RADIOSENSITISATION OF LOW HER-2 EXPRESSING HUMAN BREAST CANCER CELL LINES

By
Mogammad Baahith Hamid

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Supervisor: Professor John M Akudugu
Co-supervisor: Dr Antonio M Serafin

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature …….. Date **01 / 12 / 2014**

ABSTRACT

Breast Cancer remains one of the world's leading causes of cancer related deaths amongst women. Its treatment has evolved from invasive, highly toxic therapies to treatments that possess a higher specificity and a lower toxicity. Despite improvements in overall survival, many patients do not benefit from these agents because of acquired and/or inherent tumour resistance, which could hinder treatment efficacy. Novel treatment strategies are, therefore, warranted to address these challenges and to significantly improve patient responses. Inhibiting components of the HER-2 signalling pathway can significantly sensitise breast cancer cells to low doses of ionising radiation.

The objective of this study was to inhibit key molecular targets of the human epidermal growth factor receptor 2 (HER-2) signalling pathway and expose breast cancer cell lines to doses of radiation, so as to establish potential therapeutic targets that may be amenable to combined modality therapy, and formulate a cocktail of inhibitors to evaluate its radiosensitising capability.

This study found that pre-treatment of two breast cancer cell lines (MDA-MB-231 and MCF-7) with a HER-2 inhibitor (TAK-165) had little or no effect on radiosensitivity. However, a radiation enhancement was observed when these cells were pre-treated either with BEZ235, a dual inhibitor of phosphoinositide 3-kinase (PI3K) and mammalian target for rapamycin (mTOR), or a cocktail of TAK-165 and BEZ235.

These findings suggest that concurrent inhibition of HER-2, PI3K and mTOR during radiotherapy might improve treatment response of breast cancer patients.

OPSOMMING

Borskanker bly steeds een van die leidende oorsake van sterftes aan kanker in vrouens. Behandeling het vanaf ‘n ingrypende, hoogs toksiese terapie verander na ‘n regimen wat hoogs spesifiek met ‘n laer toksisiteit is. Nogtans trek baie pasiënte geen voordeel uit hierdie nuwe benadering nie, omdat inherente en/of verworwe tumorweerstand daarteen suksesvolle uitkomste verhoed.

Nuwe behandelingstrategieë is dus nodig om hierdie uitdagings te bekamp en om resultate in pasiënte aansienlik te verbeter.

Inhibisie van komponente van die HER-2-seinoordragkaskade kan borskankerselle gevoelig maak vir lae dosisse van geïoniseerde bestraling.

Die doelwit van hierdie studie was om sleutelteikens in die HER-2-seinoordragkaskade te inhibeer en om borskankerselle daarna aan bestralings dosisse bloot te stel. Sodoende word potensiële terapeutiese teikens wat vatbaar is vir gekombineerde modaliteitsterapie geïdentifiseer, waarna ‘n kombinasie van inhibitore geformuleer en geëvalueer kan word ten opsigte van hulle kapasiteit om gevoeligheid vir bestraling te verhoog.

Die studie bevind dat voorbehandeling met ‘n HER-2-inhibitor (TAK-165) van borskankersellyne (MDA-MB-231 en MCF-7) min of geen invloed gehad het op stralingsensitiwiteit nie. ‘n Stralingsversterking is egter geïdentifiseer toe die selle vooraf behandel is met óf BEZ-235, ‘n tweevoudige inhibitor van fosforinositied 3-

kinase (PI3K) en soogdierteiken vir rapamisien (mTOR), óf ‘n mengsel van TAK-165 en BEZ-235.

Hierdie bevindinge suggereer dat gelyktydige inhibisie van die HER-2-seinoordragkaskade, PI3K en mTOR gedurende stralingsterapie moontlik die uitkoms in borskankerpasiënte kan verbeter.

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ABBREVIATIONS

Akt	**Serine-threonine protein kinase**
ATP	**Adenosine Triphosphate**
α	**Linear coefficient of inactivation after ^{60}Co γ-irradiation**
β	**Quadratic coefficient of inactivation after ^{60}Co γ-irradiation**
cm^2	**Centimetre squared**
Co	**Cobalt**
°C	**Degree Celsius**
DMSO	**Dimethyl Sulfoxide**
DNA	**Deoxyribose Nuclei Acid**
ER	**Estrogen Receptor**
EGFR	**Epidermal Growth Factor Receptor**
FBS	**Foetal Bovine Serum**
Gy	**Grey**
HER	**Human Epidermal Growth Factor Receptor**
HER-1/ erbB1	**Human Epidermal Growth Factor Receptor 1**
HER-2 /erbB2	**Human Epidermal Growth Factor Receptor 2**
HER-3/ erbB3	**Human Epidermal Growth Factor Receptor 3**
HER-4/ erbB4	**Human Epidermal Growth Factor Receptor 4**
IC_{50}	**Inhibitory Concentration at 50%**
IGF	**Insulin Growth Factor receptor**

IgG	**Immunoglobulin G**
Inc.	**Incorporated**
µg	**Microgram**
ml	**Millilitre**
mM	**Millimolar**
min	**Minute**
nM	**Nanomolar**
mTOR	**Mammalian Target for Rapamycin**
PBS	**Phosphate Buffer Saline**
PE	**Plating Efficiency**
PI3K	**Phosphoinositide 3-kinase**
PR	**Progesterone Receptor**
PTEN	**Phosphatase and tensin homolog**
PVDF	**Poylyvinylidene Difluoride membrane**
REF	**Radiation Enhancement Factor**
RPM	**Revolutions Per Minute**
RPMI	**Roswell Park Memorial Institute**
SEM	**Standard Error of the Mean**
SD	**Standard Deviation**
SF	**Survival Fraction**
SF_2	**Surviving Fraction at 2 Gy**
SF_6	**Surviving Fraction at 6 Gy**
SSD	**Source-to-Sample Distance**
TBS	**Tris-Buffered Sline**
TLD	**Thermoluminescent Dosimetry**

TMB	**Tetramethyl benzidine**
TKIs	**Tyrosine Kinase Inhibitors**

CHAPTER 1

1.1. Introduction

Cancer continues to be a major global burden. This is largely attributed to the growth and aging of the world's population, as well as the increased adoption of cancer causing behaviours, such as smoking and leading an unhealthy lifestyle (Jemal et al., 2011). Breast Cancer is the most frequently diagnosed cancer. Worldwide, breast cancer is responsible for 32% of all cancers and 15% of all cancer related deaths in females (Salouti et al., 2011). In Africa, approximately 29 and 15 per 100 000 persons are diagnosed or dies of breast cancer, respectively (Jemal et al., 2011). In Southern Africa, higher incidence and mortality rates are observed corresponding to 38 and 19 per 100 000 persons, respectively (Jemal et al., 2011). Consequently, breast cancer has become a significant health concern in Southern Africa and the greater African continent.

Breast cancer is a disease composed of various subtypes, each possessing unique molecular characteristics, and therefore having different clinical responses and requiring unique treatment modalities (Higgins and Baselga., 2011; Park et al., 2012; Perou et al., 2000). Currently, many options are available to treat breast cancer. Although these modalities have improved treatment outcomes, many still have shortcomings as a consequence of high toxicity and tumour resistance. This clinical situation is further exacerbated by the heterogeneity of breast cancer cells. Therapeutic approaches targeting specific antigens may also fail as targets are expressed at widely varying levels within cell populations. Novel treatment strategies are thus warranted to address these clinical challenges. Radiotherapy has been

used for decades as the primary therapy for many types of cancers. Exposing cancer cells to ionising radiation has proved effective in gaining control of tumour size as it invokes lethal damage to cellular DNA and induces apoptosis (programmed cell death) (Buchholz, 2009; Duru et al., 2012; Liang et al., 2003; Pearce et al., 2001). Today, radiotherapy has become the standard treatment for post-operative breast cancer so as to decrease the risk of tumour recurrence (Pinnaro et al., 2010; Whelan., 2010). Even though the addition of radiotherapy has improved overall disease-free survival of breast cancer patients, many tumours do not respond to radiotherapy as these tumours are inherently, or have become, radioresistant (Jung et al., 2012, Li et al., 2012; Liang et al., 2003). Radiotherapy is also damaging to normal tissue and thus many patients suffer from short-term and long-term side effects (Buchholz., 2009). Breast cancer treatment has moved from invasive, non-specific, highly toxic regimens to modalities that are less invasive and less toxic to normal tissue as they are now target specific.

