; in Stage II, the cancer is confined within the prostate, but the likelihood of spread is greater than in Stage I; in Stage III, the cancer has spread beyond the prostate but not to the lymph nodes or other organs; and in Stage IV, the cancer has spread systematically beyond the prostate to the lymph nodes and other organs (Schlomm et al., 2007; Heidenreich et al., 2011).

Localised disease, i.e. Stages I and II, have various alternatives for clinical management, the first being surveillance (or watchful waiting), followed by traditional radiation therapy, and, depending on age, surgery will be employed (Heidenreich et al., 2011; Heidenreich et al., 2014; Litwin and Tan, 2017). Once the disease has spread beyond the confines of the organ i.e. Stages III and IV, the disease has

metastasised (Heidenreich et al., 2014). The approach to managing the disease must now change. Systematic clinical management with chemotherapy is then employed. There are numerous drugs, for example, enzalutamide, abiraterone and prednisone that have been used successfully in chemotherapy, improving the survival of patients, but the challenge is treatment induced resistance (de Bono et al., 2010; Bitting and Armstrong, 2013; Ryan et al., 2015; Wei et al., 2017).

Advanced prostate cancers are responsive to ADT at the beginning of treatment, but tend to become hormone refractory and resistant to therapy at a later stage (Shen and Abate-Shen, 2010; Carrión-Salip et al., 2012; Gómez-Millán et al., 2015). In addition, ADT does not entirely eliminate androgen independent cancer cells in advanced prostate cancer. Although chemotherapy with enzalutamide, abiraterone and prednisone appears to be effective for castration-resistant prostate cancers which do not respond to ADT, the median survival period is only about 4 months (Azad et al., 2015; Ryan et al., 2015; Antonarakis et al., 2015).

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.
- Surgery is invasive and can only be performed on localised prostate cancer. In addition to complications, such as incisional hernia and rectal injury, surgery has a high morbidity rate.
- 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 to ADT (Taitt, 2018).

- 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.

1.7.6. Targeted Therapy

Targeted therapy/personalised medicine has been studied in depth, especially in patients with breast and head-and-neck cancers, in addressing the challenges faced with conventional treatment (Small, 2004; Bitting and Armstrong, 2013; Hurvitz et al., 2013; Kratochwil et al., 2016). Recently, Maleka and colleagues looked at targeted therapy as an alternative to traditional radiotherapy (Maleka et al., 2015; 2019). Studies on signalling pathways, such as those involving EGFR, PI3K, Akt, and mTOR (LoPiccolo et al., 2008; Courtney et al., 2010; Choi et al., 2010; Li et al., 2012; Bitting and Armstrong, 2013), might lead to the identification of potentially effective therapeutic interventions for prostate cancer.

1.7.7. The PI3K/Akt/mTOR Signalling Pathway

The PI3K pathway is a non-linear pathway which transmits signals through protein-protein or lipid-protein interaction, playing a very critical regulatory function in cellular processes such as cell survival, protein synthesis, proliferation and glucose metabolism (Courtney et al., 2010; Mayer and Arteaga, 2015).

These are the most studied and researched pathways in cancer therapy. PI3K/Akt/mTOR leads to cell survival, and mitogen-activated protein kinase (MAPK) leads to cell proliferation. Both pathways are implicated in carcinogenesis thus making them very interesting signalling pathways in targeted therapy (Sarker et al., 2009). Prostate cancer cells utilise multiple molecular pathways to proliferate, survive and invade tissue during the course of tumour progression (Shukla et al., 2007; Sarker et al., 2009). Among several prostate signalling pathways, upregulation 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 (Carracedo et al., 2008; Sarker et al., 2009; Shen and Abate-Shen, 2010).

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.

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

Breast and head-and-neck cancer treatments have been very successful, targeting the PI3K/Akt/mTOR pathway, given its importance in cell survival signalling (Sanchez et al., 2011; Hurvitz et al., 2013). Since it is indicated that PCa has a high prevalence of activation of this pathway, its inhibition has great potential for improved clinical outcome in men with advanced prostate cancer.

The catalytic domain of the p110 subunits and mTOR belong to the phosphatidylinositol kinase-related family of kinases, and are, therefore, structurally

similar. Targeting more than one target protein in the survival pathway is the direction in which targeted therapy research is headed, and recent clinical studies have shown the benefit of switching off more than a single target protein (LoPiccolo et al., 2008; Li et al., 2012). 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 (Serra et al., 2008; Chen et al., 2018). 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 (Carracedo and Pandolfi, 2008; Carracedo et al., 2008).

LY294002, a dual PI3K-mTOR inhibitor, has been used in preclinical studies, but the disadvantage of this agent is that it is too toxic for clinical settings (Toulany et al., 2006; Shukla et al., 2007). 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 (Serra et al., 2008; Fokas et al., 2012; Potiron et al., 2013). In the clinic, NVP-BEZ235 has been well-tolerated in patients with solid tumours (Choi et al., 2010).

1.7.9. Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR) family, consists of four members, namely; ErbB1 (EGFR), ErbB2, ErbB3 and ErbB4 (Barton et al., 2001). 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.

Activation of the EGFR signalling pathway takes place when the EGFR family of peptides binds to their receptive extracellular domains (Traish and Morgentaler, 2009). Dimerisation of the receptor takes place with another member of the ErbB family or an EGFR monomer. 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. The most important and well-studied signalling route of the EGFR is the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway and (PI3K) (Sarker et al., 2009; Shen and Abate-Shen, 2010).

The ErbB receptors are expressed in a variety of epithelial tissues where they play an important role in development, proliferation and differentiation (Guérin et al., 2010). High levels of EGFR have been observed in a variety of tumours, including prostate, breast, colorectal and ovarian. Bladder cancer and renal cell cancer, too, are reported to express EGFR. ErbB2 and EGFR have been implicated in the development of numerous cancers, including PCa. 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 (Guérin et al., 2010; Ghosh et al., 2005; Carrión-Salip et al., 2012).

1.7.10. Inhibition of the EGFR

Currently, targeted therapies in oncology include the two major categories of molecules: monoclonal antibodies and tyrosine kinase inhibitors (TKI). These are new therapeutic approaches for PCa currently in the clinic. They 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. Preclinical studies have demonstrated that EGFR-targeting agents have the potential to be used in combination with cytotoxic chemotherapy and radiotherapy (Li et al., 2012). IMC-C225 (cetuximab) has been shown to enhance the effects of cytotoxic agents and radiotherapy (Liang et al., 2003; Small, 2004; Harris, 2004; Dhupkar et al., 2010; Liu et al., 2010).

1.7.11. The Role of Androgen in Prostate Cancer

The function of the prostate is initially controlled by androgens which are important in maintaining and regulating the expression of specific genes (Traish and Morgentaler, 2009; Ryan and Tindall, 2011). 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 (Ghosh et al., 2005; Carrión-Salip et al., 2012).

The majority of prostate tumours are dependent on androgens for growth and advanced prostate cancers are generally treated with ADT (Sadar, 1999; Jain et al.,

2012). However, the majority of prostate cancers have been known to develop resistance against ADT (Pienta and Bradley, 2006; 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).

Prostate cancer is a disease of androgens. Androgens control the growth and function of the prostate through a series of events involving the metabolism of testosterone into 5 α -dihydrotestosterone (5 α -DHT) by the enzyme 5 α -reductase (Chen et al., 2008). This makes androgens crucial in the maintenance of prostate homeostasis, and in the regulation of proteins like EGFR, PI3K, Akt, and mTOR, which are involved in cellular proliferation and survival (Ghosh et al., 2005; Carrión-Salip et al., 2012).

In androgen-dependent PCa, cell proliferation and survival are mainly controlled by androgens through androgen receptors (Chen et al., 2008). The AR pathway can be effectively regulated because the cells depend on androgens for survival, and are responsive to ADT. However, in androgen-independent PCa, cell proliferation and survival are facilitated by alternative pathways, such as EGFR and PI3K (Ghosh et al., 2005; Carrión-Salip et al., 2012). Interestingly, EGFR may be activated through the signalling of PI3K/Akt/mTOR, an alternative means of survival for androgen-independent cells in the absence of androgens. This consequently leads to tumour growth and invasion, and ultimately metastasis, making available treatment options ineffective.

The AR and EGFR are related to each other because of the regulatory mechanisms observed between the respective pathways (Knudsen and Penning, 2010). The EGFR, through PI3K/Akt/mTOR, is the driving force of androgen-independent PCa cell survival (Carrión-Salip et al., 2012). The PI3K/Akt/mTOR pathway also plays a central role in the development and progression of cancer by regulating cell differentiation, growth, metabolism, migration and survival.

1.7.12. Regulation of EGFR and PI3K Pathways by Androgen

The development of prostate cancer involves a series of events, including changes in the function of numerous growth regulatory signalling components such as AR, EGFR and PI3K/Akt/mTOR. Expression of EGFR in normal prostate tissue is suppressed by androgen, while in PCa it is upregulated by androgens (Traish and Morgentaler, 2009; Knudsen and Penning, 2010). This suggests that in advanced PCa, EGFR is highly expressed due to the upregulation of androgens. Increased EGFR in prostate cancer is correlated with a high Gleason score and disease progression from androgen-dependent to androgen-independent. EGFR is a transmembrane receptor and its expression varies significantly in relation to androgen dependence. Activity of EGFR predominantly phosphorylates specific tyrosine residues activating several signalling cascades, including the PI3K pathway which controls multiple biological processes of cancer.

1.7.13. Cross-Talk between AR, EGFR and PI3K Signalling Pathways in Prostate Cancer

EGFR and PI3K are positively correlated with androgen-independence (Sadar, 1999; Carrión-Salip et al., 2012). The expression of EGFR and PI3K pathway proteins increases as the PCa progresses from androgen-dependent to androgen-independent. Disease relapse and progression from androgen-dependent to independent are correlated with the expression of EGFR, a phenomenon that could be important in cellular response to therapeutic agents (Traish and Morgentaler, 2009). The increase in expression of pathway components may offer options for more effective targeted therapies in androgen-independent prostate cancer.

The AR is highly expressed in the majority of androgen-independent prostate cancers, suggesting that the AR signalling pathway is activated in the absence of androgens (Lonergan and Tindall, 2011). Cross-talk between AR and EGFR, through the PI3K pathway, has been demonstrated and supported by preclinical models (Sadar, 1999; Carracedo et al., 2008; Joshi et al., 2015). This cross-talk may present itself as an important pathway during PCa progression, giving cells a survival advantage, and might serve as a potential target for therapy. The cross-talk further emphasises the importance of combination therapy for optimum tumour control.

Androgen deprivation therapy is currently used for patients with metastatic PCa, in conjunction with regimens involving the blocking of androgen synthesis by ketoconazole and Abirateratone, lowering of AR protein levels through heat shock protein 90 (Hsp90) chaperone inhibitors, and degrading of protein with ansamycin

antibodies (Li et al., 2010; Carver et al., 2011; Schweizer et al., 2012). This combined approach has become necessary due to the inability of the ADT to completely block the survival pathway of androgen-independent tumour cells (Thamilselvan et al., 2017; Scott, 2017). Activation of the PI3K pathway is associated with androgen signalling and may, in part, be responsible for the castration-resistant phenotype observed in metastatic PCa (Bitting and Armstrong, 2013). It has been suggested that this resistance could be reversed by inhibiting the PI3K and EGFR pathways to restore the AR signalling pathway (Carver et al., 2011). This would imply the reversal of androgen-independence to androgen-dependence and should, therefore, sensitise cells to ADT. Also, inhibition of AR can activate the PI3K pathway and give rise to a survival advantage (Carver et al., 2011; Thamilselvan et al., 2017; Scott, 2017). These findings would suggest that targeting EGFR, AR, PI3K and mTOR might potentially be a useful therapeutic strategy.

CHAPTER 2: MATERIALS AND METHODS

2.1. Study Location and Ethical Consideration

All experiments were performed in the Division of Radiobiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg. The study was approved by the Health Research Ethics Committee (HREC) of the Faculty of Medicine and Health Sciences, Stellenbosch University, South Africa (HREC Reference #: X19/02/004; Appendix B).

2.2. Reagents for Colony Forming Assay

Fixative

The fixative for clonogenic cell assays consisted of a mixture of glacial acetic acid, methanol and deionised water, in the ratio of 1:1:8 (v/v/v), respectively.

Staining Solution

Colonies were stained with 0.01% Amido black (Naphthol Blue Black) in fixative.

2.3. Specific Inhibitors

NVP-BEZ235

NVP-BEZ235 (Santa Cruz Biotechnology, Texas, USA, cat # 364429) is a dual inhibitor of phosphoinositide-3-kinase (PI3K) and mammalian target of rapamycin (mTOR), with an inhibitory concentration at 50% (IC₅₀) 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). NVP-BEZ235 has a molecular weight of 469.55 and is soluble

in dimethyl sulfoxide (DMSO). It has a chemical formula of $C_{30}H_{23}N_5O$ and Figure 2.1 shows its chemical structure. NVP-BEZ235 was reconstituted as a stock solution of 106 mM in dimethyl sulfoxide (DMSO) and stored at $-20^{\circ}C$ until used.

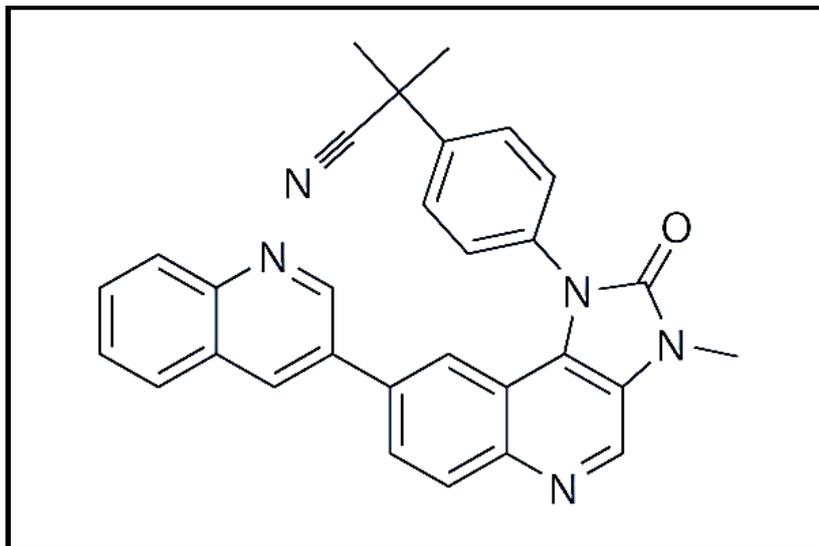


Figure 2.1: Chemical structure of NVP-BEZ235.

AG-1478

AG-1478 (Tocris Bioscience, UK, cat # 1276) 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 is soluble in dimethyl sulfoxide (DMSO). It has a chemical formula of $C_{16}H_{14}ClN_3O_2$ and Figure 2.2 shows its chemical structure. AG-1478 was reconstituted as a stock solution of 10 mM in DMSO and stored at $-20^{\circ}C$ until used.

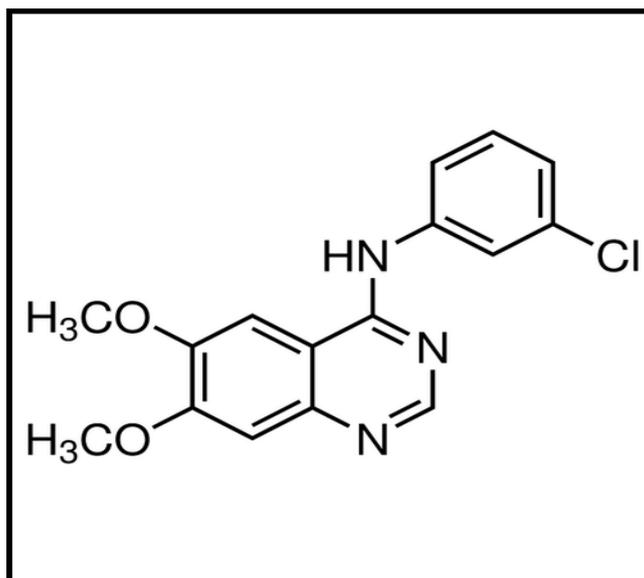


Figure 2.2: Chemical structure of AG-1478.

MDV3100

MDV3100 (KareBay Biochem, USA; cat # S1250) is an androgen receptor (AR) inhibitor with an IC₅₀ ranging from 4-21 nM. It is a potent oral antagonist to the AR that lacks any agonist activity, and prevents nuclear translocation of AR and its binding to DNA (Shen and Abate-Shen, 2010). MDV3100 has a molecular weight of 464.44 and is soluble in dimethyl sulfoxide (DMSO). It has a chemical formula of C₂₁H₁₆F₄N₄O₂S and Figure 2.3 shows its chemical structure. For this study, 5 mg stock of MDV3100 was reconstituted with 1.076 ml of DMSO, giving a concentration of 10 mM, and stored at -20°C until used.

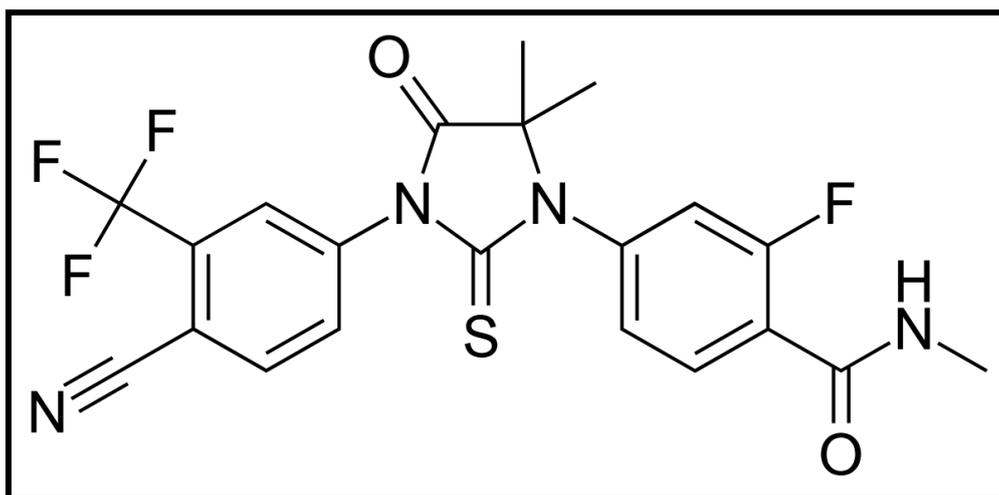


Figure 2.3: Chemical structure of MDV3100

2.4. Cell Culture Media

Dulbecco's Modified Eagle's Medium (DMEM)

Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Germany; cat # 4655) was supplemented with 10% heat-inactivated FBS (HyClone, UK, cat # SV30160.30IH), and penicillin (100 U/ml) / streptomycin (100 µg/ml) solution (Lonza, Belgium, cat # DE17-602E).

Roswell Park Memorial Institute medium (RPMI-1640)

The Roswell Park Memorial Institute medium, RPMI-1640 (Sigma-Aldrich, USA; cat # R8758), was supplemented with 0.3 mg/L L-glutamine, 2.0 mg/L sodium bicarbonate, 10% heat-inactivated FBS, and penicillin (100 U/ml) / streptomycin (100 µg/ml).

2.5. Cell lines

1542-NPTX (passage 18-31)

The benign 1542-NPTX (1542N) human prostate epithelial cell line 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), and was used to represent normal prostate tissue. The cells were grown in RPMI-1640, supplemented with 10% heat-inactivated FBS, and penicillin (100 U/ml) / streptomycin (100 µg/ml). The cell line was provided by Professor JRW Masters (Prostate Cancer Research Centre, University College London, UK).

DU145 (passage 7-22)

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 have an epithelial-like morphology, were grown in Minimum Essential Medium (MEM) (Sigma-Aldrich, Germany) supplemented with 10% heat-inactivated FBS, and penicillin (100 U/ml) / streptomycin (100 µg/ml). The cell line was obtained from Professor P Bouic (Synexa Life Sciences, Montague Gardens, South Africa).

BPH-1 (passage 2-11)

The benign prostatic hyperplasia-1 (BPH-1) cell line was established from human prostate tissue obtained by transurethral resection. Primary cell cultures were immortalised with simian virus 40 (SV40) large T-antigen (Hayward et al., 1995). For this study, even though BPH-1 cell line is not defined as a cancer, it was assumed to be of tumour-derived as it has been suggested that it may be a

precursor to PCa (Hayward et al., 1995). The BPH-1 cells have a cobblestone appearance in monolayer culture and grows in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, and penicillin (100 U/ml) / streptomycin (100 µg/ml). The cells were obtained from Professor SW Hayward (Department of Urology, University of California, USA).

LNCaP (passage 4-13)

The LNCaP cell line was established from a supraclavicular lymph node metastasis of human prostatic adenocarcinoma (Horoszewicz et al., 1983). LNCaP has a fibroblastoid morphology, low anchorage potential, is adherent, but grows in aggregates and as single cells in RPMI-1640 medium supplemented with 5% heat-inactivated FBS, and penicillin (100 U/ml) / streptomycin (100 µg/ml). The cultures require gentle handling at all times because the cells are easily dislodged by tapping, shaking or pipetting. The low anchorage potential is also responsible for the 10-20% cell loss during media changes in long-term experiments (Horoszewicz et al., 1983). To address this problem, flasks were coated with fibronectin (100 µl of fibronectin in 15 ml of sterile PBS) and incubated overnight, before use. The cells were obtained from Professor Helmut Klocker (Department of Urology, University of Innsbruck, Austria).