A deeper understanding of breast cancer cell heterogeneity has led to the identification of numerous biological markers which play valuable roles in breast cancer cell survival and proliferation. From this, effective targeted therapies which inhibit these survival and proliferative signalling markers have been developed; some are currently available in the clinic while others remain in the trial phase (Normanno et al., 2009). Trastuzumab is the first of these targeted therapeutic agents that has successfully been applied in breast cancer treatment. Trastuzumab has shown significant clinical activity in targeting the human epidermal growth factor receptor 2 (HER-2), particularly among the percentage of the breast cancer population that overexpresses this receptor. It has been suggested that trastuzumab may be able to slow down tumour growth. However, not all breast cancer subtypes express this

antigen and thus alternative treatment strategies are required to effectively treat these breast cancer subtypes (Hall and Cameron, 2009). Targeting other survival proteins, such as those of the PI3K/Akt/mTOR pathway, could prove to be beneficial for low- or non-HER-2 expressing subtypes of breast cancer. The inhibition of these proteins has also been implicated in enhancing cancer cell radiosensitisation (Awada et al., 2012; Rexer et al., 2014).

Targeted therapy has improved overall patient survival; however, its treatment efficacy has also been hindered by breast cancer cell inherent or acquired resistance (Higgins and Baselga., 2011; Hurvitz et al., 2013). It has also been reported that the efficacy of targeted therapy is limited when using these agents individually, and that using these therapeutic agents in combination would derive better patient responses (Normanno et al., 2009).

It can, therefore, be reasoned that using a cocktail of targeted therapeutic agents to inhibit more than one survival signalling targets would be more effective in treating breast cancer, than using these agents individually. The objective of the following study will be to develop a new therapeutic strategy using radiotherapy in combination with agents that target pro-survival signalling components.

1.2. Literature Review

1.2.1. Cancer

Cancer is a somatic disease characterised by the dysregulation of cell proliferation and apoptosis. Most cancers are thought to occur as a consequence of a series of DNA repair abnormalities which ultimately establish a growth advantage for the clones of cancer cells in which these repair processes have occurred (Bamford et al., 2004). These cells possess the ability to be self-sufficient in growth signals and insensitive to anti-growth signalling. Tumour cells generate their own growth signals so that they may be independent from growth stimulation from their normal tissue microenviroment. The deregulation of growth signals and their respective receptors enable cancer cells to be hyperresponsive to growth stimuli that would normally not trigger its development. It also allows these cells to remain independent from external growth factors. These characteristics assist in cancers ability to evade apoptosis and replicate continuously (Hanahan and Weinberg., 2000 and 2011).

Many cancers also sustain themselves through the initiation and development of new blood vessels (angiogenesis), which provide nutrients and oxygen. These cells may alter their energy metabolism to a state which could favour the development of new cancer cells. Cancer cells are able to spread (metastasise) from their original location to other parts of the body, or infiltrate the surrounding tissue. (Hanahan and Weinberg., 2000 and 2011). These unique characteristics could, in part, contribute to the current challenges faced in the clinic.

Cancer progression is generally described in stages using roman numerals; stage 0 is when a carcinoma is found *in situ*. Cancer is at stage I when it is localized to one part of the body and stage II cancer is diagnosed when these cells start to invade surrounding tissue on one side of the body. Stage III cancer has also been categorised as, when cancer has advanced locally and a patient is diagnosed with stage IV cancer when this disease had metastasised (Maughan et al., 2010).

Kruger and colleagues have reported that membrane vesicles released into the extracellular environment by malignant cells could play a role in tumour progression. These vesicles known as exosome-like vesicles play a major role in cell-to-cell communication. These vesicles may contain oncogenic molecules that may convey oncogenic signals to non-malignant cells. Kruger et al, has also reported that these vesicles contain molecules which could give insight into a particular cancer that the body might be suffering from, in the case of Kruger et al they looked at breast cancer. They have found that exosomes of MDA-MB-231 and MCF-7 (both breast cancer cell lines) express calmodulin, which regulates the Akt pathway and is associated with poor prognosis in breast cancer patients (Kruger et al., 2014). These exosomes could provide vital information about a specific type of cancer and may assist in the detection of cancer as well.

Approximately 200 different types of cancers exist, their characteristics depending on the tissue of origin (Madani et al., 2011). Breast cancer will be investigated in this study.

1.2.2. Breast Cancer

Originating from mammary gland epithelium, breast cancer is a heterogeneous disease composed of multiple subtypes with unique molecular features. This results in distinctive clinical responses (Park et al., 2012; Perou et al., 2000). Gene expression profiling studies have established four major molecular classifications in which these subtypes are categorised. These groups are**:** Luminal A; Luminal B; HER-2-enriched; and basal cell-like (Perou et al., 2000; Sorlie et al., 2001; 2003). Luminal A or B breast cancer are breast tumours that express progesterone receptors (PR), oestrogen receptors (ER), and low levels to no expression of the human epidermal growth factor receptor 2 (HER-2) (Adelaide et al., 2007; Phipps et al., 2008). HER-2 enriched breast cancer overexpresses the HER-2 membrane receptor (Adelaide et al., 2007). The basal cell-like subtype of breast cancer does not express PR, ER or HER-2 and is thus known as a triple negative subtype (Adelaide et al., 2007; Phipps et al., 2008). Luminal A and B subtypes are considered to have a better prognosis, as they express essential therapeutic targets required for current targeted therapies. The basal cell-like subtype constitutes about 17% of the breast cancer population and is associated with a poor prognosis as this subtype has a ‘triple negative’ phenotype, meaning it does not express ER, PR or HER-2, which are essential therapeutic targets (Foulkes, Smith and Reis-Filho., 2010). Therefore, this subtype is considered to be highly aggressive and difficult to treat. This may not only be attributed to the lack of therapeutic targets (Adelaide et al., 2007; Park et al., 2012; Phipps et al., 2008).

Approximately 20-30% of the breast cancer patient population overexpress HER-2, which is regarded as the HER-2 enriched subtype, and is associated with a poor

clinical prognosis as the amplification of HER-2 has been implicated in enhanced tumour aggressiveness, higher risk of relapse and reduced survival (Adelaide et al., 2007; Molina et al., 2001). This can potentially be attributed to HER-2 being the major component of a signalling pathway which conveys cell survival signals through transducer proteins and DNA transcription regulators (Bombonati et al., 2012; Gutierrez et al., 2011). However, a significant percentage of breast cancer patients do not overexpress HER-2, thus, cancer cells would not completely rely on HER-2 to ignite this survival pathway and a subset of patients would not benefit from anti-HER-2 therapy.

1.2.3. HER-2 Signalling Pathway

The human epidermal growth factor receptor-2 (HER-2/neu, erbB2) is a member of the transmembrane tyrosine kinase receptor family, also known as the epidermal growth factor receptor family, which consists of HER-1 (erbB1); HER-2; HER-3 (erbB3); HER-4 (erbB4) (Bombonati et al., 2012; Gutierrez et al., 2011). The HER-2 signalling pathway is a complex network consisting of three stages (Figure 1). These stages encompass the tyrosine kinase membrane receptor family and their specific ligands which activate the pathway, transducers which transmit this activating signal down the pathway, and effector proteins which regulate the expression of genes controlling tumour cell survival (Bombonati et al., 2012; Gutierrez et al., 2011).

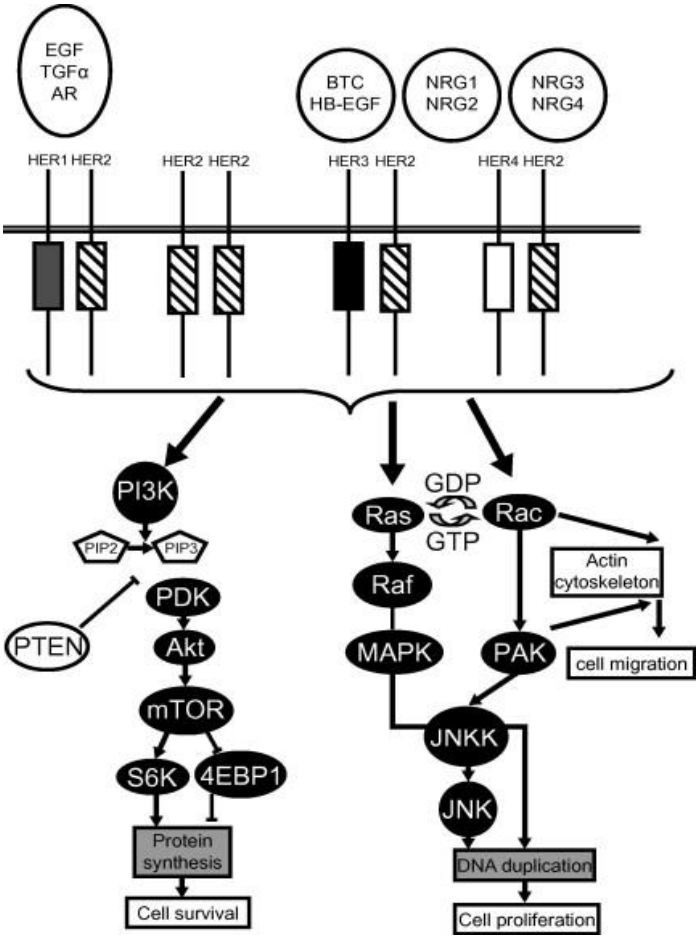

Figure 1: Human epidermal growth factor receptor family signal transduction pathways. Homodimerisation and heterodimerisation of HER-2 leads to tyrosine kinase activation and downstream signalling via the PI3K/Akt/mammalian target of rapamycin pathway, and the Ras/Raf/mitogen-activated protein kinase pathway to stimulate processes involved in cell survival and proliferation (Hurvitz et al., 2013).

HER-2 has no known ligand and thus forms hetero- and homodimers to activate the survival signalling pathway. The phosphorylation of these dimers results in the activation of a sequence of downstream transducer proteins, including phosphoinositide 3-kinase (PI3K), serine-threonine protein kinase (Akt), and mammalian target for rapamycin (mTOR).

Activation of mTOR results in the phosphorylation of protein synthesis regulators, and can lead to the differential expression of genes involved in cell survival, cell growth, and many other vital cellular functions. Dimerisation of the tyrosine kinase receptors family is not only a method of activating the survival pathway, but also a way of diversifying this activating signal to ensure that there is more than one form of dimer that could activate the HER-2 signalling pathway (Hurvitz et al., 2012). Alternate activators of the HER-2 signalling pathway have also been discovered. Nahta and colleagues have reported that HER-2 is also phosphorylated by dimerising with insulin-like growth factor-1 receptor, demonstrating that activation of the HER-2 survival pathway may not completely depend on the epidermal growth factor receptor family (Nahta et al., 2005).

1.2.4. Breast Cancer Therapy

Breast cancer subtypes, as listed in Table 1, have different epidemiological risk factors, unique biological histories and different responses to therapy. These characteristics suggest that each breast cancer subtype will not respond to the same treatment in the same manner and would thus require individualized treatment regimens. Table 1 depicts the recommended treatment regimens for specific subtypes of breast cancer (Goldhirsch et al., 2011).

Table 1: Breast cancer subtypes and their recommended therapy

Breast Cancer Subtype	Recommended Therapy:
Luminal A	Endocrine therapy
Luminal B	Endocrine Therapy, cytotoxic therapy and anti-HER-2 therapy
HER-2 enriched	Cytotoxic therapy and anti-HER-2
Basal-like	Cytotoxic therapy

(Goldhirsch et al., 2011)

The therapeutic options for breast cancer are generally determined by the tumour-node-metastasis staging. Lymphovascular spread, histologic grade, hormone receptor status, HER-2 overexpression, the presence of other disorder, as well as the menopausal state and age of the patients are also important factors to consider (Maughan et al, 2010). Table 2 depicts the typical therapeutic options for each stage of breast cancer.

Table 2: Treatment options for Breast cancer by Stage

Breast cancer Stage:	Typical Therapy:
Stage 0: in situ	No treatment or consider prophylaxis with tamoxifen or Breast-conserving surgery (consider mastectomy if extensive or multifocal and radiotherapy
Stage I and II: Early stage invasive	Breast-conserving surgery and radiotherapy
Stage III: Locally advanced	Chemotherapy followed by breast conserving therapy or mastectomy and radiotherapy
Stage IV: Metastatic	Address patient's treatment goals; radiation and bisphosphate for pain

(Maughan et al, 2010)

At stage 0 breast cancer, there are two types of carcinomas that require different treatment approaches. Lobular carcinoma *in situ* is a minor finding of an abnormal tissue growth in the lobule of the breast, and does not progress to invasive breast cancer. Ductal carcinoma *in situ* can progress to invasive breast cancer. The standard therapy for a ductal carcinoma *in situ* is breast conserving surgery followed with radiotherapy; however a mastectomy may be recommended for multifoci disease (Maughan et al., 2010).

For patients with stages I and II cancer, surgery has become the standard therapeutic regimen. Breast-conserving surgery has now become the preferred treatment over the traditional modified radical mastectomy. The outcome of this surgery is more aesthetically pleasing to the patient as only the tumour is removed leaving behind the healthy mammary tissue, unlike the radical mastectomy where the whole breast is removed. Radiotherapy is given after the breast conserving-surgery; this decreases the chances of local recurrence and improves patient survival rates. Breast conserving surgery has the highest success rate in women with early-stage breast cancer (Maughan et al., 2010).

When breast cancer has advanced into the surrounding tissue and includes tumours larger than 5 cm, extensive regional lymph node involvement, as well as the chest wall and skin, and if these tumours are considered inoperable, and the disease is classified as stage III breast cancer. Chemotherapy with surgery and radiotherapy is recommended for this stage of breast cancer. A 5-year survival rate of 55% may be achieved in patients with stage III breast cancer (Maughan et al., 2010).

Metastatic breast cancer is the final stage. Patients who relapse after being treated for stage I, II, or III breast cancer may present with metastatic disease. A 5-year survival rate of only 23.3% may be attained in of these patients. Treatment with radiation or bisphosphonates, endocrine therapy or chemotherapy may assist in managing pain. Although chemotherapy and endocrine therapy can be tolerated, they inevitably have side effects. Trastuzumab alone or in combination with chemotherapy may be used as treatment for metastatic cancer overexpressing ERBB2 (HER-2) (Maughan et al., 2010).

Breast cancer therapy has progressed from highly invasive, non-specific, extremely toxic interventions to treatment modalities that are less invasive, highly specific and less toxic. Although there has been a significant improvement in breast cancer treatment, as well as the number of treatment options available, patient benefit has been suboptimal (Verbrugge et al., 2014). Therefore, new treatment strategies are warranted that would maximise beneficial patient response. For the purposes of this study radiotherapy and targeted therapies will be the investigated.

1.2.4.1. Radiotherapy

Ionising radiation has been used for years as the primary treatment for many types of cancers (Yacoub et al., 2006). Radiotherapy can cause cell death through the generation of free radicals which induce high levels of cellular DNA damage (Buchholz 2009; Duru et al., 2012; Pearce et al., 2001).

Radiotherapy is a significant primary treatment for breast cancer as it plays an essential role in the local control of the disease (Liang et al., 2003). Randomised clinical trials have reported that the addition of radiotherapy as an adjuvant to other treatment regimens for breast cancer improves overall disease-free patient survival (Li et al., 2012; Liang et al., 2003). This therapy has become the standard treatment after breast conserving surgery for patients with early stage cancer as it reduces the risk of local recurrence and can assist in preventing the need for mastectomy (Pinnaro et al., 2010; Whelan., 2010). Despite advances in early diagnosis and treatment efficacy, death due to breast cancer remains high, and has been attributed to treatment evasion by metastatic tumours and the recurrence of primary tumours (Duru et al., 2012).