2.6. Routine Cell Culture, Cryopreservation and Maintenance

The cells were routinely maintained at 37°C in a humidified atmosphere (95% air and 5% CO₂) in SHEL LAB incubators (Sheldon Manufacturing Inc, USA). Aseptic techniques were used and all cell culture procedures were carried out in vertical laminar

flow cabinets. Cells were routinely grown in 75 cm² flasks, and passaged when culture confluency was at 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 (FBS) and 0.1 ml of DMSO, stored at -80°C overnight, and then transferred to liquid nitrogen for use at a later stage.

2.7. Proliferation Arrest of Prostatic Cells by 5 α - dihydrotestosterone (DHT)

To confirm the androgen dependence status of the cell lines used in this study, their sensitivity to androgen treatment was determined. For this, 8000-10000 cells were seeded in 24-well multiwell plates with medium containing charcoal-stripped foetal bovine serum (csFBS). By stripping of serum with activated carbon, steroid hormones, specifically 5 α -dihydrotestosterone (5 α -DHT), endogenous hormones, growth factors and cytokines are removed. This, thus, enables the *in vitro* evaluation of the effects of steroid hormones by addition of 5 α -DHT to cell cultures. The medium for 1542N cell line was supplemented with 0.5% csFBS, the BPH-1 cell line with 10% csFBS, and 5% csFBS for the LNCaP cell line, which served as the positive control. 5 α -DHT was added a day later (2 days later in the case of LNCaP) in concentrations ranging from 0.001–10 nM, for a period of 4 days. The experiment was stopped after 4 days and the cell growth determined by crystal violet assay, as described by Baker and colleagues (Baker et al., 1986). Two control flasks were set up, one with charcoal-stripped FBS and one with non-stripped FBS. The optical

density (OD) readings, expressed as a percentage of the OD of the positive controls (cultures with non-stripped FBS), were plotted against concentrations of DHT.

2.8. Irradiation of Cell Cultures

Cell lines grown in 25 cm² tissue culture flasks were irradiated using a Precision MultiRad 160 X-irradiator (Precision X-Ray Inc., Branford, CT, USA) at the Division of Radiobiology (Faculty of Medicine and Health Sciences, Stellenbosch University). The X-ray energy and tube current were 130 kV and 23 mA, respectively. Samples were irradiated at a source-to-sample distance of 65 cm, measured to the base of the experimental flasks, at a dose rate of 1.0 Gy/min. Build-up consisted of 5 ml or 10 ml (depending on experimental set-up) of medium in the flasks. For all assays cell cultures were irradiated at room temperature (22°C).

2.9. Cell Survival Assay and Radiosensitivity

Intrinsic radiation response in all cell lines was measured by clonogenic assay. Near-confluent stock cultures in exponential growth phase were trypsinised to give single-cell suspensions and counted using a haemocytometer. Cells were seeded in triplicate per experiment in 25 cm² culture flasks (Greiner, Germany; # 690160) at varying densities ranging from 300 to 100 000 cells per flask, depending on cell type, and absorbed radiation dose, and left to attach for 4–5 hours. The PCa cell lines have relatively low plating efficiencies of ~15-35%. It was, thus, necessary to plate high numbers of cells in order to obtain appropriate numbers of colonies. The cell cultures were then exposed to radiation doses ranging from 0-10 Gy and incubated

at 37°C for 10-14 days, depending on the cell line. The colonies were fixed, stained with Amido Black, and air-dried before counting, using a stereoscopic microscope (Nikon, Japan; Model # SMZ-1B), and corresponding surviving fractions calculated. The means (\pm SD) of the surviving fractions (SF) for the three experiments were plotted against irradiation dose, and cell survival curves obtained by fitting the data to the linear-quadratic equation:

$$SF = \exp[-\alpha D - \beta D^2] \quad (2.1),$$

where α and β are the linear and quadratic cell inactivation constants, respectively, and D is the dose in Gy. Cellular radiosensitivity was expressed in terms of the surviving fraction at 2 Gy (SF_2), surviving fraction at 6 Gy (SF_6), absorbed dose for 50% cell killing (D_{50}), and mean inactivation dose (\bar{D}). \bar{D} is the area under the cell survival curve and depicts radiosensitivity over low-high doses.

2.10. Target Inhibitor Toxicity Measurements

To assess the influence of the inhibitor concentration on cytotoxicity, single-cell suspensions were seeded (600-100,000 cells per flask, depending on cell line) into 25 cm² culture flasks, and incubated for 3-4 h to allow the cells to attach. The cells were exposed to their respective inhibitors; NVP-BEZ235 (0.001-1,000 nM), AG-1478 (1-100,000 nM) and MDV3100 (10–100 nM), and incubated for 7-10 days for colony formation. The colonies were fixed, stained, washed in tap water, air-dried and counted. To determine the equivalent concentration of each inhibitor for 50% cell

kill (EC_{50}), the surviving fractions (SF) were plotted as a function of log (inhibitor concentration) and fitted to a 4-parameter logistic equation of the form:

$$SF = B + \frac{T-B}{\{1-10^{[(\log EC_{50}-D)HS]}\}} \quad (2.2),$$

where B and T are the minimum and maximum of the sigmoidal curve, respectively, D is the log (Inhibitor concentration), and HS is the steepest slope of the curve. For each cell line and dose point, three independent experiments were performed.

2.11. Evaluation of Therapeutic Potential

To assess whether the various treatment protocols (X-rays or inhibitors) used in this study had a potential therapeutic benefit, a relative sensitivity (RS) was determined. For this, the D_{50} and EC_{50} of the normal cell lines (1542-NPTX) were compared with those for the respective tumour cell lines (LNCaP, BPH-1, DU145), as follows (Maleka et al., 2019):

$$RS = \frac{D_{50}(normal)}{D_{50}(tumour)} \quad or \quad \frac{EC_{50}(normal)}{EC_{50}(tumour)} \quad (2.3).$$

The criteria for no potential benefit with possible undesirable effects, no potential benefit, and potential therapeutic benefit of each agent, are $RS < 1.0$, $RS = 1.0$ and $RS > 1.0$, respectively.

2.12. Determination of Radiosensitivity Modification by Target Inhibitors

The effect of inhibitor exposure on radiosensitivity was investigated. Attached cells were treated with their respective inhibitors: 1542N (400 nM of AG-1478 and 53.82 nM of NVP-BEZ235 or 21.36 nM MDV3100); DU145 (6613 nM of AG-1478 and 16.25 nM of NVP-BEZ235 or 22.43 nM MDV3100); LNCaP (302 nM of AG-1478 and 6.10 nM of NVP-BEZ235 or 183.2 MDV3100) and BPH-1 (677 nM of AG-1478 and 6.11 nM of NVP-BEZ235 or 20.35 MDV3100). For each experiment, sets of cell culture flasks given inhibitors alone (singly and in combination) were immediately irradiated with 2, 4, 6, 8 or 10 Gy, clinically used doses per fraction, in conventional and hypofractionated radiotherapy, respectively. Unirradiated flasks, without inhibitors, served as Controls for cultures irradiated with and without inhibitors, respectively, or a cocktail of both inhibitors at the same concentration. Inhibitor-treated cell cultures were used as Controls for those receiving inhibitors and irradiation to allow for inter-experimental variations in inhibitor toxicity, as exposures to predetermined concentrations do not always yield the expected cell kill. The interaction between inhibitors and irradiation was expressed as a modifying factor (MF_{survival}), given as the ratio of surviving fractions at a dose of X-rays (or the mean inactivation dose, \bar{D}) in the absence and presence of inhibitor:

$$MF_{\text{survival}} = \frac{SF(X\text{-rays})}{SF(\text{inhibitor}+X\text{-rays})} \text{ or } \frac{\bar{D}(X\text{-rays})}{\bar{D}(\text{inhibitor}+X\text{-rays})} \quad (2.4).$$

The criteria for inhibition, no effect, and enhancement of radiosensitivity by inhibitors are $MF < 1.0$, $MF = 1.0$, and $MF > 1.0$, respectively.

2.13. Inhibitor Interaction

To test for potential interaction between inhibitors, the toxicity data obtained in Section 2.10 were fitted to the function:

$$\log(f_a/f_u) = m \times \log(D) - m \times \log(D_m) \quad (2.5),$$

where f_a and f_u are the affected and unaffected fractions of cells, respectively, to generate median-effect plots for each inhibitor. D is the concentration of inhibitor, D_m is the median-effect concentration of inhibitor, and the coefficient m is an indicator of the shape of the inhibitor concentration-effect relationship (Chou, 2006; Hamunyela et al., 2017). The shape parameter $m = 1$, >1 , and <1 are for hyperbolic, sigmoidal, and flat-sigmoidal inhibitor concentration-effect curves, respectively.

The mode of interaction between any two inhibitors was assessed by determining combination indices (CI) for each inhibitor cocktail from the fitted parameters of equation (2.3) according to the equation:

$$CI = \frac{D_1}{\left\{ D_{m1} \times \left(\frac{f_{a1}}{1-f_{a1}} \right)^{\frac{1}{m_1}} \right\}} + \frac{D_2}{\left\{ D_{m2} \times \left(\frac{f_{a2}}{1-f_{a2}} \right)^{\frac{1}{m_2}} \right\}} \quad (2.6),$$

where D_1 is the concentration of Inhibitor 1 and D_2 is the concentration of Inhibitor 2. m_1 and m_2 are the respective shape parameters. f_{a1} and f_{a2} are given as: $(1 - SF)$, as defined in equation (2.2)). D_{m1} and D_{m2} are the corresponding median-effect concentrations. Synergism, additivity, and antagonism are indicated by $CI < 1$, $CI =$

1, and $CI > 1$, respectively. Furthermore, the criteria for very strong synergism, strong synergism, and synergism are $CI < 0.1$, $0.1 \leq CI \leq 0.3$, $0.3 < CI \leq 0.7$, respectively (Chou, 2006).

2.14. Data Analysis

The GraphPad Prism computer programme (GraphPad Software, San Diego, CA, USA) was used to perform statistical analyses. For associations, linear regression analyses were used. Standard equations were used to fit nonlinear relationships. Data were presented as the mean \pm standard error of the mean (SEM) of three independent experiments, as indicated by error bars. For each experiment and data point, 3 replicates were assessed. To compare two data sets, the unpaired *t*-test was used. *P*-values were calculated from two-sided tests. A *P*-value of <0.05 indicated a statistically significant difference between the data sets.

CHAPTER 3: RESULTS

3.1. Androgen Sensitivity of DU145, LNCaP, BPH-1, and 1542N Cells

The crystal violet vital dye staining assay showed that when the DU145 cells were grown in medium with charcoal-stripped foetal bovine serum (csFBS), cell proliferation decreased by about 35% (Figure 3.1), indicating dependence on essential growth factors including androgen and other hormones. Addition of 5 α -dihydrotestosterone (5 α -DHT), at concentrations of 0.001 to 1.0 nM, appeared to induce a further concentration-dependent reduction in cell proliferation, but the decrease in proliferation was not statistically significant ($P = 0.2433$). The rate of proliferation at 1.0 nM was found to be approximately half the rate in cultures treated with medium containing non-stripped FBS. However, at higher 5 α -DHT concentrations (10 and 100 nM), cell proliferation recovered to levels comparable with those in non-stripped FBS cultures.

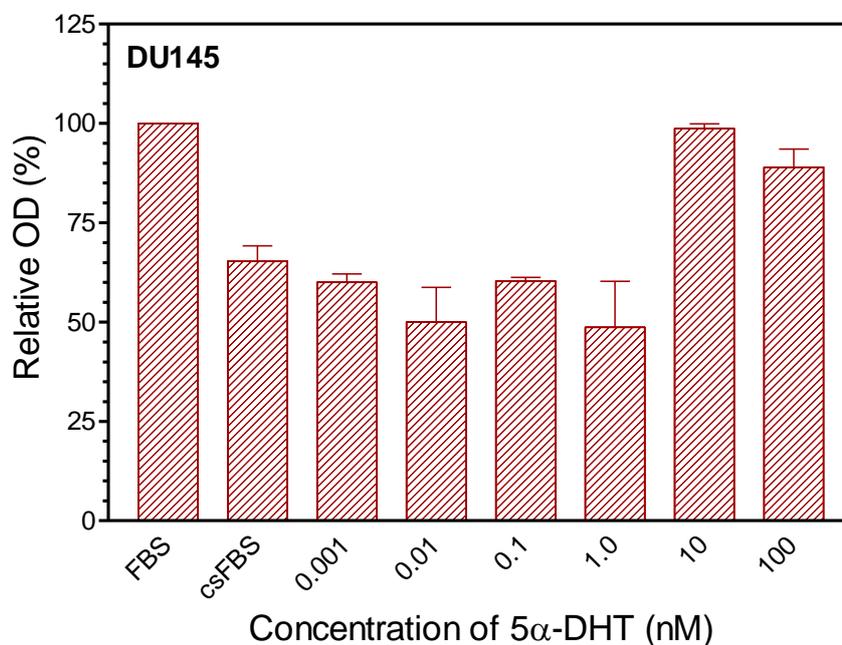


Figure 3.1: The effect of 5 α -dihydrotestosterone (DHT) addition to medium with charcoal stripped foetal bovine serum (csFBS) on the proliferation of DU145 cells, measured by crystal violet dye staining assay.

Growing the LNCaP cells in csFBS containing medium resulted in a 38% reduction in cell proliferation (Figure 3.2). When grown in csFBS containing medium, spiked with 5α -DHT, a clear 5α -DHT concentration-dependent increase in proliferation emerged, indicating dependence of these cells on androgen availability.

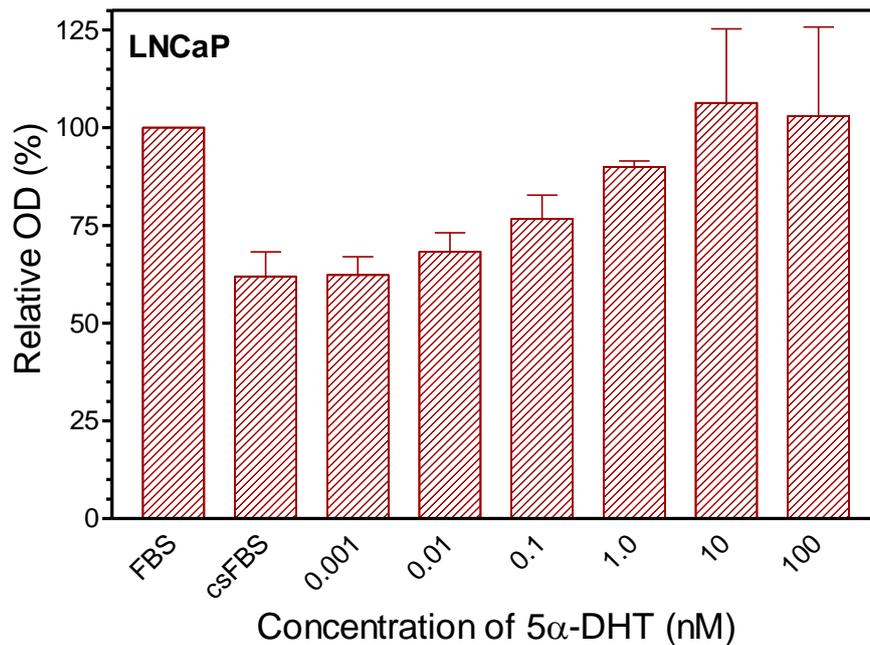


Figure 3.2: The effect of 5α -dihydrotestosterone (DHT) addition to medium with charcoal stripped foetal bovine serum (csFBS) on the proliferation of LNCaP cells, measured by crystal violet dye staining assay.

As expected, depriving the BPH-1 cells of essential growth factors when they were grown in medium supplemented with charcoal-stripped FBS, led to a 34% reduction in proliferation (Figure 3.3). Adding 5α -DHT at concentrations ranging between 0.001 and 100 nM did not result in a recovery in cell proliferation, indicating that the BPH-1 cell are insensitive to androgen treatment.

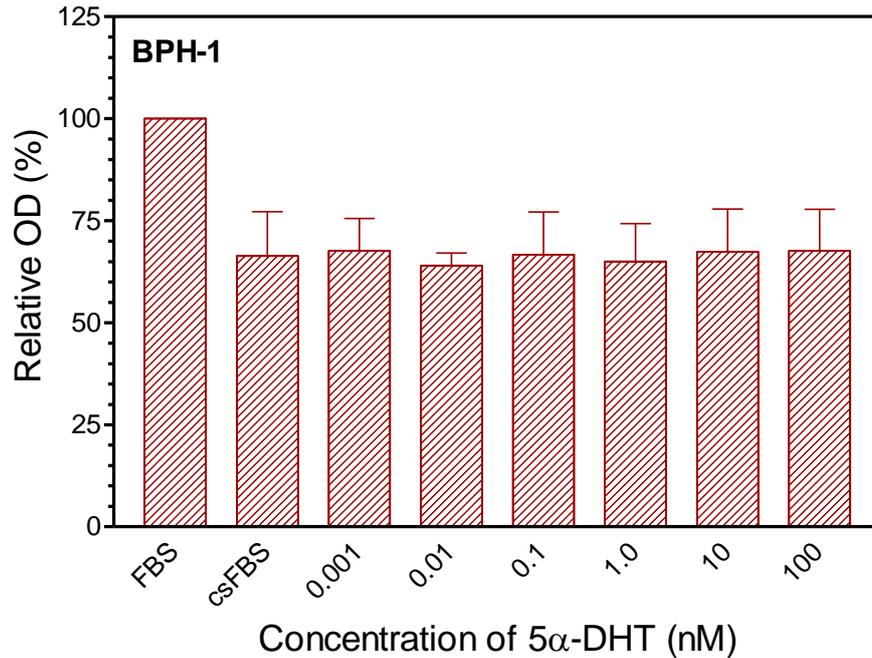


Figure 3.3: The effect of 5 α -dihydrotestosterone (DHT) addition to medium with charcoal stripped foetal bovine serum (csFBS) on the proliferation of BPH-1 cells, measured by crystal violet dye staining assay.

Interestingly, when the apparently normal 1542N cells were grown in medium supplemented with csFBS, a less than 20% reduction in proliferation was observed (Figure 3.4). As in the case of the DU145 cells, treatment of the 1542N cells with 5 α -DHT (0.001 to 0.1 nM) resulted in an additional concentration-dependent reduction in proliferation, reaching a minimum of ~66% at 0.1 nM. However, the decrease in cell proliferation did not reach statistical significance ($P = 0.3558$). Exposure of cells to higher concentrations of 5 α -DHT led to complete recovery in proliferation.

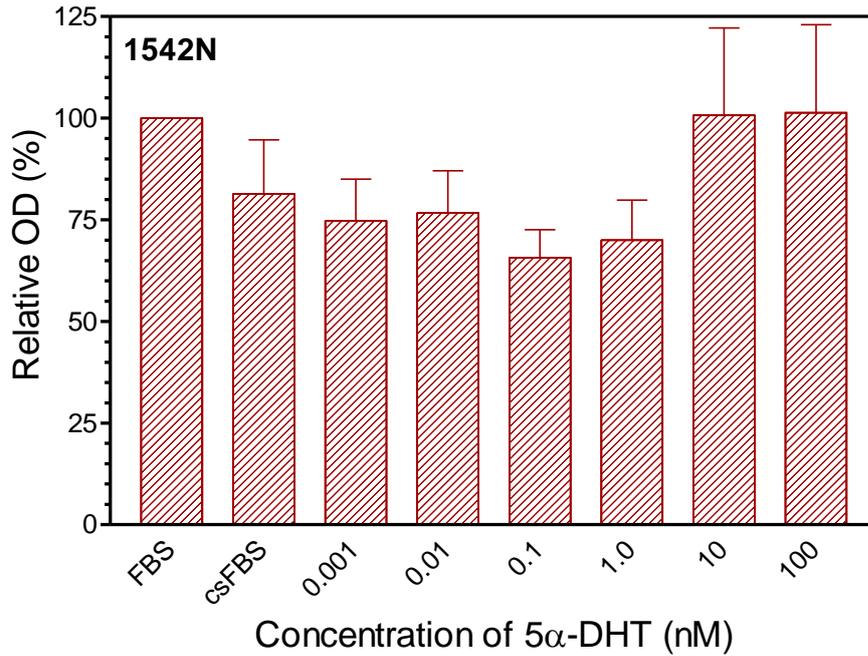


Figure 3.4: The effect of 5 α -dihydrotestosterone (DHT) addition to medium with charcoal stripped foetal bovine serum (csFBS) on the proliferation of 1542N cells, measured by crystal violet dye staining assay.

3.2. Intrinsic Radiosensitivity of 1542N, BPH-1, DU145, and LNCaP Cell Lines

Cell survival data for the human prostate carcinoma and normal cell lines (1542N, BPH-1, DU145, and LNCaP) were fitted to the linear-quadratic model, and the corresponding dose-response curves are presented in Figure 3.5.