Radioresistant breast cancer cells are able to survive during radiation exposure, thus increasing the risk of tumour recurrence. Cellular exposure to ionising radiation has been known to activate HER-2 and the downstream effectors of the HER-2 survival pathway (Escriva et al., 2008; Contessa et al., 2002). In some cases, HER-2 overexpression in cancer cells may indicate resistance to radiation by a variety of biochemical pathways through the activation of signalling molecules. Mammalian cells express various proteins in response to ionising radiation, suggesting that cells exposed to radiation can be manipulated by a particular survival signalling network (Amundson et al., 2004; Bisht et al., 2003).

A tumour may consist of several distinct subpopulations of cells that may respond differently to radiation therapy (Forrester et al., 1999). Radioresistant tumours are thus a significant clinical obstacle as they limit the effectiveness of radiotherapy (Duru et al., 2012; Li et al., 2012).

1.2.4.2. Targeted Therapy

The understanding that breast cancer is a disease composed of various subtypes, each possessing unique molecular characteristics that can lead to a variety of clinical responses, has led to the identification of various biological markers which play an essential role in signalling pathways that regulate cancer cell survival and proliferation. These markers paved the way for the development of target-based therapies that are highly specific, less invasive and less toxic than conventional treatment (Normanno et al., 2009). Tyrosine kinase inhibitors (TKIs) directed at a number of targets (HER-1, HER-2, HER-3 and IGF receptors), intracellular signalling pathway inhibitors (PI3K, Akt, mTOR), angiogenesis inhibitors, and agents that

interfere with DNA repair are among the examples of targeted therapies. Some of these treatments have been shown to be effective in managing breast cancer and have become the standard in breast cancer patient care (Higgins and Baselga., 2011).

Trastuzumab and lapatinib are two examples of clinically available targeted therapies that have become standard treatments for HER-2 positive breast cancer. Trastuzumab is a monoclonal antibody which targets the extracellular domain of the HER-2 receptor, and lapatinib is a tyrosine kinase inhibitor that targets the intracellular domain of HER-2 and epidermal growth factor receptor. Despite the overall improvement in survival seen with the addition of targeted therapy to other treatment regimens, many breast cancer patients do not benefit as a result of treatment resistance (Hurvitz et al., 2013). Acquired tumour resistance has been attributed to the loss of target expression as a result of continuous exposure to therapy and the activation of additional pathways that promote cell survival (Higgins and Baselga., 2011). The heterogeneity in the distribution of target expression can contribute to targeted treatment resistance. A particular cell population may exhibit a wide variation in the distribution of the antigen of interest, with some cells showing little or no target expression. This can lead to the inability to effectively target all cells of a specific subpopulation with toxic levels of a therapeutic agent. The phenomenon has been demonstrated for radiopharmaceuticals, chemotherapeutic drugs, and radioimmunotherapeutics (Akudugu and Howell, 2012a, b; Akudugu et al., 2011; Kvinnsland et al., 2001). Change in target morphology can also limit treatment efficacy. A change in antigen shape would make it difficult for the antigen specific treatment agent to bind to the relevant target and exert its toxic action. This is observed when breast cancer cells express the truncated HER-2 (p95HER-2). The

p95HER-2 lacks the epitope for trastuzumab, which is found in the full length version of this receptor. This makes these cells resistant to trastuzumab therapy (Zagozdzon et al., 2011). The development of resistance to targeted therapies is an ongoing challenge that may be addressed by novel treatment strategies (Higgins and Baselga, 2011). Targeted therapy is not only limited to anti-HER-2 targeted therapy; there are other options available which depends on the subtype of breast cancer. Hormone therapy stops growth hormone sensitive tumours by preventing the body from producing these hormones or by interfering with the action of these hormones, an example is aromatase inhibitors which blocks oestrogen production by disrupting the conversion of androgens to oestrogens (Maughan et al., 2010). Other targeted therapies are signal transduction inhibitors, gene expression modulators, apoptosis inducers, angiogenesis inhibitors and immunotherapies. This study will focus on another form of targeted therapy, tyrosine kinase inhibitors.

There is an immense interest in tyrosine kinase inhibitors as many of these have been shown to be effective anti-cancer agents (Moasser et al., 2001). Tyrosine kinase inhibitors are molecules that compete with adenosine triphosphate (ATP) at the binding site of the respective tyrosine kinase receptor. This results in the blockage of receptor phosphorylation and subsequent signalling to survival and proliferating pathways (Roy et al., 2009). Tyrosine kinase inhibitors have displayed an ability to affect trastuzumab resistant cancer cells and have several advantages over monoclonal therapy (Lin and Winer., 2004). These inhibitors are active against the truncated forms of HER-2 receptors *in vitro* (Lin and Winer., 2004). They are biologically available and well tolerated, when compared with trastuzumab. Also, they may be manipulated to target more than one receptor family simultaneously (Spector et al., 2007; Lin and Winer., 2004).

The overexpression of HER-2 is not the only contributing factor to trastuzumab resistance in breast cancer. The activation of the PI3K/Akt/mTOR pathway has been implicated in trastuzumab resistance (Chandarlapaty et al., 2012). These proteins, PI3K; Akt; and mTOR, have also been shown to be activated by cellular exposure to ionising radiation as they convey signals that lead to cell survival. This justifies the investigation of inhibitors which target HER-2 and the downstream PI3K/Akt/mTOR pathway, as the inhibition of these proteins would lead to the cessation of survival signals and result in improved treatment response.

1.2.4.2.1. TAK-165

TAK-165 (Millenium Pharmaceuticals, Inc, Cambridge, MA) is a tyrosine kinase inhibitor which targets HER-2 and has shown activity in breast cancer overexpressing HER-2 (Spector et al., 2007).

1.2.4.2.2. NVP-BEZ235

The PI3K pathway is a component of the HER-2 signalling pathway that a cell requires for the propagation of survival and proliferative signals. The activation of this pathway is commonly found in human cancers. The inhibition of this pathway may result in a variety of genetic and epigenetic abnormalities, which can produce PTEN mutations and the amplification of HER-2 which promotes treatment resistance. The PTEN gene provides instructions for making a protein that is found in almost all tissues in the body. The protein acts as a tumour suppressor, regulating the cycle of cell division by keeping cells from growing and dividing too rapidly or in an

uncontrolled way. Mutations in the PTEN gene result in an altered protein that has lost its tumour suppressor function. The loss of this protein's function likely permits certain cells to divide uncontrollably, contributing to the growth of cancerous tumours Barlund et al., 2000; Jefferies et al., 1997; Klos et al., 2006). Targeted therapy against specific components of this pathway appears to be effective as single agents, or in combination, in various human cancers (Brachmann et al., 2009).

NVP-BEZ235 (BEZ235; Novartis Pharma) is a low molecular weight molecule imidazoquinoline that inhibits the class 1 PI3K and mTOR catalytic activity by competing at the ATP-binding site (Maira et al., 2008). In a study by Brachmann and colleagues, it was determined that BEZ235 exhibited both antiproliferative and cytotoxic activities (Brachmann et al., 2009). While BEZ235 caused cell death in cell lines MDA-MB-453; MCF-7; MDA-MB-361; SKBr3 and HCC141, it inhibited proliferation in cell lines MDA-MB-231 and MDA-MB-468. The study further showed that BEZ235 efficiently shuts down the PI3K pathway in HER-2 amplified cells, and induces cell death via active apoptotic processes that preferentially require the activation of the initiator, caspase-2 (Brachmann et al., 2009). Ultimately, it was concluded that BEZ235 would be most effective for breast cancer with HER-2 and/or PI3K mutations. This provides a strong rationale for phase II studies using BEZ235 alone or in combination with anti-HER-2 therapy.