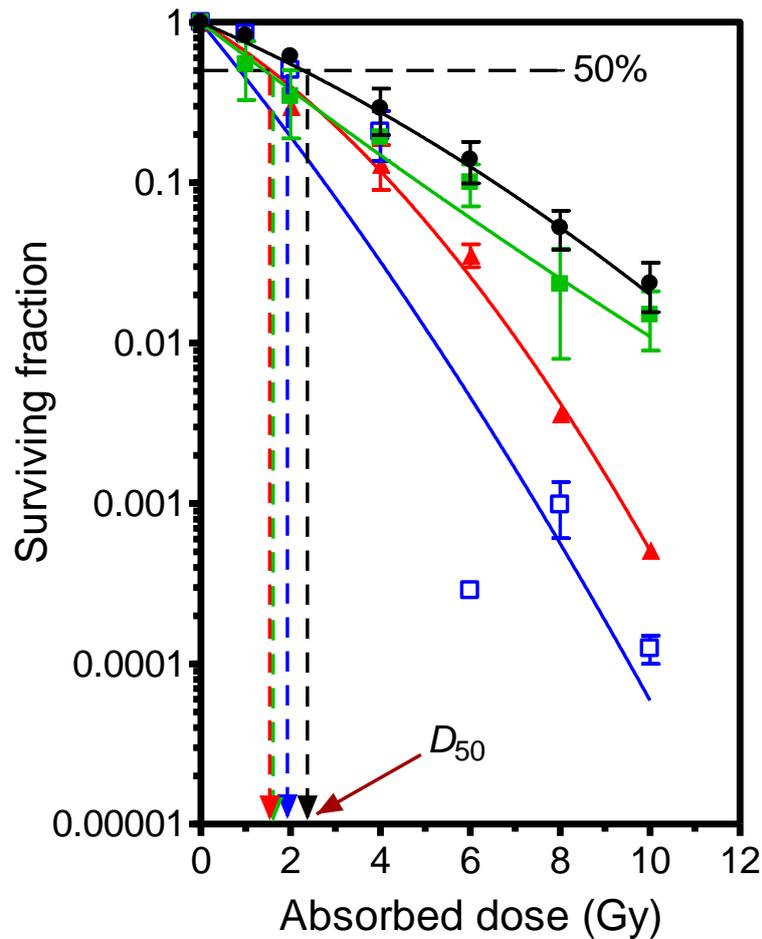


Figure 3.5: Clonogenic cell survival curves for 4 human prostate cell lines [DU145 (●), LNCaP (□), BPH-1 (▲), 1542N (■)] after X-ray irradiation. Survival curves were obtained by fitting experimental data to the linear-quadratic model. Symbols represent the mean surviving fraction \pm SEM from 3 independent experiments. The dose at which 50% of cells survive (D_{50}) is the dose at which each survival curve intersects the horizontal dashed line.

Intrinsic cellular radiosensitivity was expressed in terms of the surviving fraction at 2 Gy (SF_2), surviving fraction at 6 Gy (SF_6), radiation dose at which a cell survival of 50% (50% cell killing) was obtained (D_{50}), and mean inactivation dose (\bar{D}). The radiosensitivity parameters are summarised in Table 3.1.

Table 3.1: Summary of radiobiological parameters for the 1542N, BPH-1, DU145, and LNCaP cell lines. SF_2 and SF_6 denote the surviving fraction at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. \bar{D} denotes the mean inactivation dose (area under the cell survival curve). D_{50} the radiation absorbed dose for 50% cell killing. Data are presented as the mean \pm SEM from 3 independent experiments.

Cell Line	SF_2	SF_6	\bar{D} (Gy)	D_{50} (Gy)	α (Gy ⁻¹)	β (Gy ⁻²)
DU145	0.53 \pm 0.10	0.13 \pm 0.04	3.19 \pm 0.38	2.25 \pm 0.54	0.28 \pm 0.06	0.01 \pm 0.01
LNCaP	0.19 \pm 0.04	0.01 \pm 0.01	2.52 \pm 0.19	0.93 \pm 0.19	0.78 \pm 0.24	0.02 \pm 0.02
BPH-1	0.40 \pm 0.07	0.03 \pm 0.01	1.94 \pm 0.14	1.65 \pm 0.36	0.39 \pm 0.05	0.04 \pm 0.01
1542N	0.39 \pm 0.10	0.07 \pm 0.04	2.09 \pm 0.74	1.53 \pm 0.32	0.49 \pm 0.11	0.00 \pm 0.00

In terms of (\bar{D}), the benign prostatic hyperplasia cell line was found to be the most radiosensitive, with a mean inactivation dose of 1.94 ± 0.54 Gy. The DU145 cell line emerged as the most radioresistant, with a mean inactivation dose of 3.19 ± 0.38 Gy. The rank order of radioresistance in the cell lines was found to be BPH-1 < 1542N < LNCaP < DU145. On the other hand, when D_{50} is considered, the androgen-dependent cell line, LNCaP, emerged as more radiosensitive than its androgen-independent counterparts (1542N, BPH-1, DU145). The rank order of radioresistance in the cell lines was found to be LNCaP < 1542N < BPH-1 < DU145, with D_{50} -values of 0.93 ± 0.19 , 1.53 ± 0.32 , 1.65 ± 0.36 , and 2.25 ± 0.54 Gy, respectively. No statistically significant differences emerged between the radiosensitivity (based on \bar{D} and D_{50}) of the normal cell line when compared with those of the tumour cell lines ($0.1353 \leq P \leq 0.7893$). Given the inconsistency in radiosensitivity ranking when a single radiobiological parameter is used, it is necessary to consider using multiple

parameters to obtain a more representative reflection of relative cellular radiosensitivity. For this, a rank order was constructed based on SF_2 , SF_6 , D_{50} , and \bar{D} , as presented in Table 3.2. Using the frequency of occurrence of each cell line under each rank, the cell lines may be arranged in order of increasing radiosensitivity as: DU145 → BPH-1 → 1542N → LNCaP.

Table 3.2: Summary of relative radiosensitivity of DU145, BPH-1, LNCaP, and 1542N cell lines based on SF_2 , SF_6 , D_{50} , and \bar{D} .

Parameter	Increasing Radiosensitivity →			
SF_2	DU145	BPH-1	1542N	LNCaP
SF_6	DU145	1542N	BPH-1	LNCaP
D_{50}	DU145	BPH-1	1542N	LNCaP
\bar{D}	DU145	LNCaP	1542N	BPH-1

To determine whether treatment of the prostate tumour cell lines with X-rays has a potential therapeutic benefit, the relative sensitivities (RS), as described in Section 2.11, were determined on the basis of the D_{50} -values. The data presented in Table 3.3 summarise the relative radiosensitivities of the tumour cells. The absence of significant differences between the radiosensitivity of the apparently normal cell line and its tumour counterparts translates to relative sensitivities (RS) that do not differ significantly from unity, indicating no potential therapeutic benefit.

Table 3.3: Summary of D_{50} -values for 4 human prostate cell lines (normal: 1542N; cancer: DU145, LNCaP; benign prostate hyperplasia: BPH-1) and their relative radiosensitivities (RS) determined by clonogenic cell survival after exposure to X-rays (Equation 2.3). The 95% confidence intervals of the D_{50} -values are in parentheses. P -value indicates the level of significance in the difference between the D_{50} of the normal cell line (1542N) relative to those of the tumour cell lines (DU145, LNCaP, BPH-1).

Cell Line	D_{50} (Gy)	P -value	RS^*
1542N	1.41 ± 0.53 (0.00 - 3.01)	–	–
DU145	2.25 ± 0.54 (0.59 - 3.90)	0.1932	0.63 ± 0.28
LNCaP	0.93 ± 0.19 (0.37 - 1.50)	0.2935	1.52 ± 0.64
BPH-1	1.65 ± 0.36 (0.57 - 2.73)	0.6214	0.85 ± 0.37

*Errors were calculated using error propagation formulae for ratios.

3.3. Inhibitor Cytotoxicity

3.3.1. Cytotoxicity of EGFR Inhibitor (AG-1478)

Figure 3.6 shows that the EGFR inhibitor (AG-1478) exhibits a concentration-dependent toxicity in all cell lines, and sensitivity to inhibitor treatment was expressed in terms of equivalent concentration for 50% cell killing (EC_{50}) as the mean (\pm SEM). Treatment with AG-1478 yielded the same sensitivity ranking, as

observed for X-ray exposure (Figure 3.5), with the LNCaP showing more sensitivity than the other cell lines.

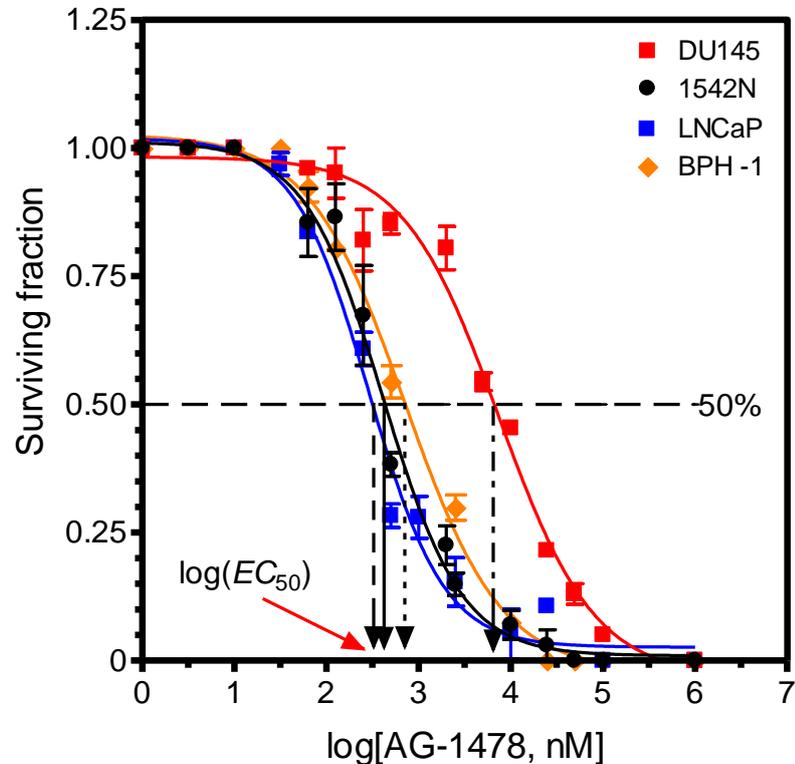


Figure 3.6: Cytotoxicity curves for EGFR inhibitor (AG-1478) treatment of 4 human prostate cell lines (DU145, 1542N, LNCaP, BPH-1). Curves were obtained by plotting cell survival as a function of log (inhibitor concentration). Cell survival was determined by the colony assay, and data were fitted to a sigmoidal equation. Data points are means \pm SEM of 3 independent experiments. The log of the concentration at which 50% of cells survive (EC_{50}) is that at which each survival curve intersects the horizontal dashed line (as indicated by red arrow).

The EC_{50} of the normal cell line (1542N) emerged as 416 ± 46 nM and was significantly lower than those of the DU145 (7431 ± 1052 nM, $P = 0.0402$, $R^2 = 0.6915$) and BPH-1 (708 ± 60 nM, $P = 0.0055$, $R^2 = 0.8815$) cell lines (Table 3.4). The EC_{50} of the relatively more sensitive LNCaP cell line did not differ significantly from that of the 1542N cell line (290 ± 31 nM, $P = 0.0783$, $R^2 = 0.5805$).

3.3.2. Cytotoxicity of PI3K and mTOR Inhibitor (NVP-BEZ235)

Inhibition of PI3K and mTOR inhibitor with NVP-BEZ235 also resulted in a concentration-dependent cell killing, as shown in Figure 3.7. The rank order of resistance to NVP-BEZ235 treatment is BPH-1 \approx LNCaP < DU145 < 1542N, with EC_{50} -values of 6.26 ± 0.59 , 6.42 ± 0.74 , 16.49 ± 2.97 , and 51.33 ± 11.81 nM, respectively. All tumour cell lines were significantly more sensitive to NVP-BEZ235 treatment than the normal cell line, as shown in Table 3.4 ($P \leq 0.0047$).

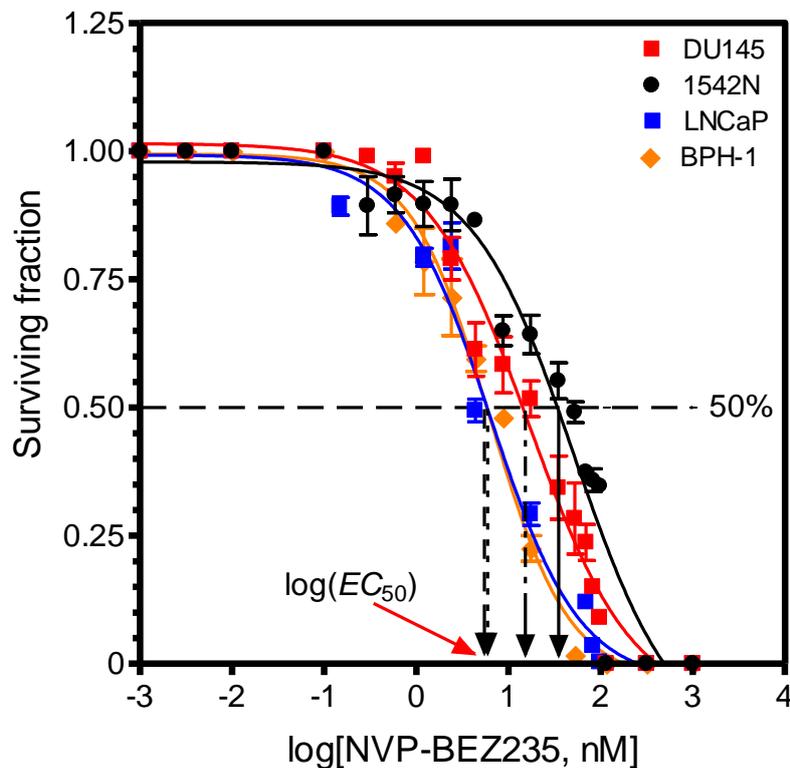


Figure 3.7: Cytotoxicity curves for PI3K and mTOR inhibitor (NVP-BEZ235) treatment of 4 human prostate cell lines (DU145, 1542N, LNCaP, BPH-1). Curves were obtained by plotting cell survival as a function of log (inhibitor concentration). Cell survival was determined by the colony assay, and data were fitted to a sigmoidal equation. Data points are means \pm SEM of 3 independent experiments. The log of the concentration at which 50% of cells survive (EC_{50}) is that at which each survival curve intersects the horizontal dashed line (as indicated by red arrow).

3.3.3. Cytotoxicity of Androgen Receptor Inhibitor (MDV3100)

The cytotoxicity of the androgen receptor inhibitor (MDV3100) was also concentration-dependent, as illustrated in Figure 3.8. The LNCaP cell line was significantly more resistant to androgen receptor inhibition than the rest of the cell lines ($P \leq 0.0008$), which were similarly sensitive to MDV3100 treatment. The rank order of increasing sensitivity to MDV3100 treatment is LNCaP \rightarrow 1542N \approx BPH-1 \approx DU145, with EC_{50} -values of 92.73 ± 10.55 , 16.92 ± 0.87 , 16.64 ± 1.20 , and 15.74 ± 0.94 nM, respectively (Table 3.4).

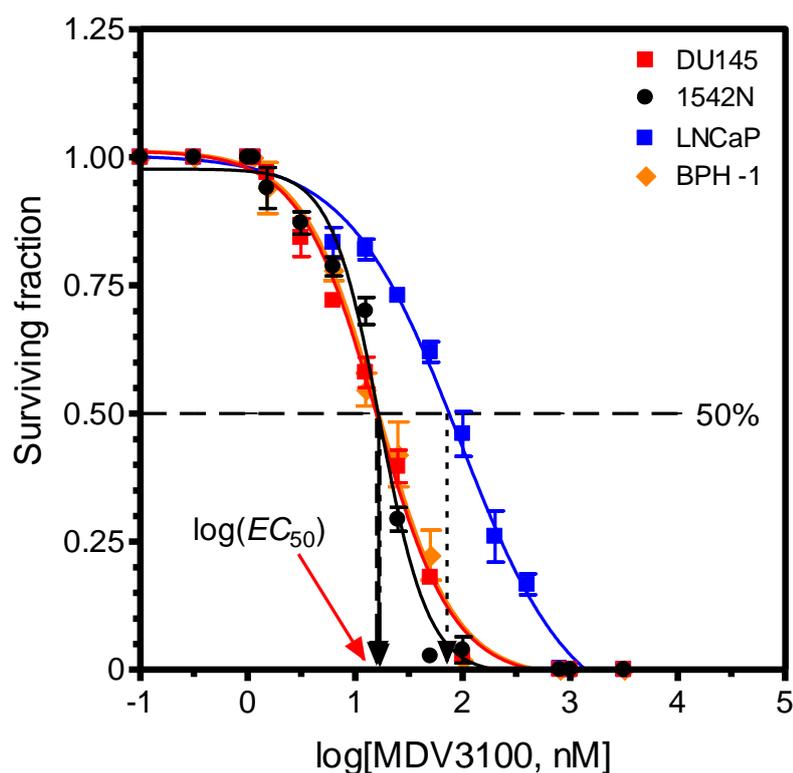


Figure 3.8: Cytotoxicity curves for PI3K and mTOR inhibitor (MDV3100) treatment of 4 human prostate cell lines (DU145, 1542N, LNCaP, BPH-1). Curves were obtained by plotting cell survival as a function of log (inhibitor concentration). Cell survival was determined by the colony assay, and data were fitted to a sigmoidal equation. Data points are means \pm SEM of 3 independent experiments. The log of the concentration at which 50% of cells survive (EC_{50}) is that at which each survival curve intersects the horizontal dashed line (as indicated by red arrow).

Table 3.4: Summary of cytotoxicity data for 2 human prostate cancer cell lines (DU145 and LNCaP), a benign prostatic hyperplasia cell line (BPH-1), and normal prostate cell line (1542N) treated with EGFR inhibitor (AG-1478), PI3K and mTOR inhibitor (NVP-BEZ235), and AR inhibitor (MDV3100). EC_{50} denotes the equivalent concentration for 50% cell survival. T and B are the maximum and minimum of the concentration-response curve, respectively (Figures 3.6-3.8). HS is the steepest slope of the curve.

Cell line	Treatment	EC_{50} (nM)	T	B	HS
DU145	AG-1478	7431±1052	0.98±0.02	-0.03±0.03	-0.84±0.08
	NVP-BEZ235	16.49±2.97	1.02±0.02	-0.08±0.05	-0.80±0.08
	MDV3100	15.74±0.94	1.01±0.01	-0.02±0.01	-1.22±0.08
LNCaP	AG-1478	290±31	1.02±0.02	0.03±0.02	-1.07±0.11
	NVP-BEZ235	6.42±0.74	0.99±0.01	-0.03±0.02	-0.91±0.08
	MDV3100	92.73±10.55	1.00±0.02	-0.10±0.04	-0.84±0.07
BPH-1	AG-1478	708±60	1.03±0.01	-0.02±0.01	-0.83±0.05
	NVP-BEZ235	6.26±0.59	0.99±0.02	-0.03±0.02	-1.02±0.09
	MDV3100	16.64±1.20	1.01±0.02	-0.02±0.02	-1.23±0.11
1542N	AG-1478	414±46	1.01±0.02	0.01±0.01	-1.02±0.10
	NVP-BEZ235	51.33±3.81	0.98±0.02	-0.16±0.09	-0.79±0.11
	MDV3100	16.92±0.87	0.98±0.01	-0.01±0.01	-1.92±0.17

To evaluate the existence of a potential therapeutic benefit when the prostate tumour cell lines are treated with the specified inhibitors, relative sensitivities (RS), as described in Section 2.11, were determined on the basis of the EC_{50} -values. The data presented in Table 3.5 summarise the relative sensitivities of the tumour cells.

Table 3.5: Summary of EC_{50} -values for 4 human prostate cell lines (normal: 1542N; cancer: DU145, LNCaP; benign prostate hyperplasia: BPH-1) and their relative radiosensitivities (RS) determined by clonogenic cell survival after exposure to X-rays (Equation 2.3). The 95% confidence intervals of the EC_{50} -values are in parentheses. P -value indicates the level of significance in the difference between the EC_{50} of the normal cell line (1542N) relative to those of the tumour cell lines (DU145, LNCaP, BPH-1).

Cell line	Treatment	EC_{50} (nM)	P -value	RS^*
1542N	AG-1478	414±46 (330-518)	-	-
	NVP-BEZ235	51.33±3.81 (32.32-81.53)	-	-
	MDV3100	16.92±0.87 (15.24-18.78)	-	-
DU145	AG-1478	7431±1052 (5554-9943)	0.0402	0.06±0.01
	NVP-BEZ235	16.49±2.97 (11.82-24.07)	0.0047	3.11±0.61
	MDV3100	15.74±0.94 (13.93-17.78)	0.9250	1.08±0.08
LNCaP	AG-1478	290±31 (233-360)	0.0783	1.43±0.22
	NVP-BEZ235	6.42±0.74 (5.09-8.10)	<0.0001	8.00±1.10
	MDV3100	92.73±10.55 (73.59-116.80)	<0.0001	0.18±0.02
BPH-1	AG-1478	708±60 (596-841)	0.0055	0.59±0.08
	NVP-BEZ235	6.26±0.59 (5.15-7.59)	0.0004	8.20±0.98
	MDV3100	16.64±1.20 (14.36-19.27)	0.9377	1.02±0.09

*Errors were calculated using error propagation formulae for ratios.

For EGFR inhibition (using AG-1478), the absence of a statistically significant difference in EC_{50} of the relatively more sensitive LNCaP cell line from that of the 1542N cell line resulted in a relative sensitivity of 1.43 ± 0.22 (Table 3.5), indicating a positive but limited therapeutic benefit. The markedly high resistance to EGFR

inhibitions exhibited by the DU145 and BPH-1 cell lines resulted in very low relative sensitivities of 0.06 ± 0.01 and 0.59 ± 0.08 , respectively.

In the case of inhibition of PI3K and mTOR (using NVP-BEZ235), the tumour cell lines, DU145, LNCaP, and BPH-1, were found to be 3- to 8-fold more sensitive than the normal cell line (1542N) with relative sensitivities of 3.11 ± 0.61 , 8.00 ± 1.10 , and 8.2 ± 0.98 , respectively (Table 3.5).

The sensitivity of the normal cell line (1542N) to androgen receptor (AR) inhibition (using MDV3100) was comparable to those of the DU145 and BPH-1 cell lines. This translated to relative sensitivities that do not differ from unity (Table 3.5). However, the LNCaP cell line emerged as highly resistant to AR inhibition, giving a relative sensitivity of 0.18 ± 0.02 .

3.4. Mode of Inhibitor Action in Cells

To determine the nature of cellular response to treatment with the EGFR, PI3K and mTOR, and AR inhibitors, the cell survival data presented in Figures 3.6-3.8 were log transformed as described in Section 2.13, to obtain median-effect plots for the inhibitors.

Figure 3.9 represents the median-effect plots for the DU145 cell line. The curves for EGFR and PI3K/mTOR inhibition are virtually parallel, with slopes of 0.78 and 0.73, respectively (Table 3.6; $m < 1.0$), indicating flat-sigmoidal concentration-effect curves. The median-effect concentrations (D_m) of AG-1478 and NVP-BEZ235 were

low, and emerged as 13.28 and 14.27 nM. On the other hand, androgen receptor inhibition in these cells yielded a much steeper curve with a slope of 1.63 ($m > 1.0$), indicating a sigmoidal response. The corresponding median-effect concentration of MDV3100 was ~370-fold higher than those of AG-1478 and NVP-BEZ235 (Table 3.6).

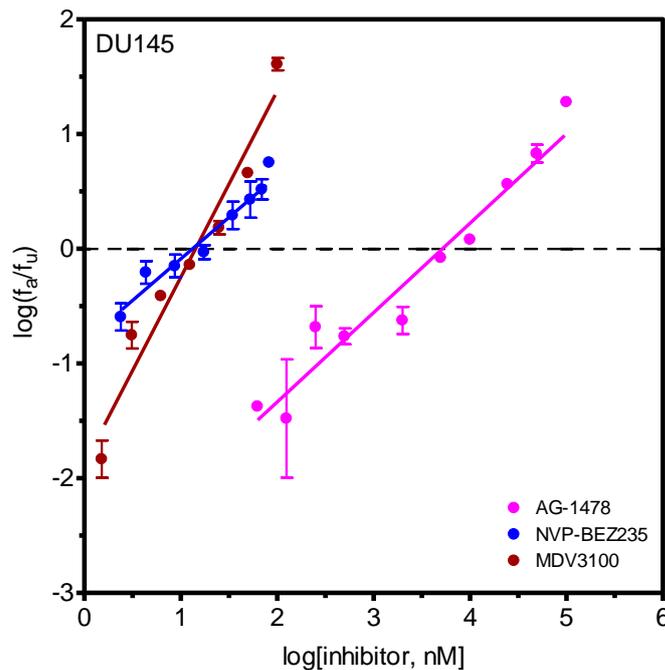


Figure 3.9: Median-effect plots for DU145 cells treated with AG-1478, NVP-BEZ235 and MDV3100 from the toxicity data presented in Figures 3.6 – 3.8. Transformed cell survival data were fitted to the function: $\log(f_a/f_u) = m \times \log(D) - m \times \log(D_m)$, where f_a and f_u are the affected and unaffected fractions of cells, respectively (Section 2.13). The coefficient m is an indicator of the shape of the inhibitor concentration-effect relationship. D and D_m are the concentration and median-effect concentration of the inhibitor.

Figure 3.10 shows the concentration-effect curves for the three inhibitors in the LNCaP cell line. Here, the curves for the PI3K/mTOR and androgen receptor inhibition are parallel and emerge with slopes of 0.81 and 0.83, respectively (Table 3.6; $m < 1.0$), implying that NVP-BEZ235 and MDV3100 display flat-sigmoidal cytotoxic effects in the LNCaP cell population. The corresponding median-effect

concentrations were 4.47 and 68.19 nM. Inhibition of EGFR in these cells with AG-1478 resulted in a steeper curve ($m = 1.27$), indicating a sigmoidal cytotoxic response. The median-effect concentration of AG-1478 in the LNCaP cell line was 368 nM.

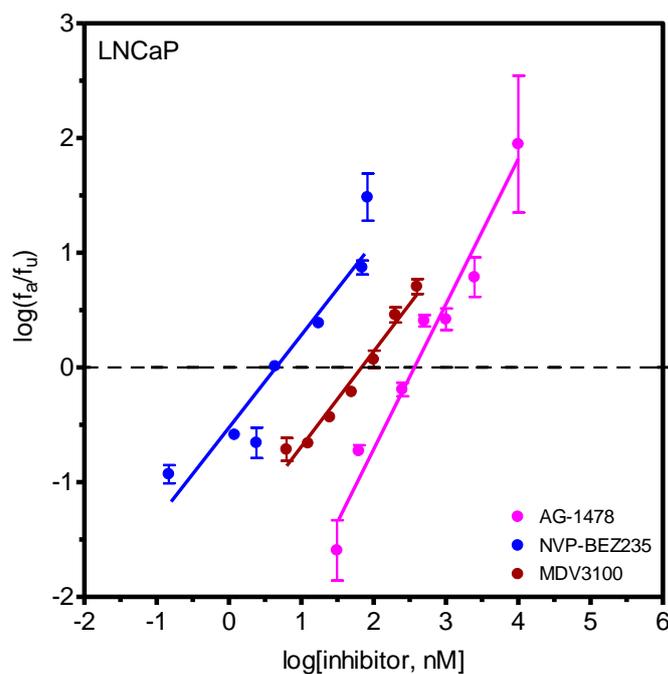


Figure 3.10: Median-effect plots for LNCaP cells treated with AG-1478, NVP-BE235 and MDV3100 from the toxicity data presented in Figures 3.6 – 3.8. Transformed cell survival data were fitted to the function: $\log(f_a/f_u) = m \times \log(D) - m \times \log(D_m)$, where f_a and f_u are the affected and unaffected fractions of cells, respectively (Section 2.13). The coefficient m is an indicator of the shape of the inhibitor concentration-effect relationship. D and D_m are the concentration and median-effect concentration of the inhibitor.

Table 3.6: Summary of parameters of median-effect plots for EGFR inhibitor (AG-1478), PI3K and mTOR inhibitor (NVP-BEZ235), and AR inhibitor (MDV3100) in 2 human prostate cancer cell lines (DU145 and LNCaP), a benign prostatic hyperplasia cell line (BPH-1), and normal prostate cell line (1542N).

Cell line	Treatment	<i>m</i>	<i>D_m</i> (nM)	Shape of concentration-effect curve
DU145	AG-1478	0.78 ± 0.06	5162 ± 3	flat-sigmoidal
	NVP-BEZ235	0.73 ± 0.07	13.28 ± 0.46	flat-sigmoidal
	MDV3100	1.63 ± 0.10	14.27 ± 0.27	sigmoidal
LNCaP	AG-1478	1.27 ± 0.11	368 ± 2	sigmoidal
	NVP-BEZ235	0.81 ± 0.07	4.47 ± 0.17	flat-sigmoidal
	MDV3100	0.83 ± 0.05	68.19 ± 0.62	flat-sigmoidal
BPH-1	AG-1478	0.95 ± 0.06	760 ± 2	flat-sigmoidal
	NVP-BEZ235	1.22 ± 0.13	4.31 ± 0.17	sigmoidal
	MDV3100	1.54 ± 0.13	14.14 ± 0.40	sigmoidal
1542N	AG-1478	1.00 ± 0.08	517 ± 2	hyperbolic
	NVP-BEZ235	0.67 ± 0.06	41.82 ± 0.74	flat-sigmoidal
	MDV3100	1.70 ± 0.13	11.35 ± 0.30	sigmoidal

PI3K/mTOR and androgen receptor inhibition in the BPH-1 cell line resulted in a sigmoidal cytotoxic response, giving shape parameters of 1.22 and 1.54, respectively (Figure 3.11; Table 3.6). The respective median-effect concentrations were found to be 4.31 and 14.14 nM. Inhibition of EGFR in BPH-1 cells yielded a flat-

sigmoidal response, with shape parameter of 0.95 and a resulting median-effect concentration of AG-1478 of 760 nM.

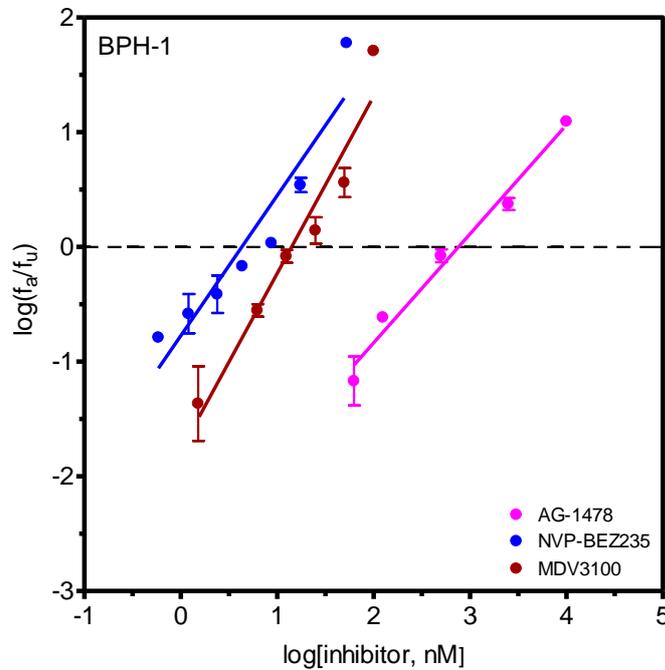


Figure 3.11: Median-effect plots for BPH-1 cells treated with AG-1478, NVP-BEZ235 and MDV3100 from the toxicity data presented in Figures 3.6 – 3.8. Transformed cell survival data were fitted to the function: $\log(f_a/f_u) = m \times \log(D) - m \times \log(D_m)$, where f_a and f_u are the affected and unaffected fractions of cells, respectively (Section 2.13). The coefficient m is an indicator of the shape of the inhibitor concentration-effect relationship. D and D_m are the concentration and median-effect concentration of the inhibitor.

Inhibition of EGFR, PI3K/mTOR, and AR in the 1542N cell line yielded hyperbolic, flat-sigmoidal, and sigmoidal cytotoxic responses, respectively (Figure 3.12). The shape parameters for treatment with AG-1478, NVP-BEZ235, and MDV3100 were 1.0, 0.67, and 1.70, respectively. The corresponding median-effect concentrations emerged as 517, 41.82, and 11.35 nM.

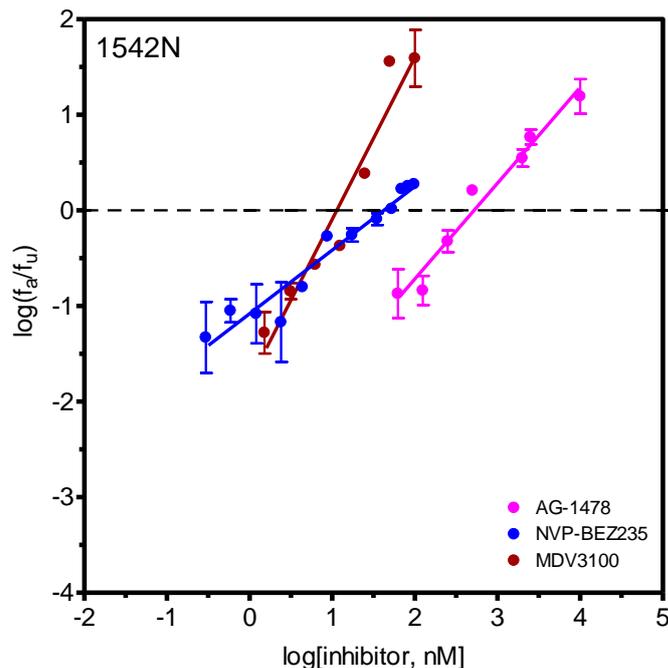


Figure 3.12: Median-effects plots for 1542N cells treated with AG-1478, NVP-BEZ235 and MDV3100 from the toxicity data presented in Figures 3.6 – 3.8. Transformed cell survival data were fitted to the function: $\log(f_a/f_u) = m \times \log(D) - m \times \log(D_m)$, where f_a and f_u are the affected and unaffected fractions of cells, respectively (Section 2.13). The coefficient m is an indicator of the shape of the inhibitor concentration-effect relationship. D and D_m are the concentration and median-effect concentration of the inhibitor.

3.5. Inhibitor Interaction

To evaluate potential interactions between the EGFR, PI3K and mTOR, and AR inhibitors in the DU145, LNCaP, BPH-1, and 1542N cell lines, combination indices (CI) were determined for two-inhibitor combinations (at EC_{50} concentrations), as described under Section 2.13. The inhibitor-inhibitor interaction data are summarised in Table 3.7. Combination of all inhibitors with each other resulted in very strong synergism ($CI < 0.1$) in the DU145 cell line.

While antagonism ($CI > 1.0$) was observed in the LNCaP cell line when the EGFR inhibitor (AG-1478) was combined with the PI3K/mTOR inhibitor (NVP-BEZ235) or the AR inhibitor (MDV3100), a very strong synergism emerged when cells were concomitantly treated with NVP-BEZ235 and MDV3100.

Table 3.7: Summary of combination indices for EGFR inhibitor (AG-1478), PI3K and mTOR inhibitor (NVP-BEZ235), and AR inhibitor (MDV3100), when used concurrently at their respective EC_{50} concentrations in 2 human prostate cancer cell lines (DU145 and LNCaP), a benign prostatic hyperplasia cell line (BPH-1), and normal prostate cell line (1542N).

Cell line	Agent 1	Agent 2		
		AG-1478	NVP-BEZ235	MDV3100
DU145	AG-1478	-	0.0082	0.0784
	NVP-BEZ235	0.0082	-	0.0787
	MDV3100	0.0784	0.0787	-
LNCaP	AG-1478	-	1.1035	1.0830
	NVP-BEZ235	1.1035	-	0.0315
	MDV3100	1.0830	0.0315	-
BPH-1	AG-1478	-	0.0998	0.0741
	NVP-BEZ235	0.0998	-	0.1591
	MDV3100	0.0741	0.1591	-
1542N	AG-1478	-	0.0096	0.1095
	NVP-BEZ235	0.0096	-	0.1028
	MDV3100	0.1095	0.1028	-

In the BPH-1 cell line, combining AG-1478 with the NVP-BEZ235 or MDV3100 resulted in a very strong synergism ($CI < 0.1$). However, concomitant treatment of cells with NVP-BEZ235 and MDV3100 was only synergistic ($0.1 \leq CI \leq 0.3$).

Similarly, Combination of all inhibitors with each other in the apparently normal cell line (1542N) yielded effects that ranged from strong to very strong synergism ($CI < 0.1$).

3.6. Radiomodulation by Inhibitors

To investigate the relationship between the fraction of cells retaining their reproductive integrity and absorbed radiation doses, and the impact of cellular exposure of inhibitors of EGFR (AG-1478), PI3K/mTOR (NVP-BEZ235) and AR (MDV3100) on radiosensitivity, the colony forming assay was used. The clonogenic cell survival data for the DU145 cell line (the most radioresistant cell line (Table 3.2) are presented in Figure 3.13. Pre-treatment with AG-1478, NVP3100, and a cocktail of both inhibitors radiosensitised these cells. However, the radiosensitisation by AG-1478 emerged only at doses higher than 6 Gy, as reflected in the modifying factors not differing markedly from unity (Table 3.8). Although the radiosensitisation by NVP-BEZ235 and the cocktail was high, it did not reach statistical significance, except for PI3K/mTOR inhibition when the parameter \bar{D} was used. The significant radiosensitisation is in line with the strong synergism seen in this cell line between these inhibitors (Table 3.7).

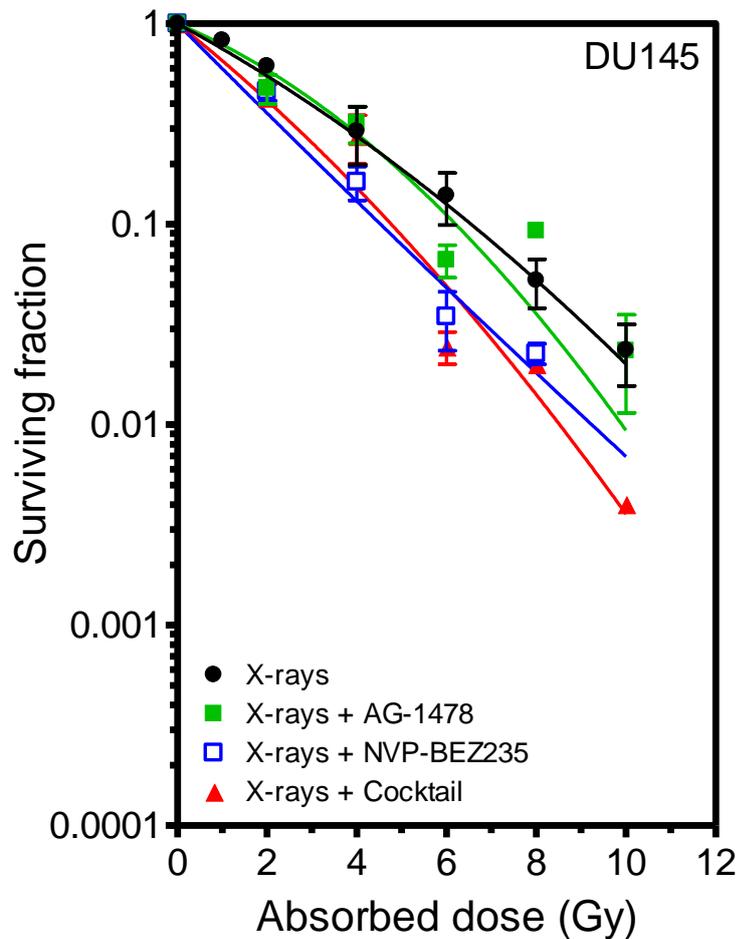


Figure 3.13: Cell survival curves for the DU145 cell line after X-ray irradiation. Cells were irradiated without or in the presence of AG-1478 and NVP-BEZ235, administered either singly or as a cocktail at EC_{50} concentrations. Symbols represent the mean surviving fraction \pm SEM from three independent experiments.

The data presented in Figure 3.14 are the cell survival curves for when PI3K/mTOR and AR were inhibited in the DU145 cell line with NVP-BEZ235 and MDV3100, singly and in combination. This inhibition resulted in radiosensitization over the entire radiation dose range, with modifying factors ranging from 1.20 to 4.33 (Table 3.9). Only the radiosensitisation by cocktail pre-treatment, when SF_6 and \bar{D} were used as radiosensitivity parameters, emerged statistically significant with modifying factors of

4.33 ($P = 0.0339$) and 1.37 ($P = 0.0422$), respectively. This level of radiosensitisation seems to be consistent with the strong synergism seen in this the cocktail (Table 3.7).

Table 3.8: Modifying factors (MF), relative to X-ray treatment alone, derived from SF_2 , SF_6 , and \bar{D} values, as described in Section 2.12 for the DU145 cell line, irradiated in the presence of AG-1478 and NVP-BEZ235, singly or as a cocktail at EC_{50} concentrations. Errors in modifying factors were calculated using error propagation formulae for ratios.

Treatment	Parameter	<i>P</i> -value	<i>MF</i>
SF_2			
X-rays	0.53 ± 0.10	-	-
X-rays + AG-1478	0.64 ± 0.19	0.6219	0.83 ± 0.29
X-rays + NVP-BEZ235	0.37 ± 0.06	0.1500	1.43 ± 0.36
X-rays + Cocktail	0.42 ± 0.08	0.3775	1.26 ± 0.34
SF_6			
X-rays	0.13 ± 0.04	-	-
X-rays + AG-1478	0.12 ± 0.02	0.8012	1.08 ± 0.38
X-rays + NVP-BEZ235	0.05 ± 0.01	0.0724	2.60 ± 0.95
X-rays + Cocktail	0.05 ± 0.02	0.1370	2.60 ± 1.31
\bar{D}			
X-rays	3.19 ± 0.38	-	-
X-rays + AG-1478	2.94 ± 0.32	0.5692	1.09 ± 0.18
X-rays + NVP-BEZ235	2.34 ± 0.15	0.0489*	1.36 ± 0.18
X-rays + Cocktail	2.51 ± 0.27	0.1867	1.27 ± 0.20

*Statistically significant difference between X-ray treatment alone and irradiation in the presence of inhibitor.

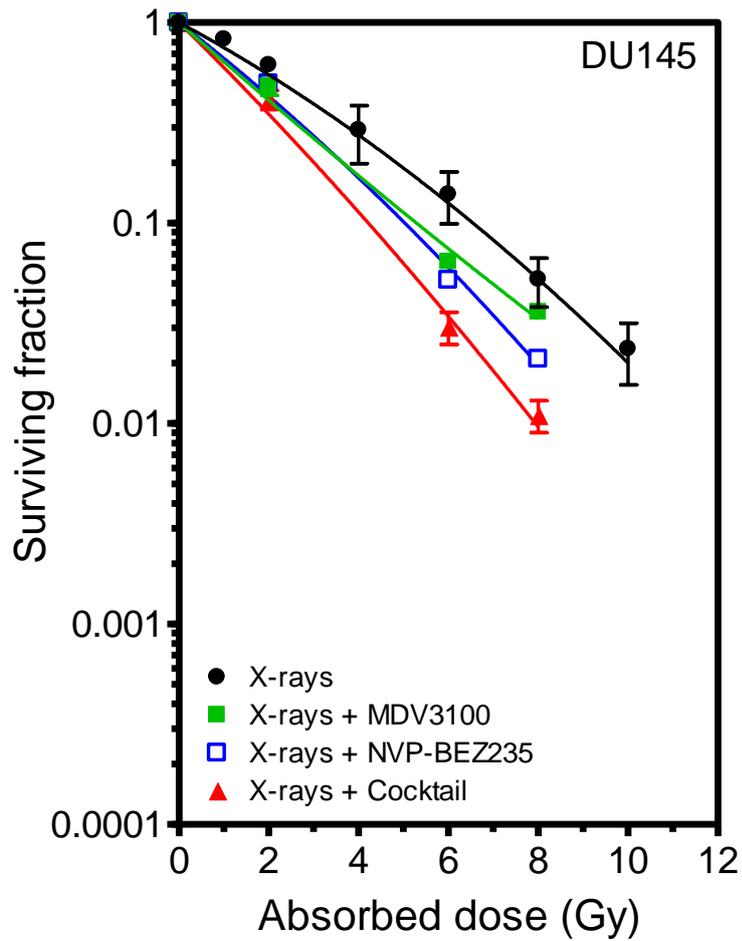


Figure 3.14: Cell survival curves for the DU145 cell line after X-ray irradiation. Cells were irradiated without or in the presence of MDV3100 and NVP-BEZ235, administered either singly or as a cocktail at EC_{50} concentrations. Symbols represent the mean surviving fraction \pm SEM from three independent experiments.