1.2.5. Combination Therapy

Combination therapy aims to overcome the limitations of monotherapy by modulating various therapeutic targets to synergistically enhance therapy effectiveness. In combination therapy, one molecule may improve the action of another by increasing

its penetration or preventing its destruction (Reece et al., 2007). A two-fold rationale has been suggested for the use of concomitant therapy. Firstly, when multiple drugs with different targets are applied, cancer cell adaption such as mutations can be delayed. Secondly, multiple drugs targeting the same cellular pathway could function synergistically for higher therapeutic efficacy and higher target selectivity (Lee and Nan, 2012).

Preclinical data show that using multiple antibodies directed against the HER family in combination with various tyrosine kinase inhibitors may lead to therapeutic strategies that selectively diminish oncogenic-related signalling and result in remarkable anti-tumour activity. This treatment strategy could possibly overcome treatment resistance (Nielsen et al., 2008).

It has been suggested that targeting the HER-2 pathway may increase the anti-tumour activity of ionising radiation (No et al., 2009). In this regard, it was shown that LY294002, a PI3K inhibitor, can inactivate the PI3K/Akt/mTOR pathway and significantly attenuate the expression of p-70S6K and phosphorylation of Akt leading to radiosensitisation of SKBR3 cells (No et al., 2009). The 70-kDa ribosomal protein S6 kinase (p-70S6K) is a serine/threonine kinase that regulates protein translation by phosphorylating ribosomal protein S6. p-70S6k is downstream of the phosphoinositide 3-kinase/AKT/mammalian target of rapamycin (mTOR) pathway, which is activated by HER-2, insulin-like growth factor receptor, and oestrogen receptor in breast cancer. Patients with tumours having increased p-70S6K phosphorylation have worse disease-free survival and increased metastasis, which shows the importance of p-70S6K in breast malignancy. This suggests that selective targeting of downstream signalling in combination with radiation as a potential therapeutic approach may defeat resistance to HER-2 therapy.

Serra and colleagues reported that when the PI3K pathway is inhibited by BEZ235, the activity of compensatory pathways such as those involving the HER gene, increases (Serra et al., 2011). This suggests that PI3K and HER-2 activities are connected through a feedback loop. When the phosphorylation PI3K pathway is inhibited, the cell increases the phosphorylation of HER-2 so as to send the signal downstream to increase phosphorylation of PI3K and increase signalling to Akt and mTOR. It is thus conceivable that when HER-2 phosphorylation is inhibited, the cell increases the phosphorylation of PI3K through alternate signalling proteins. Thus targeting both these proteins using a combined therapeutic modality would be effective in inhibiting tumour cell growth. BEZ235, which targets PI3K and mTOR, in combination with lapatinib and trastuzumab, which targets HER-2, emerged more effective in reducing cell proliferation than when these agents were used individually (Serra et al., 2011). Deregulation of the PI3K/Akt pathway sensitises cells to radiation (Riesterer et al., 2004). The current study will seek to manipulate the radiosensitivity of breast cancer cell lines MDA-MB-231 and MCF-7 by exposing them to a cocktail of TAK-165 and BEZ235. This will assist in deciphering if exposing breast cancer cells to a cocktail of survival pathway inhibitors can enhance cellular radiosensitivity, and provide insights into formulating novel therapeutic approaches.

1.3. Problem Statement

Contemporary breast cancer treatment modalities have improved patient outcomes. However, many do not benefit from these approaches as various clinical challenges still exist. Inherent and acquired resistance to treatment are just two of these challenges (Higgins and Baselga., 2011; Hurvitz et al., 2013). The inability to effectively target malignant cells with toxic levels of a single agent has been attributed to the heterogeneous expression of target antigens. This phenomenon has been demonstrated for radiopharmaceuticals, chemotherapeutic drugs and radioimmunotherapeutics (Akudugu and Howell, 2012a, b; Akudugu et al., 2011; Kvinnsland et al., 2001). Treatment of most cancers with a single agent has, therefore, had limited success and novel treatment strategies are crucial in addressing these therapeutic challenges.

1.4. Hypothesis

Inhibiting components of the HER-2 signalling pathway can significantly sensitise breast cancer cells to low doses of ionising radiation.

1.5. Aims and Objectives

This study aimed to identify potential therapeutic targets in breast cancer cell lines through the use of known inhibitors of the HER-2 survival pathway and the downstream components of this pathway. Additionally, to evaluate the effect these inhibitors would have individually and combined as radiosensitizers.

To achieve these specific aims this study's objectives are as follows**:**

1. To determine the relative survival of MDA-MB-231 and MCF-7 cell lines after exposure to ionising radiation doses ranging from 0 - 10 Gy to determine the normal radiosensitivity of these cell lines.
2. Treat the aforementioned breast cancer cells with a dose of TAK-165 or BEZ235, and expose them to ionising radiation.
3. Determine cell survival after treating these cells with an inhibitor and ionising radiation exposure, also to determine target protein expression.
4. To make-up inhibitor cocktails.
5. Treat cells with inhibitor cocktails and expose them to ionising radiation.
6. To determine cell survival after their aforementioned therapeutic approach and determine radiation enhancement factors.

CHAPTER 2

2. Materials and Methods

2.1. Cell lines

2.1.1. MCF-7

MCF-7 is a human mammary adenocarcinoma-derived cell line established from a metastatic lesion. MCF-7 is classified as a luminal A breast cancer subtype (Subik, et al. 2010). It has an epithelial-like morphology, is adherent and grows as a monolayer in Roswell Park Memorial Institute medium (RPMI-1640) (Sigma-Aldrich, USA), supplemented with 10% heat-inactivated foetal bovine serum (FBS) (HyClone, UK), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Lonza, Belgium). A frozen vial of cells was obtained from Professor S. Prince (University of Cape Town, South Africa).

2.1.2. MDA-MB-231

MDA-MB-231 is a human mammary adenocarcinoma-derived cell line established from a metastatic lesion. MDA-MB-231 is classified as a basal-like breast cancer subtype (Subik, et al. 2010). It has an epithelial-like morphology, is adherent and grows as a monolayer in RPMI-1640 medium (Sigma-Aldrich, USA), supplemented with 10% heat-inactivated foetal bovine serum (FBS) (HyClone, UK), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Lonza, Belgium). The cells were obtained from Professor S. Prince (University of Cape Town, South Africa).

2.2. Cell Culture Maintenance

All cell cultures were kept at 37ºC in a humidified atmosphere of 95% air and 5% CO_2 in SHEL LAB incubators (Sheldon Manufacturing Inc., USA), and procedures were carried out in vertical laminar flow cabinets using aseptic techniques. Cells were routinely grown in 75 cm^2 flasks, and were passaged when culture confluency was between 80 to 90%. For cryopreservation, cells were trypsinised, pelleted by centrifugation (4000 RPM for 5 minutes), resuspended in a mixture of 0.9 ml foetal bovine serum and 0.1 ml of dimethyl sulfoxide (DMSO), stored at -80°C overnight, and then transferred into liquid nitrogen for use at a later stage. The MCF-7 and MDA-MB-231 (MDA) cell lines were used in all experiments at passage numbers ranging from 39-49 and 18-28, respectively.

2.3. Irradiation Procedure

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

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

2.4. Clonogenic Cell Survival Assay

To determine the relative radiosensitivities of the breast cancer cell lines used in this study, clonogenic cell survival assays were performed. Near-confluent stock cultures were washed with sterile phosphate buffered saline (PBS), trypsinised, and the cells counted using a haemocytometer. The cells were then seeded in triplicate per experiment in 25 cm^2 culture flasks at numbers ranging from 500 - 10 000 per flask, depending on radiation absorbed dose, and left to settle for 3 - 5 hours before being exposed to ionising radiation. Cells were irradiated to graded doses ranging from 0 - 10 Gy. After an appropriate incubation period (usually 7-10 days), the colonies were fixed by decanting the medium in the flask, and replacing that with 10 ml of fixative, consisting of 100 ml glacial acetic acid, 100 ml methanol and 800 ml distilled water, for 10 minutes. The fixative was then decanted and replaced with 10 ml of Amido Black stain, consisting of 10 ml of 0.01% amido black in 1 litre of fixative. The colonies were left to stain for 10 minutes. The stain was then decanted, and the flasks left to dry. The colonies were counted using a dissection microscope. The means (± SD) of the surviving fractions for three experiments were plotted against the irradiation dose, cell survival curves were obtained by fitting the data to the linear-quadratic survival equation:

$$Survival\ (S) = e^{-(\alpha D + \beta D^2)} \quad (1)$$

where *S* is the surviving fraction, α and β are the linear and quadratic cell inactivation constants, respectively, and *D* is the dose in Gy (Fertil et al., 1984).

2.5. Target Inhibitors

BEZ235 is a dual inhibitor of PI3K and mTOR; and has a molecular weight of 469.55 and chemical formula $C_{30}H_{23}N_5O$ (Santa Cruz Biotechnology, Texas, USA). The inhibitor was dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 106 mM, which was stored at -20°C until used. BEZ235 has an inhibitory concentration (IC_{50}) of ~7 nM for p110δ activity in breast cancer cell lines: CCL-247, HTB-38 and HTB-20 (Maira et al., 2008).

TAK-165 is an inhibitor of the human epidermal growth factor receptor 2 (HER-2) tyrosine kinase; and has a molecular weight of 468.47, and chemical formula $C_{25}H_{23}F_3N_4O_2$ (Tocris, UK). It was also dissolved in DMSO to a stock of 21 mM and stored at 4ºC until needed. TAK-165 is known to have an IC_{50} of 6 nM for HER-2 activity in the BT474 breast cancer cell line (Nagasawa et al., 2006).

2.6. Effect of inhibitors on radiation response

Radiosensitisation induced by inhibitors, BEZ235 and TAK-165, added 30 minutes prior to irradiation was assessed by clonogenic assay in the MCF-7 and MDA-MB-231 cell lines. Cell cultures were exposed to inhibitors for the duration of the colony formation experiments. For HER-2 inhibition, breast cancer cells were exposed to TAK-165 at a concentration of 30 nM (~4 $\times$ IC_{50} for HER-2 activity in the BT474 breast cancer cell line (Nagasawa et al., 2006)). To inhibit PI3K and mTOR, BEZ235 was administered at a concentration of 17.5 nM (~2.5 $\times$ IC_{50} for p110δ activity in breast cancer cell lines: CCL-247, HTB-38 and HTB-20 (Maira et al., 2008)). The use of the relatively high concentrations of TAK-165 was to ensure adequate inhibition of the targets. A cocktail of TAK-165 and BEZ235 was also formulated to inhibit all three critical targets of the HER-2 signalling pathway. These agents were used at the same concentration in combination as when used individually. The final concentration of DMSO in cell cultures was 0.2%. Control cultures were not treated with DMSO vehicle, as vehicle concentrations up to 5% has been shown to be non-toxic in the MCF-7 cell line (Gao and Meléndez, 2010). Also, no cytotoxicity was demonstrated at a 10% DMSO concentration in Caco2/TC7 colon cell cultures (Da Violante et al., 2002).

Cells were seeded in 25 cm^2 tissue culture flasks in numbers ranging from 1000 - 6000 cells per flask, depending on radiation dose. After cell attachment and inhibitor treatment, the flasks were irradiated to 0, 2 and 6 Gy with ^{60}Co γ-irradiation. After an incubation period of 7 - 10 days, colonies were fixed, stained, and counted. The corresponding surviving fractions were calculated. Unirradiated cell cultures, with and without inhibitors, served as controls.

Radiosensitisation by inhibitors was expressed as a radiation enhancement factor (REF), given by the ratio of surviving fractions, with and without inhibitor:

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

or

$$\mathrm{REF}_{6\mathrm{Gy}} = \frac{\mathrm{SF\ (6\ Gy)}}{\mathrm{SF\ (6\ Gy+inhibitor)}} \qquad (3)$$

The criteria for inhibition, no effect, and enhancement of radiosensitivity by inhibitors were REF < 1.0, REF ≈ 1.0 and REF > 1.0, respectively (Akudugu and Slabbert., 2008).

2.7. Protein Extraction

Total protein extraction from all cell lines was performed on ice (4°C). Cells were harvested at 0 and 6 hours after radiation with 0 and 2 Gy. The medium was decanted and the cells rinsed with cold PBS. Cells were then mechanically harvested as a cell suspension in fresh growth medium by gentle scraping with a cell scraper. The cell suspensions were then pelleted by centrifugation (4000 RPM for 5 minutes), washed and resuspended in cold PBS, followed by a second centrifugation. The pellets were then resuspended in 250 µl TBS/1%Triton X-100 extraction buffer supplemented with 10 µl of protease inhibitor cocktail and 10 µl phosphatase inhibitor cocktail (Sigma-Aldrich, USA), and placed on a tube roller mixer overnight at 4°C. Lysates were clarified by centrifugation (15000 RPM for 15

minutes) to remove cellular debris, and the supernatants were collected and stored at -80°C.

2.8. Protein Concentration Determination

Protein determination was performed on ice (4°C) by means of the bicinchoninic acid colorimetric assay kit, manufactured by Pierce (Rockford, IL, USA). Samples were diluted in TBS/1%Triton X-100 buffer (1:1 and 1:5), and 10µl of the diluted protein placed in a 96-well multiwell plate. Two hundred microliters of working reagent (Reagent A, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide, and Reagent B containing 4% cupric sulphate were mixed in a 51:1 ratio, to produce a clear light green solution) was added to each well, and the plate incubated at 37°C for 30 minutes. A purple colour developed. The reaction was stopped by placing the multiwell plate at 4°C for 10 minutes before the absorbance values were measured with a Labtech L-4000 microplate reader (Sussex, UK) at a wavelength of 570 nM.

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

2.9. SDS-Polyacrylamide Gel electrophoresis

2.9.1. Preparation of Acrylamide Gels

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine changes in the expression of PI3K, mTOR and HER-2 proteins. This method separates proteins by the relative distance they travel across a polyacrylamide gel matrix, based on their molecular weights.

A 10% separating gel, with a 5% stacking gel, was used for optimum resolution of the PI3K, mTOR and HER-2 proteins. Briefly, the separating gel was prepared by mixing volumes of the following components**:** 5.9 ml of distilled water, 5 ml of 30% acrylamide/Bis solution 29:1 (3.3%), 3.8 ml of 1.5 M Tris (pH 8.8) and 150 µl of 10% SDS. One hundred and fifty microlitres of freshly prepared 10% ammonium persulfate and 40 µl of tetramethylethylenediamine (TEMED) were added to initiate the polymerisation reaction. The stacking gel was prepared as follows**:** 2.7 ml of distilled water, 670 µl of 30% acrylamide/Bis solution 29:1 (3.3%), 500 µl of 1.5 M Tris (pH 6.8) and 40 µl of 10% SDS. 40 µl of freshly prepared 10% ammonium persulfate and 20 µl of TEMED were added to initiate the polymerisation reaction. Using a Pasteur pipette, isopropanol was gently layered on the top of the resolving gel to allow polymerisation to proceed in the absence of oxygen.

Samples were boiled at 95°C for 5 minutes in Laemmli sample buffer (Bio-Rad, USA) before loading onto the gel. Equivalent amounts of protein (60 µg) were loaded per lane. Twenty microlitres of a precision pre-stained 10 - 250 kDa molecular weight marker (Bio-Rad, USA) was loaded in the first lane to assist with the orientation and size determination of separated proteins.

Proteins were fractioned by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a Mini-PROTEAN® Tetra cell vertical electrophoresis system (Bio-Rad, USA) at a constant voltage of 150 V (AA Hoefer Power Pac PS300-B, USA) until the gel front reached the bottom of the gel.

2.10. Western Blotting and Immunoblot

To establish whether the targets of interest are expressed by the cell models used, unirradiated and irradiated cells were assayed for protein levels, by Western blotting with antibodies specific for HER-2, PI3K and mTOR.