Table 3.9: Modifying factors (MF), relative to X-ray treatment alone, derived from SF_2 , SF_6 , and \bar{D} values, as described in Section 2.12 for the DU145 cell line, irradiated in the presence of MDV3100 and NVP-BEZ235, singly or as a cocktail at EC_{50} concentrations. Errors in modifying factors were calculated using error propagation formulae for ratios.

Treatment	Parameter	<i>P</i> -value	<i>MF</i>
SF_2			
X-rays	0.53 ± 0.10	-	-
X-rays + MDV3100	0.41 ± 0.02	0.1538	1.29 ± 0.25
X-rays + NVP-BEZ235	0.43 ± 0.02	0.2139	1.23 ± 0.23
X-rays + Cocktail	0.38 ± 0.06	0.1711	1.39 ± 0.34
SF_6			
X-rays	0.13 ± 0.04	-	-
X-rays + MDV3100	0.08 ± 0.01	0.1538	1.63 ± 0.54
X-rays + NVP-BEZ235	0.06 ± 0.01	0.0879	2.17 ± 0.76
X-rays + Cocktail	0.03 ± 0.01	0.0339*	4.33 ± 1.97
\bar{D}			
X-rays	3.19 ± 0.38	-	-
X-rays + MDV3100	2.67 ± 0.13	0.1544	1.20 ± 0.15
X-rays + NVP-BEZ235	2.67 ± 0.09	0.1376	1.20 ± 0.15
X-rays + Cocktail	2.33 ± 0.12	0.0422*	1.37 ± 0.18

*Statistically significant difference between X-ray treatment alone and irradiation in the presence of inhibitor.

Figure 3.15 displays results for the androgen dependent cell line (LNCaP), which is the most radiosensitive cell line according to Table 3.2. In general, pre-treatment of

these cells with AG-1478, NVP-BEZ235, or their cocktail either had no effect or tended to enhance cell survival, as can be deduced from the modifying factors presented in Table 3.10. The absence of a significant radiosensitisation by the inhibitor cocktail supports the finding that the mode of interaction between AG-1478 and NVP-BEZ235 in this cell line is antagonistic (Table 3.7).

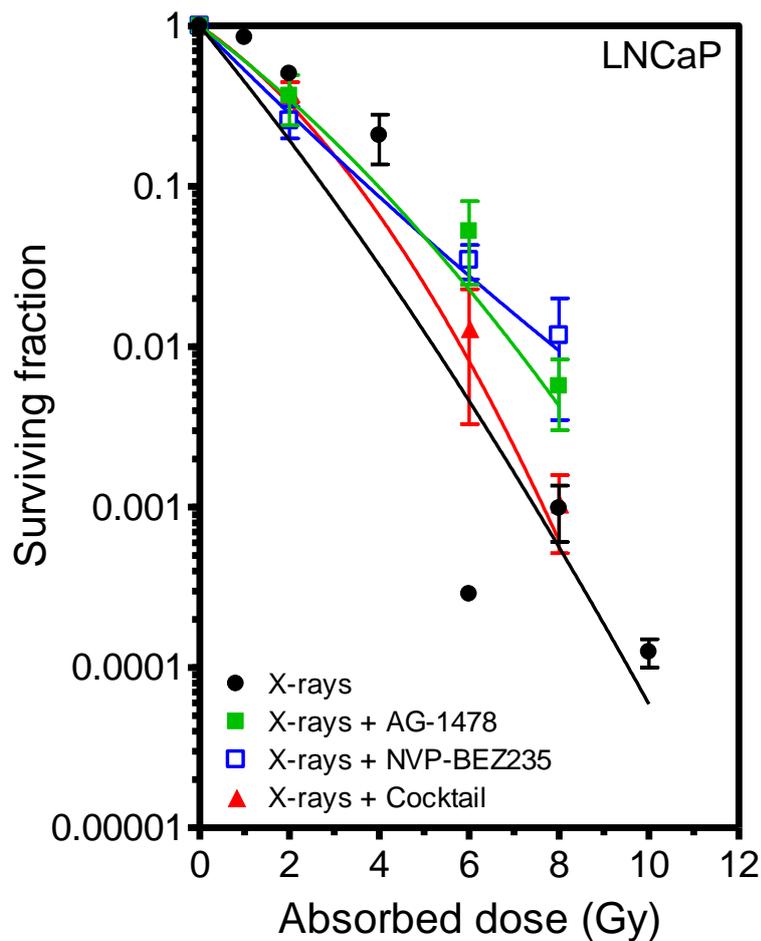


Figure 3.15: Cell survival curves for the LNCaP cell line after X-ray irradiation. Cells were irradiated without or in the presence of AG-1478 and NVP-BEZ235, administered either singly or as a cocktail at EC_{50} concentrations. Symbols represent the mean surviving fraction \pm SEM from three independent experiments.

Table 3.10: Modifying factors (MF), relative to X-ray treatment alone, derived from SF_2 , SF_6 , and \bar{D} values, as described in Section 2.12 for the LNCaP cell line, irradiated in the presence of AG-1478 and NVP-BEZ235, singly or as a cocktail at EC_{50} concentrations. Errors in modifying factors were calculated using error propagation formulae for ratios.

Treatment	Parameter	<i>P</i> -value	<i>MF</i>
SF_2			
X-rays	0.49 ± 0.16	-	-
X-rays + AG-1478	0.36 ± 0.08	0.4159	1.36 ± 0.54
X-rays + NVP-BEZ235	0.29 ± 0.07	0.2145	1.69 ± 0.69
X-rays + Cocktail	0.36 ± 0.12	0.4660	1.36 ± 0.64
SF_6			
X-rays	0.02 ± 0.01	-	-
X-rays + AG-1478	0.03 ± 0.01	0.5343	0.67 ± 0.40
X-rays + NVP-BEZ235	0.03 ± 0.01	0.4175	0.67 ± 0.40
X-rays + Cocktail	0.01 ± 0.01	0.4331	2.00 ± 2.23
\bar{D}			
X-rays	2.73 ± 0.29	-	-
X-rays + AG-1478	2.26 ± 0.30	0.2703	1.21 ± 0.21
X-rays + NVP-BEZ235	1.88 ± 0.21	0.0433*	1.45 ± 0.22
X-rays + Cocktail	2.21 ± 0.20	0.1396	1.24 ± 0.17

*Statistically significant difference between X-ray treatment alone and irradiation in the presence of inhibitor.

However, pre-treatment of the LNCaP cells with the androgen receptor inhibitor, MDV3100, either alone or in combination with NVP-BEZ235 resulted in enhanced radiosensitivity (Figure 3.16). In terms of SF_2 and SF_6 , the modifying factors were found to range between 2.72 and 21.30, with the cocktail tending to yield high radiosensitisation (Table 3.11). The extent of radiosensitisation seen in the cocktail pre-treated cell cultures supports strong synergism between NVP-BEZ235 MDV3100 (Table 3.7). The modifying factors obtained from \bar{D} were lower (Table 3.11).

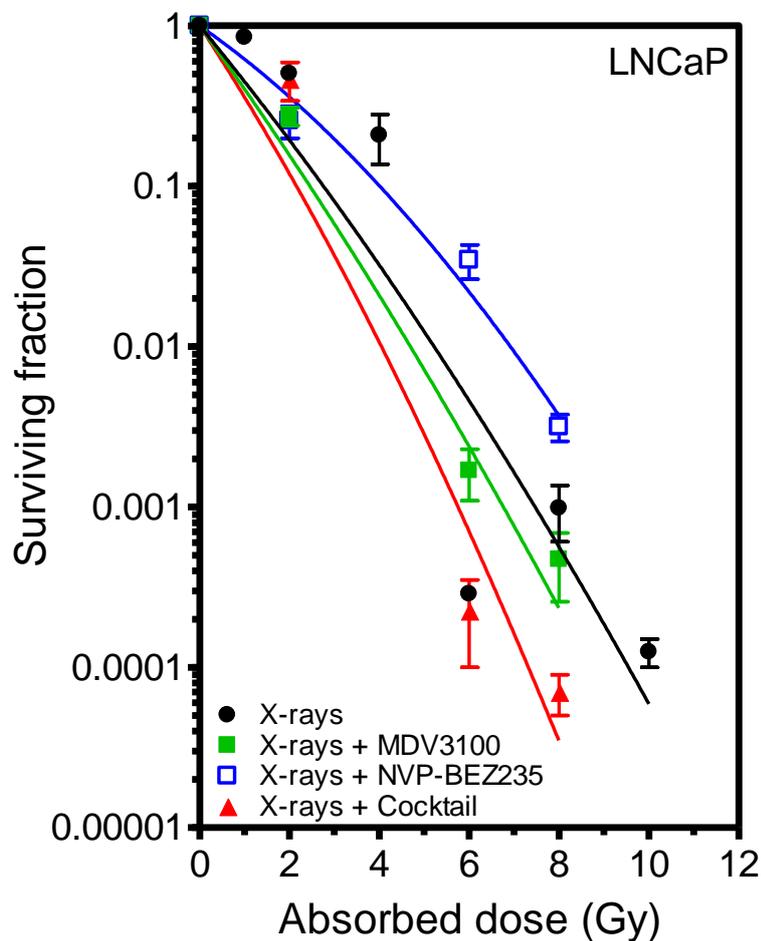


Figure 3.16: Cell survival curves for the LNCaP cell line after X-ray irradiation. Cells were irradiated without or in the presence of MDV3100 and NVP-BEZ235, administered either singly or as a cocktail at EC_{50} concentrations. Symbols represent the mean surviving fraction \pm SEM from three independent experiments.

Table 3.11: Modifying factors (MF), relative to X-ray treatment alone, derived from SF_2 , SF_6 , and \bar{D} values, as described in Section 2.12 for the LNCaP cell line, irradiated in the presence of MDV3100 and NVP-BEZ235, singly or as a cocktail at EC_{50} concentrations. Errors in modifying factors were calculated using error propagation formulae for ratios.

Treatment	Parameter	<i>P</i>-value	<i>MF</i>
SF_2			
X-rays	0.49 ± 0.16	-	-
X-rays + MDV3100	0.18 ± 0.07	0.0785	2.72 ± 1.38
X-rays + NVP-BEZ235	0.39 ± 0.11	0.5580	1.26 ± 0.54
X-rays + Cocktail	0.12 ± 0.01	0.0298*	4.08 ± 1.37
SF_6			
X-rays	0.0213 ± 0.0067	-	-
X-rays + MDV3100	0.0027 ± 0.0009	0.0506	7.89 ± 3.62
X-rays + NVP-BEZ235	0.0230 ± 0.0047	0.8487	0.93 ± 0.29
X-rays + Cocktail	0.0010 ± 0.0001	0.1152	21.30 ± 7.03
\bar{D}			
X-rays	2.73 ± 0.29	-	-
X-rays + MDV3100	1.83 ± 0.11	0.0168*	1.49 ± 0.18
X-rays + NVP-BEZ235	1.88 ± 0.20	0.0399*	1.45 ± 0.22
X-rays + Cocktail	2.40 ± 0.37	0.4466	1.14 ± 0.21

*Statistically significant difference between X-ray treatment alone and irradiation in the presence of inhibitor.

Overall, pre-treatment of the benign prostatic hyperplasia cell line (BPH-1) to NVP-BE235 or a cocktail of NVP-BE235 and AG-1478 did not result in a significant change in radiosensitivity (Figure 3.17; Table 3.12). However, pre-treatment of these cells with AG-1478 alone appeared to lead to enhanced radioresistance, on the basis of the mean inactivation dose (Table 3.12). The absence of a marked enhancement in radiosensitivity in the cocktail pre-treated cultures does not support the strong synergy seen between NVP-BE235 and AG-1478 (Table 3.7).

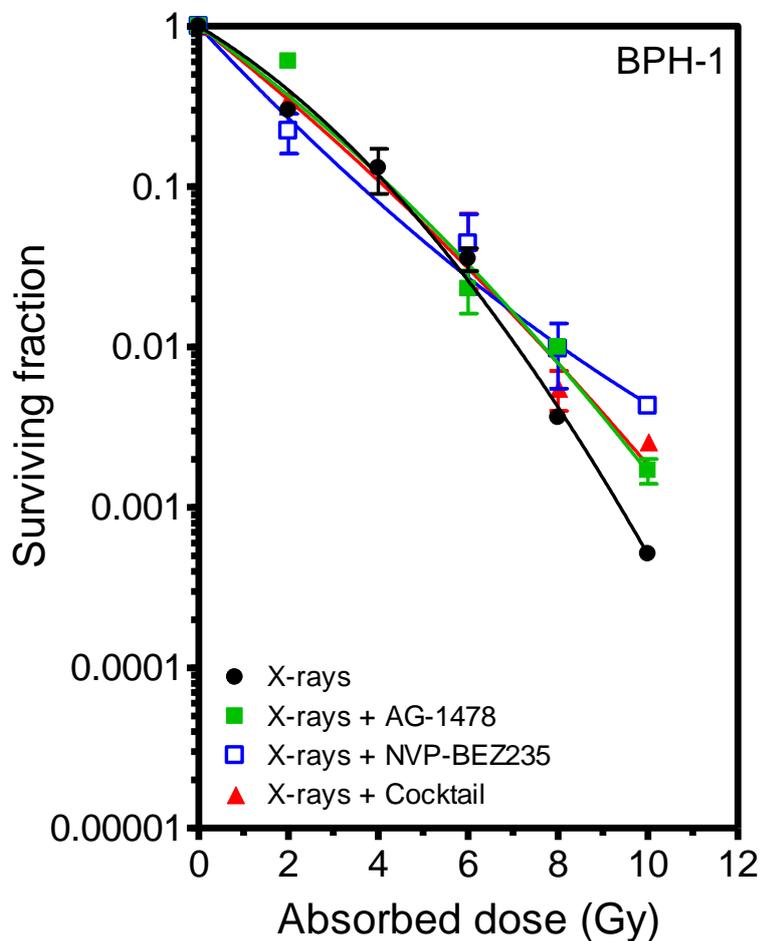


Figure 3.17: Cell survival curves for the BPH-1 cell line after X-ray irradiation. Cells were irradiated without or in the presence of AG-1478 and NVP-BE235, administered either singly or as a cocktail at EC_{50} concentrations. Symbols represent the mean surviving fraction \pm SEM from three independent experiments.

Table 3.12: Modifying factors (*MF*), relative to X-ray treatment alone, derived from SF_2 , SF_6 , and \bar{D} values, as described in Section 2.12 for the BPH-1 cell line, irradiated in the presence of AG-1478 and NVP-BEZ235, singly or as a cocktail at EC_{50} concentrations. Errors in modifying factors were calculated using error propagation formulae for ratios.

Treatment	Parameter	<i>P</i> -value	<i>MF</i>
SF_2			
X-rays	0.40 ± 0.07	-	-
X-rays + AG-1478	0.39 ± 0.06	0.8196	1.03 ± 0.24
X-rays + NVP-BEZ235	0.29 ± 0.10	0.3718	1.38 ± 0.53
X-rays + Cocktail	0.40 ± 0.08	0.9944	1.00 ± 0.27
SF_6			
X-rays	0.03 ± 0.01	-	-
X-rays + AG-1478	0.04 ± 0.01	0.3869	0.75 ± 0.31
X-rays + NVP-BEZ235	0.04 ± 0.02	0.6367	0.75 ± 0.45
X-rays + Cocktail	0.04 ± 0.02	0.4640	0.75 ± 0.45
\bar{D}			
X-rays	1.94 ± 0.14	-	-
X-rays + AG-1478	2.91 ± 0.06	0.0011*	0.67 ± 0.05
X-rays + NVP-BEZ235	1.84 ± 0.28	0.7556	1.05 ± 0.18
X-rays + Cocktail	2.25 ± 0.15	0.1533	0.86 ± 0.08

*Statistically significant difference between X-ray treatment alone and irradiation in the presence of inhibitor.

Similar results were obtained when the BPH-1 cells were pre-treated with MDV3100 or a cocktail of MDV3100 and NVP-BEZ235 (Figure 3.18), and do not reflect the synergy demonstrated between these inhibitors (Table 3.7).

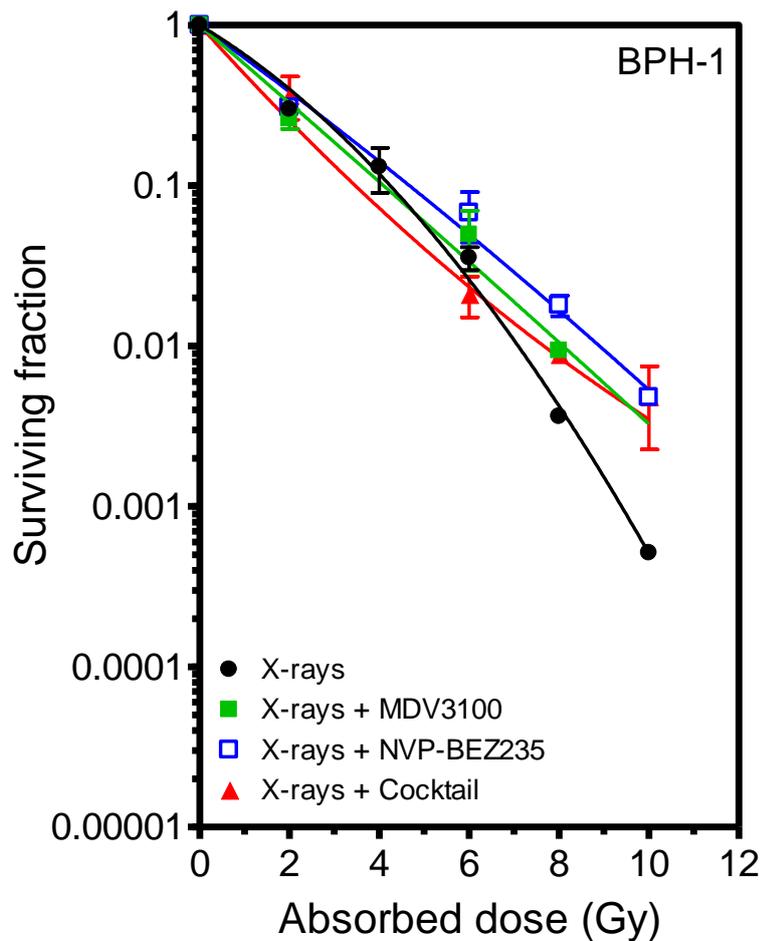


Figure 3.18: Cell survival curves for the BPH-1 cell line after X-ray irradiation. Cells were irradiated without or in the presence of MDV3100 and NVP-BEZ235, administered either singly or as a cocktail at EC_{50} concentrations. Symbols represent the mean surviving fraction \pm SEM from three independent experiments.

Table 3.13: Modifying factors (MF), relative to X-ray treatment alone, derived from SF_2 , SF_6 , and \bar{D} values, as described in Section 2.12 for the BPH-1 cell line, irradiated in the presence of MDV3100 and NVP-BEZ235, singly or as a cocktail at EC_{50} concentrations. Errors in modifying factors were calculated using error propagation formulae for ratios.

Treatment	Parameter	<i>P</i>-value	<i>MF</i>
SF_2			
X-rays	0.40 ± 0.07	-	-
X-rays + MDV3100	0.34 ± 0.06	0.4570	1.18 ± 0.29
X-rays + NVP-BEZ235	0.39 ± 0.08	0.8570	1.03 ± 0.28
X-rays + Cocktail	0.26 ± 0.03	0.0640	1.54 ± 0.32
SF_6			
X-rays	0.03 ± 0.01	-	-
X-rays + MDV3100	0.04 ± 0.01	0.4338	0.75 ± 0.31
X-rays + NVP-BEZ235	0.06 ± 0.02	0.1485	0.50 ± 0.24
X-rays + Cocktail	0.02 ± 0.01	0.5175	1.50 ± 0.90
\bar{D}			
X-rays	1.94 ± 0.14	-	-
X-rays + MDV3100	1.94 ± 0.13	0.9848	1.00 ± 0.10
X-rays + NVP-BEZ235	2.15 ± 0.17	0.3358	0.90 ± 0.10
X-rays + Cocktail	2.19 ± 0.31	0.4913	0.89 ± 0.14

Pre-treatment of the apparently normal prostate cell line (1542N) with the PI3K/mTOR and AR inhibitors, singly or in combination, only seemed to induced radiosensitisation at radiation doses higher than 4 Gy (Figure 3.20). In fact, the PI3K/mTOR inhibitor (NVP-BEZ235) with showed a radioprotective effect in these cells at lower doses. From the modifying factors presented in Table 3.14, no significant effect was apparent for all treatment permutations. Again, absence of a marked effect in the cocktail pre-treated cultures does not support the strong synergy shown between AG-1478 and NVP-BEZ235 (Table 3.7).

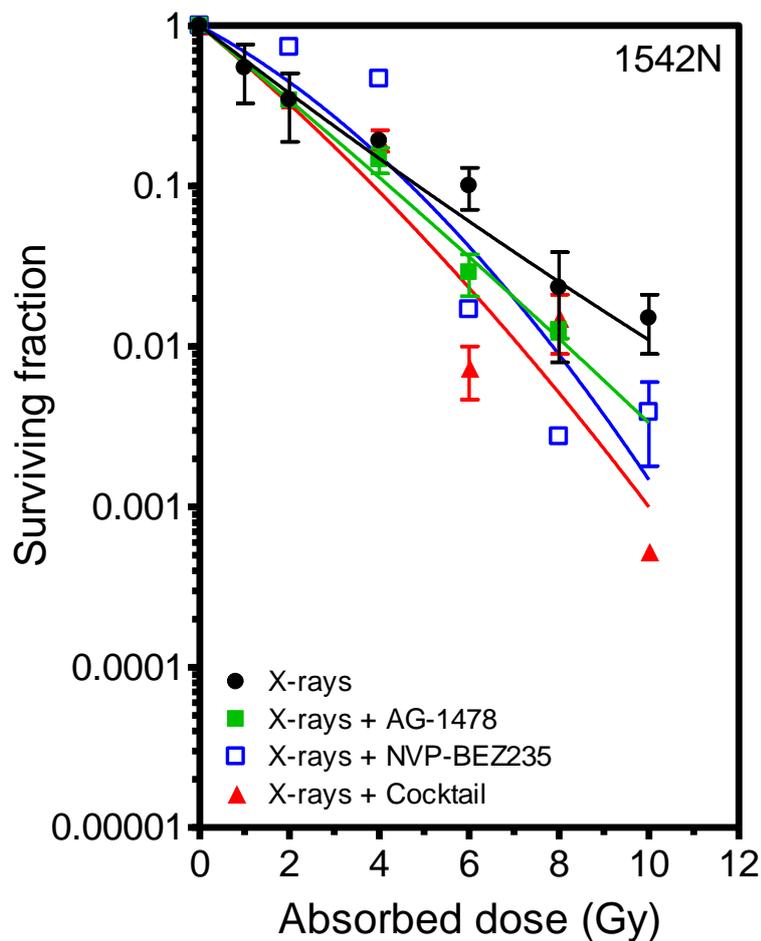


Figure 3.19: Cell survival curves for the 1542N cell line after X-ray irradiation. Cells were irradiated without or in the presence of AG-1478 and NVP-BEZ235, administered either singly or as a cocktail at EC_{50} concentrations. Symbols represent the mean surviving fraction \pm SEM from three independent experiments.

Table 3.14: Modifying factors (MF), relative to X-ray treatment alone, derived from SF_2 , SF_6 , and \bar{D} values, as described in Section 2.12 for the 1542N cell line, irradiated in the presence of AG-1478 and NVP-BEZ235, singly or as a cocktail at EC_{50} concentrations. Errors in modifying factors were calculated using error propagation formulae for ratios.

Treatment	Parameter	<i>P</i>-value	<i>MF</i>
SF_2			
X-rays	0.39 ± 0.10	-	-
X-rays + AG-1478	0.34 ± 0.02	0.5568	1.15 ± 0.30
X-rays + NVP-BEZ235	0.45 ± 0.05	0.4857	0.87 ± 0.24
X-rays + Cocktail	0.33 ± 0.06	0.5345	1.18 ± 0.37
SF_6			
X-rays	0.07 ± 0.04	-	-
X-rays + AG-1478	0.04 ± 0.01	0.3180	1.75 ± 1.09
X-rays + NVP-BEZ235	0.04 ± 0.01	0.4242	1.75 ± 1.09
X-rays + Cocktail	0.03 ± 0.01	0.2014	2.33 ± 1.54
\bar{D}			
X-rays	2.09 ± 0.44	-	-
X-rays + AG-1478	1.59 ± 0.37	0.4769	1.32 ± 0.46
X-rays + NVP-BEZ235	3.46 ± 0.17	0.0694	0.60 ± 0.13
X-rays + Cocktail	2.12 ± 0.11	0.9631	0.99 ± 0.21

Similarly, pre-treatment of the 1542N cells with MDV3100 or a cocktail of MDV3100 and NVP-BEZ235 did not result in significant radiomodulatory effects (Figure 3.20; Table 3.15). This finding is also not in line with the strong synergism shown between MDV3100 and NVP-BEZ235 (Table 3.7).

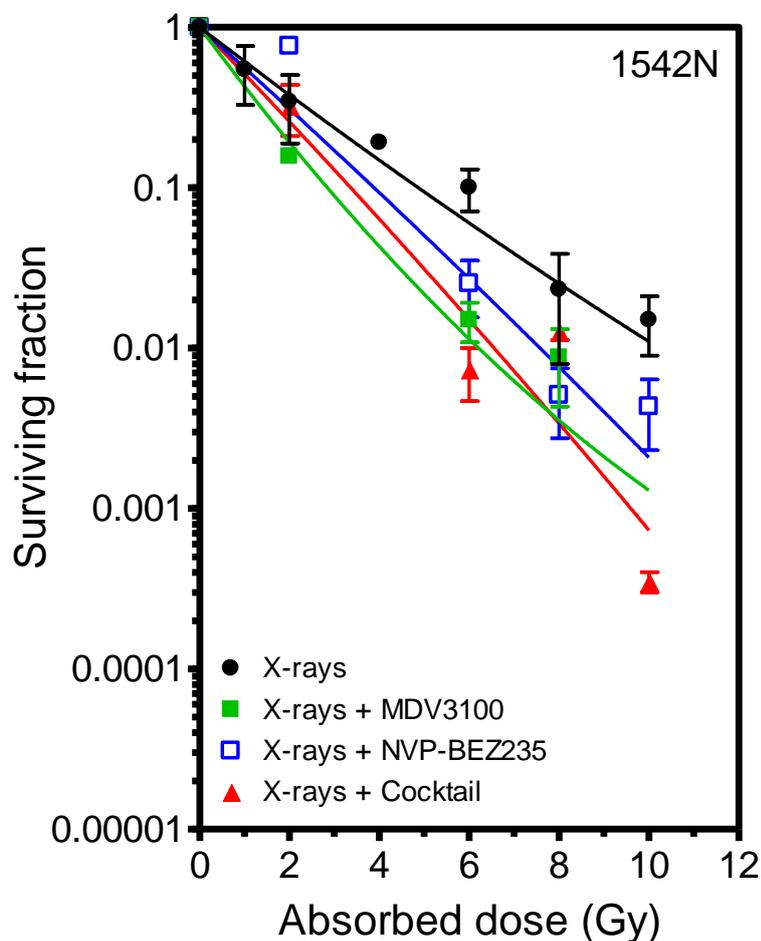


Figure 3.20: Cell survival curves for the 1542N cell line after X-ray irradiation. Cells were irradiated without or in the presence of MDV3100 and NVP-BEZ235, administered either singly or as a cocktail at EC_{50} concentrations. Symbols represent the mean surviving fraction \pm SEM from three independent experiments.

Table 3.15: Modifying factors (MF), relative to X-ray treatment alone, derived from SF_2 , SF_6 , and \bar{D} values, as described in Section 2.12 for the 1542N cell line, irradiated in the presence of MDV3100 and NVP-BEZ235, singly or as a cocktail at EC_{50} concentrations. Errors in modifying factors were calculated using error propagation formulae for ratios.

Treatment	Parameter	<i>P</i>-value	<i>MF</i>
SF_2			
X-rays	0.39 ± 0.10	-	-
X-rays + MDV3100	0.20 ± 0.04	0.0720	1.95 ± 0.63
X-rays + NVP-BEZ235	0.32 ± 0.07	0.5236	1.22 ± 0.41
X-rays + Cocktail	0.22 ± 0.06	0.1355	1.77 ± 0.66
SF_6			
X-rays	0.07 ± 0.04	-	-
X-rays + MDV3100	0.01 ± 0.01	0.1274	7.00 ± 8.06
X-rays + NVP-BEZ235	0.03 ± 0.01	0.2681	2.33 ± 1.54
X-rays + Cocktail	0.02 ± 0.01	0.1303	3.50 ± 2.66
\bar{D}			
X-rays	2.09 ± 0.44	-	-
X-rays + MDV3100	1.53 ± 0.04	0.3414	1.37 ± 0.29
X-rays + NVP-BEZ235	3.39 ± 0.13	0.0742	0.62 ± 0.13
X-rays + Cocktail	2.01 ± 0.33	0.9043	1.04 ± 0.28

CHAPTER 4: DISCUSSION

4.1. Androgen Sensitivity of 1542N, BPH-1, DU145, and LNCaP Cells

Prostate cancer presents itself as a heterogeneous disease (Shah et al., 2004; Dicken et al., 2018), meaning the same tumour expresses different genotypes giving clinicians a headache in managing the disease, because treatment is not a “one size fits all” approach. Conventional therapy still plays a pivotal role in the management of PCa, however there is room for improvement as quality of life and survival rate of patients is compromised. Targeted therapy has been investigated in the last 3 decades and promising outcomes have been brought forward (Hurvitz et al., 2013; Kratochwil et al., 2016). Interestingly, the biological processes leading to the development of PCa might provide the answers in offering tailored medicine. For the purpose of this study, the androgen sensitivity of 4 prostate cell lines was investigated (DU145, LNCaP, BPH-1, 1542N). As mentioned, PCa is a disease of hormones and its progression is driven largely by hormones, and so the majority of prostate cancers are androgen dependent (Chen et al., 2008; Ryan and Tindall, 2011; Jain et al., 2012). Consequently, PCa patients benefit from androgen deprivation therapy by surgical or chemical castration, and combination therapy, too, has yielded results in recent clinical trials (Thamilselvan et al., 2017; Scott, 2017).

The challenge, then, is the androgen independent stage, where prostate tumour growth and survival does not depend on androgens only, but on other avenues, for example, the PI3K and EGFR pathways. The molecular basis for the switch from androgen dependence to androgen independence in prostate cancer is largely unknown and has been under investigation for decades (Tilley et al., 1990; Chen et al., 1992; Yuan et al., 1993; Serafin et al., 2001; 2003). However, there is

speculation, such as the feedback loop, also known also as “cross talk”, between survival pathways (Carracedo and Pandolfi, 2008; Carracedo et al., 2008; Joshi, 2015). For example, inhibition of AR might activate the (PI3K, mTOR or EGFR), increase in proliferation and survival, resulting in castration-resistant prostate cancer (CRPC) and hormone-refractory prostate cancer (HRPC) (Lonergan and Tindall, 2011; Jain et al., 2012).

The androgen sensitivity assay shows that addition of 5α -dihydrotestosterone (5α -DHT), at concentrations of 0.001 to 100 nM, to cell culture medium has no significant effect on BPH-1 cell growth (Figure 3.3). However, when the LNCaP cells were cultured in charcoal-stripped medium, spiked with 5α -DHT over the same concentration range, proliferation was enhanced and fully restored at 10 nM of 5α -DHT (Figure 3.2). These findings are consistent with those of Serafin et al. demonstrating that BPH-1 and LNCaP cell lines are androgen independent and dependent, respectively (Serafin et al., 2001). Contrary to the observation that the DU145 and 1542N cell lines are androgen independent (Serafin et al., 2001; 2002), this study shows that 5α -DHT (at 0.001 – 1.0 nM) appeared to inhibit cell proliferation in these cell lines (Figures 3.1 and 3.4). Interestingly, at higher 5α -DHT concentrations (10 – 100 nM), proliferation in the DU145 and 1542N cells recovered to normal levels. These results seem to suggest that the DU145 and 1542N cell line are not androgen independent as initially thought (Serafin et al., 2001; 2002). In other studies by Kaighn and Tilley and colleagues the PC-3 and DU145 cell lines were seen to not express an androgen receptor and were, therefore, not dependent on androgens for growth (Kaighn et al., 1979; Tilley et al., 1990). The reason for this disparity is not known, but the current finding that cell proliferation recovered in the

DU145 and 1542N cell lines (Figures 3.1 and 3.4) at 5α -DHT concentrations of 10 - 100 nM, thus linking them to androgen dependence, seems to suggest that androgen may have a dual effect on these cells. Does this perhaps suggest that these cell lines have switched from an androgen independent status to an androgen dependent status? This result could be beneficial, especially for androgen independent cancers, which are resistant to treatment, and it would open the door to available treatment options. Especially the ADT which is the standard treatment protocol for androgen dependent cancers.

As for the LNCaP cell line, defined as androgen dependent, our results concur with work done by Serafin and colleagues (Serafin et al; 2002), also with increasing concentrations of 5α -DHT at 10 to 100 nM, similar results were observed by (Guo et al., 2000; Chen et al., 1992). The LNCaP cell line is believed to have a point mutation in the ligand-binding domain of the receptor gene, and, hence, is responsive to androgens (Veldscholte et al., 1990; 1992).

4.2. Therapeutic Benefit of X-rays and Inhibitors

Using the clonogenic survival assay, it is demonstrated for 4 human prostate cell lines that there is no significant therapeutic advantage when D_{50} -values of the tumour cell lines (LNCaP, BPH-1, DU145) were compared with that of the apparently normal cell line (1542N) (Figure 3.5, Table 3.3). The ranking order, from most radiosensitive to least radiosensitive using X-ray irradiation, is LNCaP>1542N>BPH-1>DU145, and is in close agreement with radiosensitivity data reported previously for these cell lines following cobalt-60 γ -ray exposure (Serafin et al., 2003). The absence

of a therapeutic benefit may be explained by the fact that the D_{50} -values are derived at 50% cell survival which coincides with the shoulder region of the survival curves, and may not differ markedly. For instance, a comparison of doses at the level of 10% survival, yields relative sensitivities of 1.77, 1.18, and 0.78, for the LNCaP, BPH-1, and DU145 cell lines, respectively, indicating a potential benefit for the former two. However, the rationale for choosing D_{50} and EC_{50} was to enable comparison of the relative sensitivities (RS) of the cell lines at the same level of survival, when as low as possible radiation dose or inhibitor concentration is administered. It should also be acknowledged that molecular assays have been developed applying multigene expression profiles to predict tumour radiosensitivity by comparisons with clonogenic survival data from established cell lines (Eschrich et al., 2009; Torres-Roca, 2012), that likely have the most potential for clinical implementation. Such methods have been shown to be statistically predictive of tumour response in oesophageal and rectal cancers, and of locoregional control in head and neck cancers (Eschrich et al., 2009). As such, there is the need to explore avenues like the inhibition of EGFR, PI3K, and mTOR, described here.

The cytotoxic effects of AG-1478 (EGFR inhibitor), NVP-BE235 (PI3K and mTOR inhibitor), and MDV3100 (AR inhibitor) are concentration-dependent (Figures 3.6-3.8). For AG-1478, EC_{50} values ranged from 290 - 7431 nM (Figure 3.6, Table 3.5), giving relative sensitivities of less than 1.0 for the androgen-independent BPH-1 cell line (Figure 3.3) and the DU145 cell line in which androgen appears to exert both anti- and pro-proliferative effects (Figure 3.1). This clearly shows that the normal cells (1542N) are more sensitive to EGFR inhibition than their tumour counterparts and use of AG-1478 for treatment of prostate cancer might lead to undesirable

outcomes. However, inhibiting EGFR in the androgen-dependent LNCaP cells (Figure 3.2) showed a small therapeutic benefit, with a relative sensitivity of 1.52 (Table 3.5). The significant level of resistance to EGFR inhibition seen in the DU145 and BPH-1 cell lines (relative sensitivities of 0.06 and 0.59, respectively), relative to the LNCaP cell line, is likely due to the fact that EGFR expression in the former is over 5-fold that in the latter (Sherwood et al., 1998; El Sheikh et al., 2004; Pignon et al., 2009). Higher EGFR expression levels would require significantly larger concentrations of inhibitor to achieve a given proportion of cell killing. On the other hand, EGFR expression in the androgen-dependent LNCaP cells is low and comparable to that in the normal 1542N cells (Hastie et al., 2005), consistent with the observed relative sensitivity of 1.43 ± 0.22 (Table 3.5).

For NVP-BEZ235 treatment, EC_{50} values ranged from 6.26 – 51.33 nM for all cell lines (Figure 3.7, Table 3.5), and are consistent with those recently reported for human breast cell lines (Hamunyela et al., 2017). Here, the normal cell line (1542N) is clearly the most resistant to PI3K and mTOR inhibition, making the tumour cell lines 3 to 8 times more sensitive (Table 3.5). This resistance can be attributed to NVP-BEZ235 being specifically more toxic to malignant cells, as reported elsewhere (McMillin et al., 2009). The sensitivity ranking of the malignant cell lines (DU145 and LNCaP) may be related to the extent to which NVP-BEZ235 inhibits the activity of key components of the PI3K/mTOR pathway, such as, PDK1^{Ser241}, Akt^{Thr308}, Akt^{Ser473}, GSK3 β ^{Ser19}, Foxo1^{Ser256}, S6K^{Ser235/236}, 4EBP1^{Thr27/66}, and MDM2^{Ser166}. On average, inhibition of activity of these components by a dual PI3K/mTOR inhibitor (XL765) has been shown to be about 2-fold more effective in the LNCaP cell line than the DU145 cell line (Gravina et al., 2015). The clonogenic cell survival data

presented here are consistent with this, with the LNCaP cells being 2.6-fold more sensitive than the DU145 cells (Table 3.5). However, the similarity in NVP-BEZ235 cytotoxicity in LNCaP and BPH-1 cells (EC_{50} of 6.42 ± 0.74 and 6.26 ± 0.59 , respectively) cannot be corroborated by the finding that NVP-BEZ235 is about 10-fold less effective in inhibiting cell proliferation than the latter cell line (Gravina et al., 2015). This disparity is likely due to differences in experimental design and endpoints. While the clonogenic cell survival assay described here takes about 2 weeks and reflects delayed effects of PI3K and mTOR inhibition, the cell growth assay of Gravina and colleagues lasts only 24 hours and could miss such effects (Gravina et al., 2015).

For MDV3100 treatment, EC_{50} values ranged from 15.74 – 92.73 nM for all cell lines (Figure 3.8; Table 3.5). Here, the androgen dependent cell line (LNCaP) seems to be the most significantly resistant to MDV3100, with a low relative sensitivity of 0.18. Similar resistance to MDV3100 treatment was also seen in CRPC, and LNCaP and C4-2 cells (Kuruma et al., 2013; Korpál et al., 2013). Interestingly, the traditional androgen-independent cell lines (DU145 and BPH-1) showed higher sensitivity to MDV3100 treatment. The rank order of increasing sensitivity to MDV3100 treatment is: LNCaP → 1542N \approx BPH-1 \approx DU145, with EC_{50} -values of 92.73, 16.92, 16.64, and 15.74 nM, respectively (Table 3.4). The significant level of resistance to MDV3100 inhibition seen in the LNCaP cell line relative to the 1542N, BPH-1 and DU145 cell lines, is likely due to the fact that androgen receptor (AR) expression in the LNCaP is higher than androgen independent cell lines (Kuruma et al., 2013; Korpál et al., 2013). Higher AR expression levels would require significantly larger concentrations

of inhibitor to effectively block receptor activity and result in a given proportion of cell killing.

Remission of prostate cancer after androgen deprivation therapy (ADT) is usually due to the initiation of CRPC. At this stage, tumours grow even when androgen levels are low. The central mechanism driving progression of CRPC is AR activation (Snoek et al., 2009). However, the question is how this is possible with low levels of androgen. Could it be due to AR gene amplification, activation of a promiscuous pathway, increase in AR sensitivity, or altered expression of co-regulators (Guo et al., 2009; Shiota et al., 2011)? These potential mechanisms are driven by AR activation, resulting in CRPC progression (Snoek et al., 2009). Therefore, targeting the androgen receptor remains a critical element in investigating novel strategies for CRPC therapies (Feldman and Feldman, 2001).

4.3. Inhibitor Interaction

Systemic toxicity experienced by patients that undergo chemotherapy for prostate cancer (PCa) is of significant concern in the clinics. Although chemotherapy drugs like IMC-C225 (cetuximab), enzalutamide, abiraterone, prednisone, and docetotaxel have yielded great results in improving cancer patient survival (Liu et al., 2010; Gomez et al., 2015; Silberstein et al., 2016; Byeon et al., 2019; Halfdanarson et al., 2019), tumour resistance and systemic toxicity remain key challenges. Therefore, therapeutic approaches combining a number of agents to achieve optimum therapeutic benefit with minimal toxicity have been proposed (Akudugu et al., 2011; 2012; Pasternack et al., 2014). Furthermore, Hamunyela and colleagues

demonstrated that a cocktail of PI3K/mTOR and human epidermal growth factor receptor 2 (HER-2) inhibitors radiosensitise breast cancer cells which have shown to be resistant to trastuzumab (Hamuneyela et al., 2015). Subsequently, it was demonstrated that a strong synergism exists between small molecule inhibitors of HER-2, PI3K, mTOR and Bcl-2 in human breast cancer cells (Hamuneyela et al., 2017). In CRPC, a combination of AKT inhibitor (AZD6363) with antiandrogen (bicalutamide) resulted in synergistic inhibition of cell proliferation and induction of apoptosis (Thomas et al., 2013). Antibody-drug conjugates (ADC) and potent antiandrogens (enzalutamide and abiraterone) also yielded synergistic effects when anti-proliferative activity was evaluated in LNCaP and C4-2 cell lines (Murga et al., 2015).

In preparing drug cocktails for combination therapy, it is highly desirable to achieve as low as possible drug doses within the cocktails. An ideal therapeutic cocktail should have the following attributes: (1) constituent drugs should not antagonise each other; (2) adequate synergy should exist between constituent drugs; (3) the safety profiles constituent drugs should not overlap, and (4) the therapeutic effectiveness of any constituent drug should not worsen adverse event signatures of the others. In this study, the modes of interaction of the dual inhibitor of PI3K and mTOR (NVP-BEZ235), EGRF inhibitor (AG-1478), and AR inhibitor (MDV3100) in *in vitro* cultures of four human prostate cell lines (DU145, LNCaP, BPH-1 and 1542N) were evaluated, as described elsewhere (Chou, 2006; Hamuneyela et al., 2017). For this, the following cocktails were used: Cocktail 1 (AG-1478 and NVP-BEZ235), Cocktail 2 (NVP-BEZ235 and MDV3100), and Cocktail 3 (MDV3100 and AG-1478). The combination indices (*CI*) for each cocktail in the DU145, BPH-1 and 1542N cell

lines ranged between 0.0082 and 0.1591 (Table 3.6), indicating strong to very strong synergism for each inhibitor combination.