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

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

2.11. Ponceau S Staining of PVDF Membranes

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

2.12. Immuno-detection

The membranes were blocked in Tris buffered saline with Tween 20 (TBST) containing 5% non-fat milk (Elite fat free milk powder, Clover SA (Pty) Ltd) for 25 minutes at room temperature, followed by two washes in Tris buffered saline (TBS)/Tween 20 solution for 5 minutes each, before the primary antibodies were added and membranes left rotating overnight at 4°C. The final concentration of each specific primary antibody on the membrane was 1:1000, as per supplier's instructions. Incubation in primary antibodies against PI3K, Anti-PI3 Kinase p110 beta antibody (ab5593) (Abcam, UK), HER-2, Neu 3B5: sc-33684 (Santa Cruz Biotechnology Inc., USA), mTOR, mTOR (7C10) Rabbit mAb (Cell Signaling Inc., USA) was followed by 3 five-minute washes in TBS/Tween 20 solution, before the addition of a secondary antibody goat anti-rabbit IgG horseradish peroxidase antibody (Santa Cruz Biotechnology, USA) and incubated for 2 hours at room temperature. The concentration of each specific secondary antibody was also 1:1000, as per supplier's instructions. The membranes were then washed several times in TBS/Tween 20 solution, before protein detection.

2.13. Western Blotting Detection System

Following Western blot transfer the immobilised proteins were detected using the tetramethylbenzidine membrane peroxidase substrate system (KPL Inc., USA). This is a sensitive colorimetric detection method for the presence of peroxidase conjugates. Immersion of the membrane in TMB for 15 minutes at room temperature permitted easy visualisation of the blue bands.

2.14. Data Analysis

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

CHAPTER 3

3. Results

3.1. Radiosensitivity

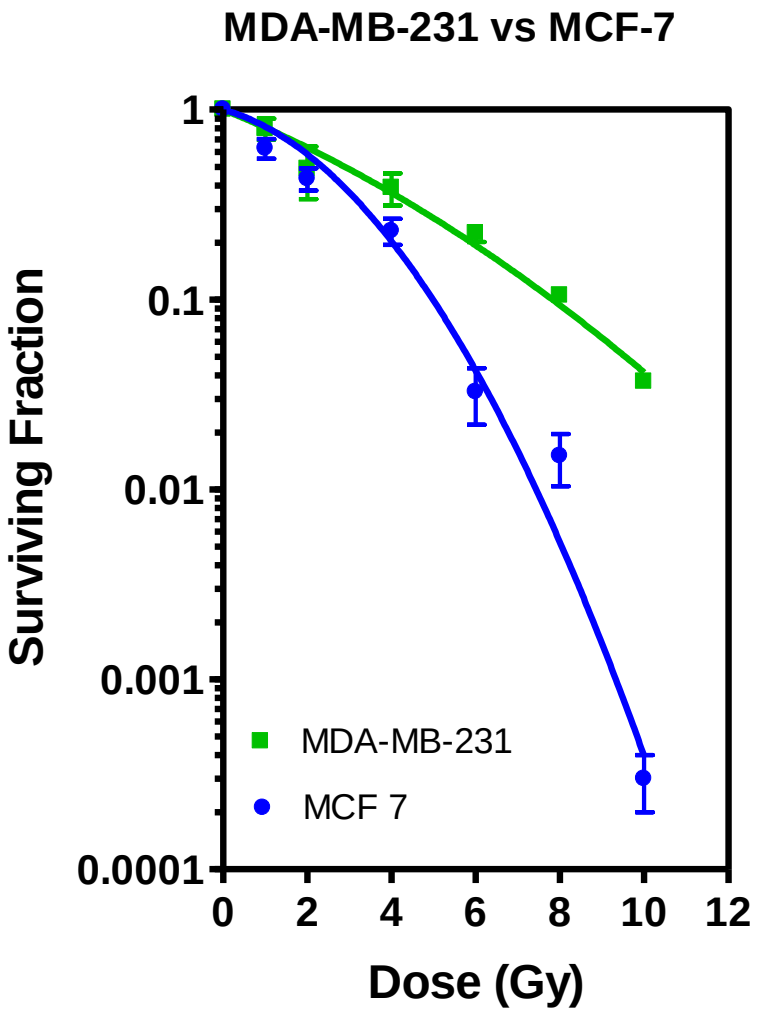

Figure 2: Clonogenic survival curve for the breast carcinoma cell lines MDA-MB-231 and MCF-7 after ^{60}Co γ-irradiation. Symbols represent the mean (±SEM) surviving fraction from 3 independent experiments. The survival curve was obtained by fitting experimental data to the linear-quadratic model.

The relative radiosensitivity of MDA-MB-231 (MDA) and MCF-7 was evaluated using the clonogenic cell survival assay. Cell survival data for the human breast carcinoma cell lines were fitted to the linear-quadratic model and are presented in Figure 2.

From the dose-response curves presented in Figure 2, it is apparent that the breast carcinoma cell line MCF-7 is more radiosensitive than the MDA-MB-231 cell line. This is consistent with the relatively steeper survival curve for MCF-7 than for MDA-MB-231. The α- and β-coefficients for the MCF-7 and MDA-MB-231 cell lines were 0.14 ± 0.10 Gy^{-1} and 0.06 ± 0.01 Gy^{-2} and 0.21 ± 0.04 Gy^{-1} and 0.01 ± 0.01 Gy^{-2}, respectively. For comparison, intrinsic cellular radiosensitivity was expressed in terms of the surviving fraction at 2 Gy (SF_2). The MDA-MB-231 cell line was found to be marginally more radioresistant than MCF-7, on the basis of SF_2. The SF_2-values emerged as 0.64 ± 0.09 and 0.58 ± 0.02 for the MDA-MB-231 and MCF-7 cell lines, respectively ($P = 0.0528$).

3.2. Cytotoxicity of Inhibitors

To assess the cytotoxicity of the PI3K, mTOR and HER-2 inhibitors, plating efficiencies (PE) for cell cultures were determined in the presence of the inhibitors, either singly or in combination, and compared with those obtained for cells cultured without inhibitors. The PE-values for the MDA cell line were found to be 0.2867 ± 0.1105 and 0.2500 ± 0.0700 in the absence and presence of BEZ235, respectively, and was the same as that observed ($P = 0.7931$). Treatment of these cells with 17.5 nM BEZ235 does not result in cell kill, based on colony formation. The PE-value for TAK-165 emerged as 0.1967 ± 0.0694 and was not significantly different from the PE

without inhibitor (P = 0.5282), corresponding to no cell kill. The plating efficiency in MDA cultures treated with a cocktail of the two inhibitors was 0.2967 ± 0.1102, and was the same as that observed in untreated cultures (P = 0.9520).

For the MCF-7 cell line, the plating efficiencies were 0.1967 ± 0.0120 and 0.0559 ± 0.0228 in the absence and presence of BEZ235, respectively, and were significantly different (P = 0.0055). BEZ235 induced a 72% cell kill in this cell line. The plating efficiency in TAK-165 treated MCF-7 cultures was 0.0820 ± 0.0006 and differed significantly from that obtained for untreated cultures (P = 0.0007). TAK-165 treatment results were about 58% cell kill in MCF-7 cultures. The PE-value for MCF-7 cells following treatment with a cocktail of the two inhibitors was found to be 0.0530 ± 0.0231 and was also significantly different from the PE for untreated cultures (P = 0.0052), giving a 73% cell kill indicating that the addition of TAK-165 and BEZ235 does not seem to have an effect on cell adherence.

3.3. Modulation of Radiosensitivity of MDA and MCF-7 at 2 Gy

To assess whether blocking the activities of HER-2, PI3K and mTOR with specific inhibitors results in changes in cellular radiosensitivity, cell cultures were treated with TAK-165 (against HER-2) and BEZ235 (against PI3K and mTOR), and subsequently irradiated with 2 Gy. The cell survival data for MDA-MB-231 and MCF-7 are shown in Figures 3 and 4.