Dual inhibition of the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MEK) and PI3K/mTOR pathways in DU145, LNCaP and PC-3 cell lines with AZD6244 and GSK2126458, respectively, also showed synergistic effects (Park et al., 2015). Synergistic effects have also been demonstrated for combination therapy castration-resistant prostate cancer with docetaxel and thymoquinone (Dirican et al., 2014). The very strong synergism observed for Cocktail 1 in DU145, BPH-1 and 1542N cells, Cocktail 2 in DU145 and LNCaP cells, and Cocktail 3 in DU145 and BPH-1 cells is consistent with that observed in human breast cell lines (Hamuneyela et al., 2017). Clinically, synergisms have also been demonstrated between AZD6363, bicalutamide, ADC, Enzalutamide and abiraterone in prostate cancer (Thomas et al., 2013; Murga et al., 2015).

The scope of the current study cannot demonstrate that synergy observed may not be accompanied by an aggravation of adverse effects of the cocktails, as the interaction profiles were derived at high inhibitor concentrations. Besides, the data also show high synergy in the apparently normal cell line. However, the *CI*-values for Cocktails 1 and 3 in the androgen-dependent LNCaP cell line were greater than 1.0 (Table 3.6), indicating antagonism between the constituent inhibitors. Inhibitor combinations with features akin to those of these cocktails may not be beneficial in the management of androgen-dependent cancers. Similar antagonism has been demonstrated in LNCaP and PC-3 cell lines when the AR and glucocorticoids receptor (GR) were dually inhibited (Wu et al., 2017).

4.4. Radiomodulation by Inhibitors

4.4.1. Radiomodulation by AG-1478 and NVP-BEZ235

Radiosensitisation of prostate cancer cells, specifically LNCaP, DU145, and BPH-1 to radiation may be achieved by targeting pathways that are implicated in radioresistance, such as the PI3K/mTOR, EGFR and AR pathways (An et al., 2007; Skvortsova et al., 2008; Jain et al., 2012). This approach could lead to the enhancement of the efficacy of radiotherapy in CRPC. Many pre-clinical studies and clinical trials have tested this hypothesis, and clinically beneficial outcomes in numerous cancers have been documented (Li et al., 2010; Fokas et al., 2012; Bonner et al., 2006; Maira et al., 2008; Cho et al., 2010; Carver et al., 2011; Haffty et al., 2006; Kassam et al., 2009; Foulkes et al., 2010). We and others have reported the benefit of targeting these pathways in an effort to develop novel treatment approaches for breast and prostate cancer (Maleka et al., 2015; 2019; Hamunyela et al., 2015; 2016; 2017; Hamid et al., 2016).

Research in breast, head and neck, and lung cancer, involving clinical trials which explore targeted and combination therapies, has yielded a wealth of knowledge. Examples of these research efforts are the following: (1) the targeting of EGFR overexpression in these cancers with receptor-blocking monoclonal antibodies (such as, cetuximab), or small molecule EGFR inhibitors (such as, gefitinib) (Liang et al., 2003; Liu et al., 2010); (2) the inhibition of the PI3K/mTOR pathway with LY294002, NVP-BEZ235 and NVP-BGT226 (Toulany et al., 2006; Fokas et al., 2012); and (3) the inhibition of the androgen receptor pathway in triple negative breast cancer with

MDV3100 (Speers et al., 2017). These studies have demonstrated that such pathway inhibition sensitises cancer cells to the cytotoxic effect of ionising radiation. However, the extent of similar studies in prostate cancer, especially CRPC, is minimal or non-existent.

Radiotherapy has remained a very important treatment modality in PCa management, but tumour resistance continues to be a major clinical challenge. In this study, the importance of conventional X-ray therapy has been demonstrated in three PCa cell lines and an apparently normal prostate cell line. The ranking of the cell line in order of increasing radiosensitivity is: DU145 → BPH-1 → 1542N → LNCaP, with only the androgen-dependent cell line being relatively more radiosensitive than the normal cell line. This would imply minimal to no therapeutic benefit of conventional radiotherapy for a majority of prostate cancers. On average, pre-treatment of tumour cell lines with the EGFR inhibitor (AG-1478) resulted in minimal or no radiosensitisation. However, pre-treatment of the with the PI3K/mTOR inhibitor (NVP-BEZ235), showed desirable results, especially at low therapeutic doses (e.g. 2 Gy), where radioprotection was seen in 1542N cells, while the tumour cells were radiosensitised by 38 - 69%. A similar radioprotection was previously demonstrated in this cell line and normal gut tissue (Potiron et al., 2013; Maleka et al., 2015). The present data suggest that combining X-rays with AG-1478 or NVP-BEZ235 at high radiation doses may not be appropriate, as the normal cell line shows high levels of radiosensitisation compared to the rest of the cell line. For the tumour cell lines, the results from a combination of radiation and a cocktail of AG-1478 or NVP-BEZ235 do not seem to differ greatly from those obtained from single inhibitor treatment prior to irradiation. This suggests that dual targeting of the EGFR

and PI3K/mTOR pathways may not have a therapeutic advantage over single pathway targeting.

4.4.2. Radiomodulation by MDV3100 and NVP-BEZ235

To explore the role of the androgen receptor (AR), which has been thought to be the core driving force for prostate cancer progression, the radiomodulatory effect of the AR inhibitor (MDV3100) was also evaluated in conjunction with PI3K/mTOR inhibitor (NVP-BEZ235).

In the androgen-dependent cell line LNCaP, MDV3100 showed the greatest radiosensitisation of almost 3-fold at low radiation doses and ~8-fold at high doses (Table 3.11). This may be attributed to the high levels of AR expression in these cells (Guo et al., 2000). These results are similar to those of the apparently normal cell line (1542N), which was radiosensitised up to 2- and 7-fold at low and high radiation doses, respectively. By definition, the 1542N cell line is androgen-independent. However, the current findings demonstrate that as the concentration of 5 α -DHT increases in 1542N cell cultures, so does the cells shift from an androgen-independent state to being androgen-dependent (Figure 3.2). In the traditionally androgen-independent cell lines (DU145 and BPH-1), the radiosensitisation by MDV3100 pre-treatment was minimal. This can be expected as these cell lines have been shown to express low levels of the androgen receptor (Guo et al., 2000). However, a switch from androgen-independent to androgen-dependent was apparent in the DU145 cell line (Figure 3.1), making the role of the AR pathway in radiation resistance unclear.

For cancers that behave like LNCaP, a cocktail of MDV3100 and NVP-BEZ235 could be an effective adjuvant to radiotherapy, as these cells were radiosensitised by cocktail pre-treatment up to 4- and 21-fold at low and high doses, respectively (Table 3.11). A potential benefit is also apparent in treating tumours that may be MDV3100-resistant, as seen in the DU145 cell line, with MDV3100/NVP-BEZ235 cocktail prior to high doses of radiation. MDV3100-resistant prostate cancers constitute a significant clinical challenge (Kuruma et al., 2013).

From the data presented here, it can be concluded that combined targeting of the PI3K/mTOR and AR pathways could potentially be an effective therapeutic strategy for androgen-dependent (whether intrinsic or switched on at high androgen levels) cancers.

CHAPTER 5: CONCLUSION

5. Conclusion

In conclusion, these data demonstrate that increasing the levels of 5 α -DHT might redefine the androgen sensitivity of human prostate cell lines, as 5 α -DHT was seen to both inhibit and promote cell proliferation in the DU145 and 1542N cell lines. The reason for this disparity is currently not known. However, this observation might be useful in designing therapeutic interventions for both androgen-dependent and independent cancers. As it has been documented that ADT is adversely affected by tumour independence on androgen, switching cells from androgen-independent to androgen-dependent might be of therapeutic benefit when administering ADT. A cocktail of PI3K/mTOR and AR inhibitor, as an adjuvant radiotherapy, may also be beneficial in the management of intrinsically androgen-dependent cancers.

In the cell lines used in this study, the potential benefit of EGFR targeting is limited. It is also important to note that use of a cocktail of AG-1478 and NVP-BEZ235, or single inhibitors at high radiation doses may result in high levels of radiosensitisation of normal tissue. The cocktails, consisting of constituents at EC₅₀, resulted in strong to very strong synergism when inhibitor interaction was tested in the cell lines, with the exception of the LNCaP cell line which demonstrated antagonistic effects. Use of an immortalised normal prostate cell line instead of normal cells derived from radiation dose limiting organs, such as the bladder and rectum, can significantly influence potential therapeutic benefit. Nonetheless, it is worth noting that unmodified normal cell lines are limited in their capacity to successfully complete intended clonogenic assays, as described in this study. These findings might assist in the

design of more effective treatment approaches for cancers that typically display resistance to radiotherapy and chemotherapy.

6. Limitations of the Study

- Normal and benign prostate cells do not divide indefinitely. For the assays described in dissertation, the apparently normal cell line (1542N) and the benign prostatic hyperplasia cell line (BPH-1) had to be transformed in order to be able to proliferate indefinitely. Therefore, data obtained with these cell lines might not give a true representation of the clinical response of normal tissue and benign tumours.
- The use of a cocktail of AG-1478 and NVP-BEZ235, or single inhibitors, at large fractions of radiation absorbed doses (e.g. 6 Gy), which are clinically relevant in the treatment of PCa, might result in undesirably high levels of radiosensitisation of normal tissue.
- The interaction profiles of the respective inhibitors, most of which were synergistic, were derived at high inhibitor concentrations (EC_{50}). Given that this synergy was also seen in the apparently normal cells, determination of the mode of inhibitor interaction at lower concentrations might inform the design of a more appropriate treatment approach.

7. Possible Future Avenues for Research

- Androgen-independent prostate cancer is an untreatable form of prostate cancer in which the normal dependence on androgens for growth and survival

has been bypassed. It would be interesting to further investigate the possibility of switching cancer cells from androgen-independent to androgen-dependent and evaluating their subsequent response to various therapies.

- The very strong synergism demonstrated with the respective cocktails at EC_{50} which shows high synergism also in the apparently normal, which is not desirable. Validating the synergism throughout the PCa cell lines with relatively low EC_{50} would be highly desirable, as to limit adverse effects to the apparently normal cell line.
- Furthermore investigate the role of androgens in chemoresistance and radio resistance. And determine major factors affecting radiosensitivity, such as DNA repair capacity, and determine the specific genes that are responsible in enhancing and promoting radioresistance. As it has been reported by Polkinghorn and colleagues (Polkinghorn et al. 2013) that there is a close interplay between androgen receptor signalling and cellular DNA damage response machinery.

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Appendix A: Publications

Peer-reviewed Papers (attached)

1. Maleka S, Serafin A, Akudugu J. PI3K and mTOR inhibitor, NVP-BEZ235, is more toxic than X-rays in prostate cancer cells. *Int J Radiat Res* 17(1):37-45, 2019. DOI: 10.18869/acadpub.ijrr.17.1.37

Published Abstracts

1. Maleka S, Akudugu JM, Serafin AM. A comparison of the effectiveness of targeted therapy and radiotherapy in human prostate cell lines. *Physica Medica: Eur J Med Phys* 41, S9-S10, 2017; DOI:10.1016/S1120-1797(17)30297-1

Conference Contributions

1. Maleka S, Serafin AM, Akudugu JM. A comparison of the effectiveness of targeted therapy and radiotherapy in human prostate cell lines. SAAPMB Congress, Durban, South Africa, September 2017 (oral).
2. Maleka S, Serafin AM, Akudugu JM. A comparison of the effectiveness of targeted therapy and radiotherapy in human prostate cell lines. 18th SASMO/SASCRO Congress, Johannesburg, South Africa, August 2017 (oral).
3. Maleka S, Serafin AM, Akudugu JM. Evaluation of cytotoxicity of BEZ-235 and AG-1478 in prostate cancer cells. 60th Academic Year Day, Faculty of Medicine and Health Sciences, University of Stellenbosch, Tygerberg, South Africa, August 2016 (oral).
4. Maleka S, Serafin A, Akudugu J. The specific inhibitor of PI3K and mTOR (BEZ-235) has potential radio-protective effect. 17th SASMO/SASCRO Congress, Cape Town, South Africa, August 2015 (oral).

PI3K and mTOR inhibitor, NVP-BEZ235, is more toxic than X-rays in prostate cancer cells

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ABSTRACT

Background: Radiotherapy and adjuvant androgen deprivation therapy have historically been the first treatment choices for prostate cancer but treatment resistance often limits the capacity to effectively manage the disease. Therefore, alternative therapeutic approaches are needed. Here, the efficacies of radiotherapy and targeting the pro-survival cell signaling components epidermal growth factor receptor (EGFR), phosphoinositide 3-kinase (PI3K), and mammalian target of rapamycin (mTOR), with their respective inhibitors are compared. **Materials and Methods:** The cytotoxic effects of inhibitors of PI3K and mTOR (NVP-BEZ235) and EGFR (AG-1478), and X-rays, were evaluated in prostate cell lines (LNCaP: cancer; DU145: cancer; BPH-1: benign prostatic hyperplasia; 1542N: apparently “normal”) using a colony forming assay. The cells were exposed to a range of X-ray doses or varying concentrations of the inhibitors, to obtain cell survival curves from which relative sensitivities (RS) of the tumor cell lines were derived as the ratio of their sensitivities to that of the “normal” cell line. **Results:** The LNCaP cells trended to be more sensitive to X-rays and AG-1478 exposure than 1542N cells, with RS-values of 1.65 ± 0.48 ($P=0.1644$) and 1.37 ± 0.22 ($P=0.0822$), respectively. NVP-BEZ235 emerged as very cytotoxic in all tumor cell lines, yielding RS-values of 3.69 ± 0.83 (DU145; $P=0.0025$), 8.80 ± 1.73 (LNCaP; $P<0.0001$), and 8.76 ± 1.70 (BPH-1; $P=0.0011$). **Conclusion:** These findings demonstrated that targeted therapy, specifically that using NVP-BEZ235, might result in a more effective treatment modality for prostate cancer than conventional radiotherapy.

Keywords: Radiotherapy, targeted therapy, prostate cancer, PI3K, mTOR, EGFR.

► Original article

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INTRODUCTION

In addition to androgen therapy, radiotherapy is a first line option for the treatment of prostate cancer. Radiotherapy is a highly effective treatment option for localized prostate cancer with manageable treatment-related side effects^(1,2). A major challenge in radiotherapy is normal tissue toxicity and patients failing to achieve long-term tumor control. On the other hand, androgen therapy does not benefit patients with androgen-independent cancers as these tumors do not respond to treatment⁽³⁾. The prognosis for localized and regional prostate disease is good. However, with almost one million new cases of

prostate cancer (PCa) and over a quarter of a million prostate cancer-related deaths recorded per year worldwide, PCa remains the second most common cancer in men, with increasing incidences and mortality rates globally, and also in sub-Saharan Africa^(4,5). With the abovementioned problems, new treatment strategies are needed to address these therapeutic dilemmas.

Targeted therapies have emerged as alternative treatment modalities to overcome the issue pertaining to treatment resistance and normal tissue toxicity^(6,7,8,9). The use of different omics techniques has led to progress in the molecular classification of both early and late stage prostate cancer which may manifest itself

in targeted, personalized therapies. NVP-BE235, a dual inhibitor of PI3K and mTOR, is cytotoxic and yet has the potential to protect normal tissue⁽¹⁰⁻¹²⁾ and sensitize cancer cells to radiotherapy^(10,11,13,14,15,16,17).

PI3K pathway-mediated cross-talk between the androgen receptor (AR), which plays a pivotal role in prostate malignancy, and EGFR, has been demonstrated, and underpinned by preclinical models⁽¹⁸⁻²⁰⁾. This cross-talk may present itself as an important mechanism during PCa progression, giving cells a survival advantage, and might serve as a potential target for cancer therapy⁽²¹⁾.

In search of alternatives to radiotherapy, this study compares the efficacies of radiotherapy and targeting PI3K, mTOR, and EGFR with specific inhibitors NVP-BE235 and AG-1478 using four human prostate cell lines (DU145, LNCaP, BPH-1 and 1542N). The potential therapeutic benefit of each agent (radiation or inhibitor) is discussed.

MATERIALS AND METHODS

Cell lines and culture maintenance

The apparently “normal” 1542N cell line (passage number: 18-25) was derived from the normal prostate epithelial tissue of a patient with primary adenocarcinoma of the prostate, and immortalised with the E6 and E7 genes of the human papilloma virus 16⁽²²⁾. The cells were a gift from Prof JRW Masters (Prostate Cancer Research Centre, University College London, UK). The epithelial cell line, BPH-1 (benign prostatic hyperplasia-1) (passage number: 2-7), was established from human prostate tissue obtained by transurethral resection. Primary cell cultures were immortalised with simian virus 40 (SV40) large T-antigen⁽²³⁾. The cells were obtained from Professor SW Hayward (Department of Urology, University of California, USA). Although the link between BPH and PCa remains largely controversial, there is ample evidence to suggest that the former is a precursor of the latter^(24,25,26,27). Therefore, the BPH-1 cell line is considered as “malignant” in

the current study. The LNCaP cell line (passage number: 4-9) was established from a supraclavicular lymph node metastasis of human prostatic adenocarcinoma⁽²⁸⁾, and was obtained from Professor Helmut Klocker (Department of Urology, University of Innsbruck, Austria). These cell lines were grown in Roswell Park Memorial Institute medium, RPMI-1640 (Sigma-Aldrich, USA; cat #: R8758) supplemented with 10% (5% for LNCaP) heat-inactivated foetal bovine serum (FBS) (HyClone, UK; cat #: SV30160.30IH), penicillin (100 U/ml) and streptomycin (100 mg/ml) (Lonza, Belgium; cat #: DE17-602E). The malignant DU145 (passage number: 7-18) cells were derived from a metastatic lesion of the central nervous system⁽²⁹⁾, and were a gift from Prof P Bouic (Synexa Life Sciences, Montague Gardens, South Africa). Cells were routinely grown in Minimum Essential Medium (MEM) (Sigma-Aldrich, Germany). Growth media were supplemented with 10% heat-inactivated foetal bovine serum (FBS) (HyClone, UK; cat #: SV30160.30IH), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Lonza, Belgium; cat #: DE17-602E). All cell cultures were grown as monolayers in 75-cm² flasks (Greiner Bio-One, Germany; cat #: 658170) and were maintained by incubation at 37°C in a humidified (relative humidity: 84%) atmosphere (95% air and 5% CO₂). Cell cultures were used for experiments upon reaching 70-90% confluence.

Inhibitors

NVP-BE235 (C₃₀H₂₃N₅O; M_w = 469.55; Santa Cruz Biotechnology, TX, USA, cat # 364429) is a dual inhibitor of phosphoinositide-3-kinase (PI3K) and mammalian target of rapamycin (mTOR), with an inhibitory concentration at 50% (IC₅₀) of ~5 nM for PI3K and 6 nM for mTOR, and shown to have IC₅₀-values ranging from ~12 to 17 nM for inhibiting *in vitro* proliferation and survival of prostate cancer cell lines, PC3M, PC3, and DU145^(11,30). AG-1478 (C₁₆H₁₄ClN₃O₂.HCl; M_w = 352.22; Tocris Bioscience, UK, cat # 1276) is a specific inhibitor of EGFR with an IC₅₀ of 3 nM, and has been shown to have an IC₅₀ of 1 µM for inhibiting *in*

in vitro proliferation of a non-small cell lung cancer cell line, NCI-H2170⁽³¹⁾. Stock solutions of NVP-BEZ235 (106 mM) and AG-1478 (10 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C until used.

Cell culture irradiation and inhibitor treatment

Monolayer cell cultures of DU145, 1542N, LNCaP, and BPH-1 in exponential growth were trypsinized to give single-cell suspensions and were plated for X-ray exposure (300-100000 cells per flask, adjusted for irradiation dose), and for inhibitor treatment (1000-4000 cells per flask, adjusted for inhibitor concentration) into 25-cm² culture flasks (Nest Biotechnology, China; cat #: 707001). The cell cultures in 10 ml of growth medium were incubated for 4-5 h (4-7 h for LNCaP) to allow the cells to attach. The LNCaP cell line has altered adhesion properties (low anchoring potential) which explains why the cells are left to settle for a longer period of time⁽²⁸⁾. The attached cells were then irradiated with X-rays or treated with inhibitors of PI3K and mTOR (NVP-BEZ235) and EGFR (AG-1478), respectively. For X-ray exposure, cell cultures were irradiated at room temperature (22°C) to 0-10 Gy using a Faxitron MultiRad 160 X-irradiator (Faxitron Bioptics, Tucson, AZ) at a dose rate of 1 Gy/min. Cell cultures were treated with inhibitors, without media replacement. Cells were exposed to NVP-BEZ235 (0.001-1,000 nM) and AG-1478 (1-100,000 nM) after appropriate dilution of stock solutions in cell culture medium.

Radiosensitivity and inhibitor toxicity measurement

The colony assay was used to measure intrinsic cellular radiation response, and cytotoxicity of inhibitors. Briefly, irradiated and inhibitor-treated cells were incubated for 10 days (BPH-1 and DU145) and 14 days (LNCaP and 1542N) to form colonies. To test for possible inhibitor solvent toxicity, two sets of control (untreated) cultures were prepared for each experiment. One set was exposed to DMSO at a final concentration corresponding to that of the highest inhibitor concentration, and the

resulting plating efficiencies of the control culture sets compared. The experiments were stopped by decanting the growth medium, washing with phosphate buffered saline, and fixing with glacial acetic acid:methanol:water (1:1:8, v/v/v). The colonies were stained with 0.01% amido black in fixative, washed in tap water, air-dried, and counted using a stereoscopic microscope (Nikon, Japan; Model #: SMZ-1B). Three independent experiments were performed for each radiation dose and inhibitor concentration, and the mean surviving fractions were calculated. Surviving fractions (*SF*) were calculated according to the formula, $SF = n_{col}(t) / \{ [n_{col}(u) / n_{cell}(u)] \times n_{cell}(t) \}$, where $n_{cell}(t)$ and $n_{cell}(u)$ represent the number of cells plated in treated (irradiated or inhibitor treated) and untreated (control) cultures, respectively. $n_{col}(t)$ and $n_{col}(u)$ are the corresponding number of colonies counted. No inhibitor solvent related toxicity was observed in control (untreated) cultures containing the highest concentration of DMSO (0.000066% for NVP-BEZ235 at 1:10000 dilution of stock; and 0.1% for AG-1478 taken directly from stock).

Cell survival data for X-ray exposure were fitted to the linear-quadratic (LQ) model to generate survival curves (equation (1)), and cellular radiosensitivity, expressed in terms of the absorbed radiation dose at which 50% cell killing occurred (D_{50}), was determined.

$$S = \exp [-\alpha D - \beta D^2] \quad (1)$$

where S is the surviving fraction, α and β are the linear and quadratic coefficients, respectively, and D is the absorbed dose in Gy.

To determine the equivalent concentration of each inhibitor for 50% cell killing (EC_{50}), the surviving fractions were plotted as a function of log (inhibitor concentration) and were fitted to a 4-parameter logistic equation describing a sigmoidal curve (equation (2))^(16,33,34).

$$SF = B + \frac{T-B}{\{1-10^{[(\log EC_{50}-D)HS]}\}} \quad (2)$$

where B and T are the minimum and maximum of the sigmoidal curve, respectively, D is the log(inhibitor concentration), and HS is the

steepest slope of the curve.

To determine whether a treatment agent (X-rays or inhibitor) had a potential therapeutic benefit, a relative sensitivity (RS) was derived by comparing the D_{50} and EC_{50} of the “normal” prostate cell line, (1542N), with those of the tumor cell lines (DU145, LNCaP, BPH-1) as follows:

$$RS = \frac{D_{50}(\text{“normal”})}{D_{50}(\text{tumor})} \text{ or } \frac{EC_{50}(\text{“normal”})}{EC_{50}(\text{tumor})} \quad (3)$$

The criteria for no potential benefit with possible undesirable effects, no potential benefit, and potential therapeutic benefit of each agent are $RS < 1.0$, $RS = 1.0$ and $RS > 1.0$, respectively.

Data analysis

Statistical analyses were performed using the GraphPad Prism (GraphPad Software, San Diego, CA, USA) computer program. The relationship between cell survival and X-ray dose was described using a linear-quadratic equation. A 4-parameter logistic equation describing a sigmoidal curve was used to describe the relationship between inhibitor cytotoxicity (cell survival) data and inhibitor concentration. Standard equations were used to fit nonlinear relationships. All data presented in figures and used in curve fitting were calculated as the means (\pm SEM) from three independent experiments. For each experiment and data point, 3 replicates were assessed. To compare two data sets, the unpaired t -test was used. P -values and coefficients of determination, R^2 , were calculated from two-sided tests. A P -value of < 0.05 indicated a statistically significant difference between the data sets.

RESULTS

Intrinsic cellular radiosensitivity

Cell survival data for the human prostate carcinoma and “normal” cell lines were fitted to the linear-quadratic model, and the corresponding dose-response curves are

presented in figure 1. Intrinsic cellular radiosensitivity was expressed in terms of the radiation dose at which a cell survival of 50% (50% cell killing) was obtained (D_{50}), and was presented as the mean (\pm SEM). The androgen-dependent cell line, LNCaP, emerged as more radiosensitive than its androgen-independent counterparts (1542N, BPH-1, DU145). The rank order of radioresistance in the cell lines was found to be LNCaP < 1542N < BPH-1 < DU145, with D_{50} -values of 0.93 ± 0.19 , 1.53 ± 0.32 , 1.65 ± 0.36 , and 2.25 ± 0.54 Gy, respectively. No statistically significant differences emerged between the radiosensitivity of the “normal” cell line when compared with those of the tumor cell lines as presented in table 1 ($0.1644 \leq P \leq 0.7797$). This translated to relative sensitivities (RS) that do not differ significantly from unity. The corresponding RS -values for the DU145, LNCaP, and BPH-1 cell lines were 0.68 ± 0.21 , 1.65 ± 0.48 , and 0.93 ± 0.28 , respectively (table 1).

Cytotoxicity of EGFR inhibitor (AG-1478)

Figure 2 shows that the EGFR inhibitor AG-1478 exhibits a concentration-dependent toxicity in all cell lines, and sensitivity to inhibitor treatment was expressed in terms of equivalent concentration for 50% cell killing (EC_{50}) as the mean (\pm SEM). Treatment with AG-1478 yielded the same sensitivity ranking, as observed for X-ray exposure (figure 2), with the LNCaP showing more sensitivity than the other cell lines. The EC_{50} of the “normal” cell line (1542N) emerged as 400 ± 38 nM and was significantly lower than those of the DU145 (6613 ± 1510 nM, $P = 0.0147$, $R^2 = 0.8088$) and BPH-1 (677 ± 41 nM, $P = 0.0079$, $R^2 = 0.8588$) cell lines. The EC_{50} of the relatively more sensitive LNCaP cell line did not differ significantly from that of the 1542N cell line (302 ± 19 nM, $P = 0.0822$, $R^2 = 0.5712$). This resulted in very low relative sensitivities of 0.06 ± 0.02 and 0.59 ± 0.07 for the DU145 and BPH-1 cell lines (table 2), as determined from equation (3), respectively. The relative sensitivity of the LNCaP cell line was 1.33 ± 0.15 .

Cytotoxicity of PI3K and mTOR inhibitor (NVP-BEZ235)

Inhibition of PI3K and mTOR inhibitor with NVP-BEZ235 also resulted in a concentration-dependent cell killing, as shown in figure 3. The rank order of cytotoxicity following NVP-BEZ235 treatment is LNCaP \approx BPH-1 < DU145 < 1542N, with EC_{50} -values of 6.10 ± 0.40 , 6.11 ± 0.64 , 16.25 ± 4.72 , and 53.82 ± 2.95

nM, respectively. All tumor cell lines were significantly more sensitive to NVP-BEZ235 treatment than the “normal” cell line as shown in table 2 ($P \leq 0.0025$). The tumor cell lines, DU145, LNCaP, and BPH-1, were found to be 3- to 8-fold more sensitive than the “normal” cell line (1542N) with relative sensitivities of 3.31 ± 0.98 , 8.82 ± 0.75 , and 8.81 ± 1.04 , respectively (equation (3), table 2).

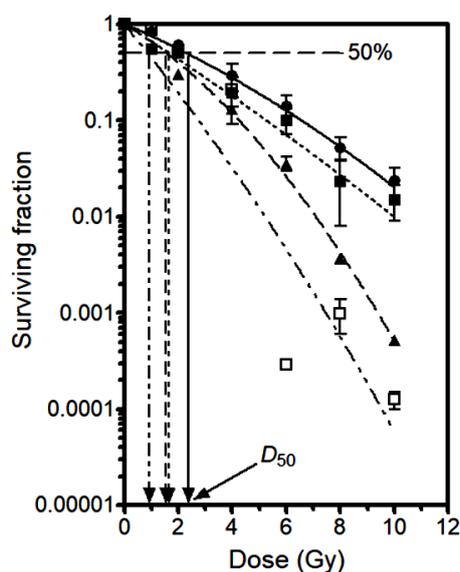


Figure 1. Clonogenic cell survival curves for 4 human prostate cell lines [DU145 (●), LNCaP (□), BPH-1 (▲), 1542N (■)] after X-ray irradiation. Survival curves were obtained by fitting experimental data to the linear-quadratic model. Symbols represent the mean surviving fraction \pm SEM from 3 independent experiments. Standard errors are not transformed into a logarithmic scale. The dose at which 50% of cells survive (D_{50}) is the dose at which each survival curve intersects the horizontal dashed line.

Table 1. Summary of cytotoxicity data for 4 human prostate cell lines (“normal”: 1542N; cancer: DU145, LNCaP; benign prostate hyperplasia: BPH-1) and relative radiosensitivity determined by clonogenic cell survival after exposure to X-rays. D_{50} denotes the absorbed radiation dose required to yield a 50% cell killing (figure 1). The 95% confidence intervals of the D_{50} -values are in parentheses. P -value indicates the level of significance in the difference between the D_{50} of the “normal” cell line (1542N) relative to those of the tumor cell lines (DU145, LNCaP, BPH-1). Relative sensitivity (RS) is the ratio of the D_{50} of the “normal” cell line to those of the tumor cell lines. α and β are the linear and quadratic coefficients of the respective cell survival curves obtained from the LQ-model (equation (1)).

Cell line	α (Gy^{-1})	β (Gy^{-2})	D_{50} (Gy)	P -value	RS
1542N	0.49 ± 0.11	0.00 ± 0.00	1.53 ± 0.32 (1.17-2.17)	–	–
DU145	0.28 ± 0.06	0.01 ± 0.01	2.25 ± 0.54 (0.59-3.90)	0.2261	0.68 ± 0.21
LNCaP	0.78 ± 0.24	0.02 ± 0.02	0.93 ± 0.19 (0.37-1.50)	0.1644	1.65 ± 0.48
BPH-1	0.39 ± 0.05	0.04 ± 0.01	1.65 ± 0.36 (0.57-2.73)	0.7797	0.93 ± 0.28

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Table 2. Summary of cytotoxicity data for 4 human prostate cell lines (1542N, DU145, LNCaP, BPH-1) treated with EGFR inhibitor (AG-1478) and PI3K and mTOR inhibitor (NVP-BE235). EC_{50} denotes the equivalent concentration for 50% cell survival (figures 2 and 3). The 95% confidence intervals of the EC_{50} -values are in parentheses. P -value indicates the level of significance in the difference between the EC_{50} of the “normal” cell line (1542N) relative to those of the tumor cell lines (DU145, LNCaP, BPH-1). Relative sensitivity (RS) is the ratio of the EC_{50} of the “normal” cell line to those of the tumor cell lines.

Cell line	Treatment	EC_{50} (nM)	P -value	RS
1542N	AG-1478	400 ± 38 (237-563)	–	–
	NVP-BE235	53.82 ± 2.95 (41.13-66.50)	–	–
DU145	AG-1478	6613 ± 1510(116-13110)	0.0147	0.06 ± 0.02
	NVP-BE235	16.25 ± 4.72 (11.83-36.57)	0.0025	3.31 ± 0.98
LNCaP	AG-1478	302 ± 19 (220-384)	0.0822	1.33 ± 0.15
	NVP-BE235	6.10 ± 0.40 (4.39-7.81)	<0.0001	8.82 ± 0.75
BPH-1	AG-1478	677 ± 41 (499-855)	0.0079	0.59 ± 0.07
	NVP-BE235	6.11 ± 0.64 (3.96-14.17)	0.0011	8.81 ± 1.04

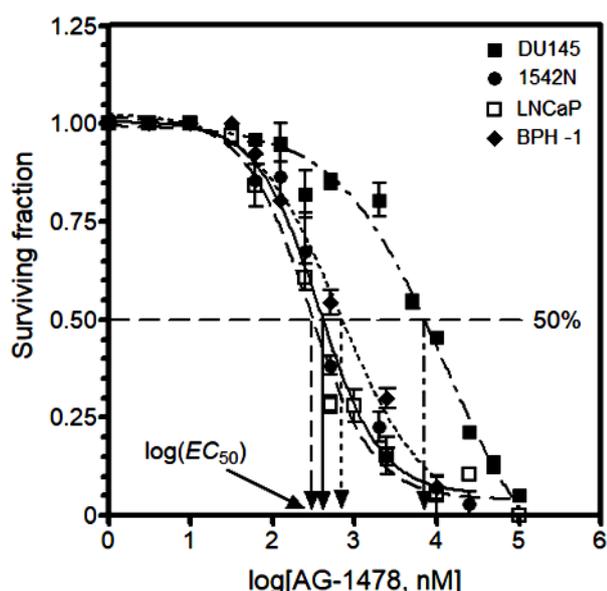


Figure 2. Cytotoxicity curves for EGFR inhibitor (AG-1478) treatment of 4 human prostate cell lines (DU145, 1542N, LNCaP, BPH-1). Curves were obtained by plotting cell survival as a function of log (inhibitor concentration). Cell survival was determined by the colony assay, and data were fitted to a sigmoidal equation. Data points are means ± SEM of 3 independent experiments. The concentration at which 50% of cells survive (EC_{50}) is that at which each survival curve intersects the horizontal dashed line.

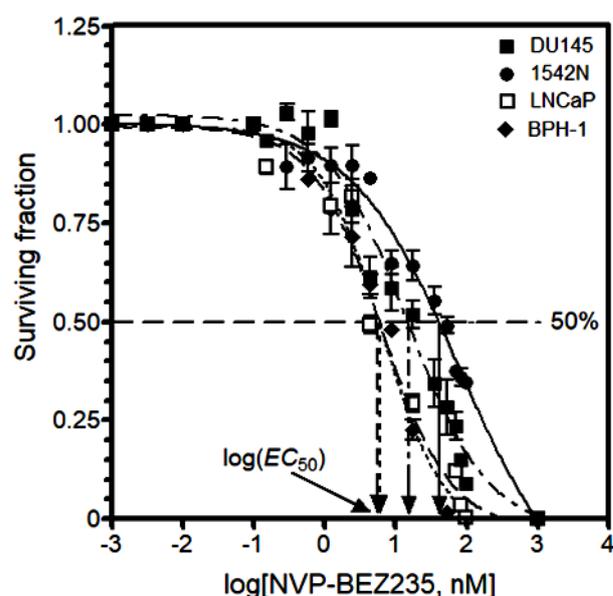


Figure 3. Cytotoxicity curves for PI3K and mTOR inhibitor (NVP-BE235) treatment of 4 human prostate cell lines (DU145, 1542N, LNCaP, BPH-1). Curves were obtained by plotting cell survival as a function of log (inhibitor concentration). Cell survival was determined by the colony assay, and data were fitted to a sigmoidal equation. Data points are means ± SEM of 3 independent experiments. The concentration at which 50% of cells survive (EC_{50}) is that at which each survival curve intersects the horizontal dashed line.

DISCUSSION

Using the clonogenic survival assay, it is demonstrated for 4 human prostate cell lines that there is no significant therapeutic advantage when D_{50} -values of the tumor cell lines (LNCaP, BPH-1, DU145) were compared with that of the “normal” cell line (1542N)

(figure 1, table 1). The ranking order, from most radiosensitive to least radiosensitive using X-ray irradiation, is LNCaP>1542N>BPH-1>DU145, and is in close agreement with radiosensitivity data reported previously for these cell lines following cobalt-60 γ -ray exposure⁽³⁵⁾. The absence of a therapeutic benefit may be explained by the fact that the D_{50} -values are

derived at 50% cell survival which coincides with the shoulder region of the survival curves, and may not differ markedly. For instance, a comparison of doses at the level of 10% survival, yields relative sensitivities of 1.77, 1.18, and 0.78, for the LNCaP, BPH-1, and DU145 cell lines, respectively, indicating a potential benefit for the former two. However, the rationale for choosing D_{50} and EC_{50} was to enable comparison of the relative sensitivities (RS) of the cell lines at the same level of survival, when as low as possible radiation dose or inhibitor concentration is administered. It should also be acknowledged that molecular assays have been developed applying multigene expression profiles to predict tumor radiosensitivity by comparisons with clonogenic survival data from established cell lines^(36,37), that likely have the most potential for clinical implementation. Such methods have been shown to be statistically predictive of tumor response in esophageal and rectal cancers, and of locoregional control in head and neck cancers⁽³⁷⁾. As such, there is the need to explore avenues like the inhibition of EGFR, PI3K, and mTOR, described here.

The cytotoxic effects of AG-1478 (EGFR inhibitor) and NVP-BEZ235 (PI3K and mTOR inhibitor) are concentration-dependent (figures 2 and 3). For AG-1478, EC_{50} values ranged from 302–6613 nM (figure 2, table 2), giving relative sensitivities of less than 1.0 for the androgen-independent DU145 and BPH-1 cells. This clearly shows that the “normal” cells (1542N) are more sensitive to EGFR inhibition than their tumor counterparts and use of AG-1478 for treatment of prostate cancer might lead to undesirable outcomes. However, inhibiting EGFR in the androgen-dependent LNCaP cells showed a small therapeutic benefit, with a relative sensitivity of 1.33 (table 2). The significant level of resistance to EGFR inhibition seen in the DU145 and BPH-1 cell lines (relative sensitivities of 0.06 and 0.59, respectively) relative to the LNCaP cell line is likely due to the fact that EGFR expression in the former is over 5-fold that in the latter^(38,39,40). Higher EGFR expression levels would require significantly larger concentrations of inhibitor to achieve a given proportion of cell killing. On the other

hand, EGFR expression in the androgen-dependent LNCaP cells is low and comparable to that in the “normal” 1542N cells⁽⁴¹⁾, consistent with the observed relative sensitivity of 1.33 ± 0.15 .

For NVP-BEZ235 treatment, EC_{50} values ranged from 6.10–53.82 nM for all cell lines (figure 3, table 2), and are consistent with those recently reported for human breast cell lines⁽¹⁶⁾. Here, the “normal” cell line (1542N) is clearly the most resistant to PI3K and mTOR inhibition, making the tumor cell lines 3 to 8 times more sensitive (table 2). This resistance can be attributed to NVP-BEZ235 being specifically more toxic to malignant cells, as reported elsewhere⁽⁴²⁾. The sensitivity ranking of the malignant cell lines (DU145 and LNCaP) may be related to the extent to which NVP-BEZ235 inhibits the activity of key components of the PI3K/mTOR pathway, such as, PDK1^{Ser241}, Akt^{Thr308}, Akt^{Ser473}, GSK3b^{Ser19}, Foxo1a^{Ser256}, S6K^{Ser235/236}, 4EBP1^{Thr27/66}, and MDM2^{Ser166}. On average, inhibition of activity of these components by a dual PI3K/mTOR inhibitor (XL765) has been shown to be about 2-fold more effective in the LNCaP cell line than the DU145 cell line⁽⁴³⁾. The clonogenic cell survival data presented here are consistent with this, with the LNCaP cells being 2.7-fold more sensitive than the DU145 cells (Table 2). However, the similarity in NVP-BEZ235 cytotoxicity in LNCaP and BPH-1 (EC_{50} of 6.10 ± 0.40 and 6.11 ± 0.64 , respectively) cannot be corroborated by the finding that NVP-BEZ235 is about 10-fold less effective in inhibiting cell proliferation than the latter cell line⁽⁴³⁾. This disparity is likely due to differences in experimental design and endpoints. While the clonogenic cell survival assay described here takes about 2 weeks and reflects delayed effects of PI3K and mTOR inhibition, the cell growth assay of Gravina *et al.* lasts only 24 hours and could miss such effects⁽⁴³⁾.

Use of an immortalized “normal” prostate cell line instead of normal cells derived from radiation dose limiting organs, such as the bladder and rectum, can significantly influence potential therapeutic benefit. Nonetheless, it is worth noting that unmodified normal cell lines

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are limited in their capacity to successfully complete intended clonogenic assays, as described in this study.

In conclusion, these data demonstrate that concomitant inhibition of PI3K and mTOR may have a higher therapeutic benefit in the treatment of androgen-dependent and -independent prostate cancers, compared to conventional radiotherapy or EGFR-targeted therapy. The findings might assist in the design of more effective treatment approaches for cancers that typically display resistance to radiotherapy and chemotherapy.

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Conflicts of interest: Declared none.

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Appendix B: Ethics Letter



UNIVERSITEIT
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Approval Notice

New Application

18/04/2019

Project ID :9296

HREC Reference # X19/02/004

Title: RADIOSENSITISATION OF ANDROGEN-DEPENDENT AND INDEPENDENT TUMOUR CELLS AS A THERAPEUTIC STRATEGY IN PROSTATE CANCER

Dear Mr Sechaba Maleka,

The **New Application** received on 22/02/2019 15:44 was reviewed by members of **Health Research Ethics Committee** via **expedited** review procedures on 18/04/2019 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Period:18 April 2019 to 17 April 2020.

Please remember to use your project ID (9296)on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Translation of the informed consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see [Forms and Instructions](#) on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

The HREC will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: <https://www.westerncape.gov.za/general-publication/health-research-approval-process>. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: [Forms and Instructions](#) on our HREC website <https://applyethics.sun.ac.za/ProjectView/Index/9296>

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Mrs. Melody Shana ,

Coordinator,

HREC1

National Health Research Ethics Council (NHREC) Registration Number:

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:
IRB0005240 (HREC1)•IRB0005239 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the [World Medical Association \(2013\). Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects](#); the South African [Department of Health \(2006\). Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa \(2nd edition\)](#); as well as the Department of Health (2015). Ethics in Health Research: Principles, Processes and Structures (2nd edition).

The Health Research Ethics Committee reviews research involving human subjects conducted or supported by the Department of Health and Human Services, or other federal departments or agencies that apply the Federal Policy for the Protection of Human Subjects to such research (United States Code of Federal Regulations Title 45 Part 46); and/or clinical investigations regulated by the Food and Drug Administration (FDA) of the Department of Health and Human Services.