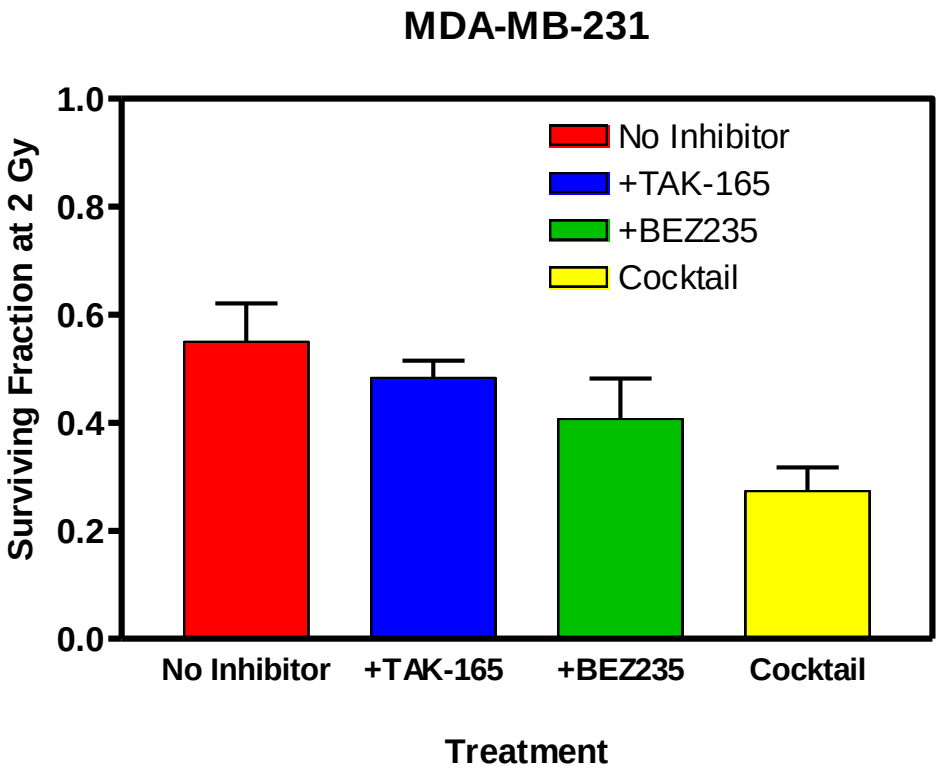

Figure 3: Clonogenic cell survival at 2 Gy of ^{60}Co γ-irradiation for a human breast carcinoma cell line (MDA-MB-231) following single or combined (cocktail) treatment with TAK-165 (HER-2 inhibitor) and BEZ235 (PI3K and mTOR inhibitor).

Treatment with TAK-165 alone sensitised MDA cells, resulting in a reduction in SF_2 from 0.55 ± 0.07 to 0.48 ± 0.03. The radiosensitisation was not statistically significant (P = 0.4395). A higher radiosensitisation was observed when MDA cells were pre-treated with BEZ235, with SF_2 decreasing from 0.55 ± 0.07 to 0.41 ± 0.08 (P = 0.2377). The SF_2 values in the presence of the two agents were not significantly different (P = 0.4006). When the cells were treated with a cocktail of TAK-165 and BEZ235, the surviving fraction at 2 Gy emerged as 0.27 ± 0.04 and was significantly lower than that obtained for TAK-165 treatment (P = 0.0181). A higher, but not

significantly different, radiosensitisation was obtained for cocktail treatment, compared with that for BEZ235 (*P* = 0.2006).

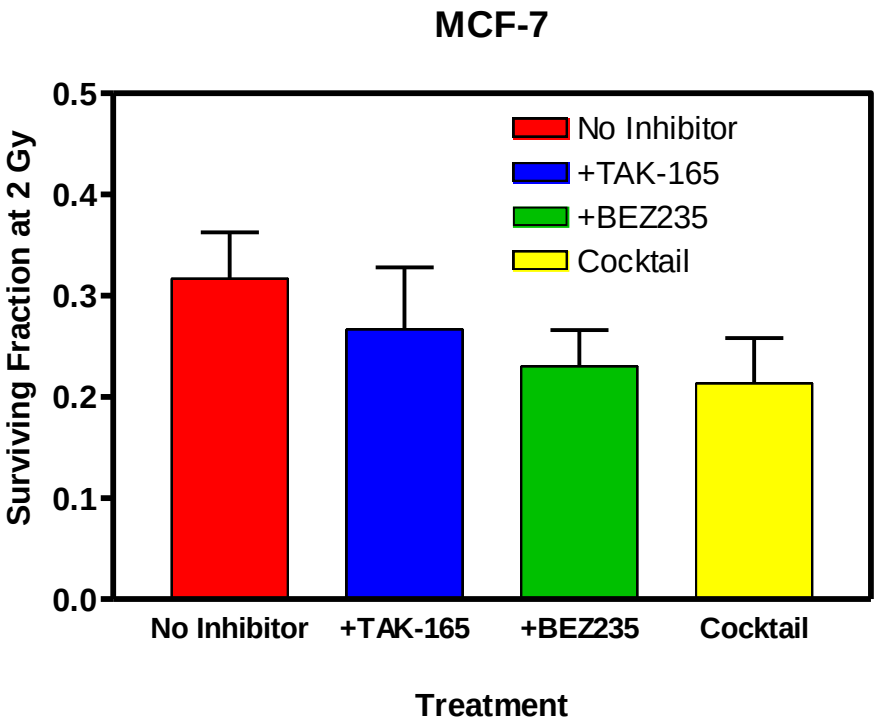

Figure 4: Clonogenic cell survival at 2 Gy of ^{60}Co γ-irradiation for a human breast carcinoma cell line (MCF-7) following single or combined (cocktail) treatment with TAK-165 (HER-2 inhibitor) and BEZ235 (PI3K and mTOR inhibitor).

The corresponding data for the MCF-7 cell line is presented in Figure 4. MCF-7 cells pre-treated with TAK-165 alone resulted in a reduction in surviving fraction at 2 Gy from 0.32 ± 0.05 to 0.27 ± 0.06 (*P* = 0.5452), indicating no significant cellular sensitisation. An increase in radiosensitisation was observed when cells were pre-treated with BEZ235 and exposed to 2 Gy of ionising radiation, resulting in a decrease in SF_2 from 0.32 ± 0.05 to 0.23 ± 0.04 (*P* = 0.2081). Cellular treatment with a BEZ235/TAK-165 cocktail appeared to have an effect on the radiosensitivity of the MCF-7 cells, resulting on a surviving fraction at 2 Gy of 0.32 ± 0.05 to 0.21 ± 0.04 (*P*

= 0.1798). Although the trend indicates an increase in radiosensitivity when cells were treated with a cocktail of BEZ235 and TAK-165, statistically there was no difference (P>0.05).

3.4. Modulation of Radiosensitivity of MDA and MCF-7 at 6 Gy

To investigate whether the apparent radiosensitisation seen in both breast carcinoma cell lines exist at higher fractional doses, as may be encountered in hypofractionated treatments, cell culture were also treated with the inhibitors of HER-2, PI3K and mTOR, irradiated to 6 Gy, and were assessed for clonogenic survival. The cell survival data are presented in Figures 5 and 6 for MDA-MB-231 and MCF-7, respectively.

The data in Figure 5 show no difference in cell survival when MDA-MB-231 cells were pre-treated with TAK-165 and exposed to 6 Gy. The surviving fractions at 6 Gy (SF_6) were 0.17 ± 0.02 to 0.16 ± 0.03 for control and inhibitor treated cell cultures, respectively (P = 0.8554). When cells were pre-treated with BEZ235 alone, SF_6 decreased from 0.17 ± 0.02 to 0.11 ± 0.02 (P = 0.1242). BEZ235 appeared to be a better radiosensitiser than TAK-165 of MDA cells at 6 Gy. Cocktail pre-treatment showed a higher, but not significantly different, sensitising effect than TAK-165 and BEZ235 individually, giving an SF_6 of 0.09 ± 0.02 ($P \geq 0.0835$).

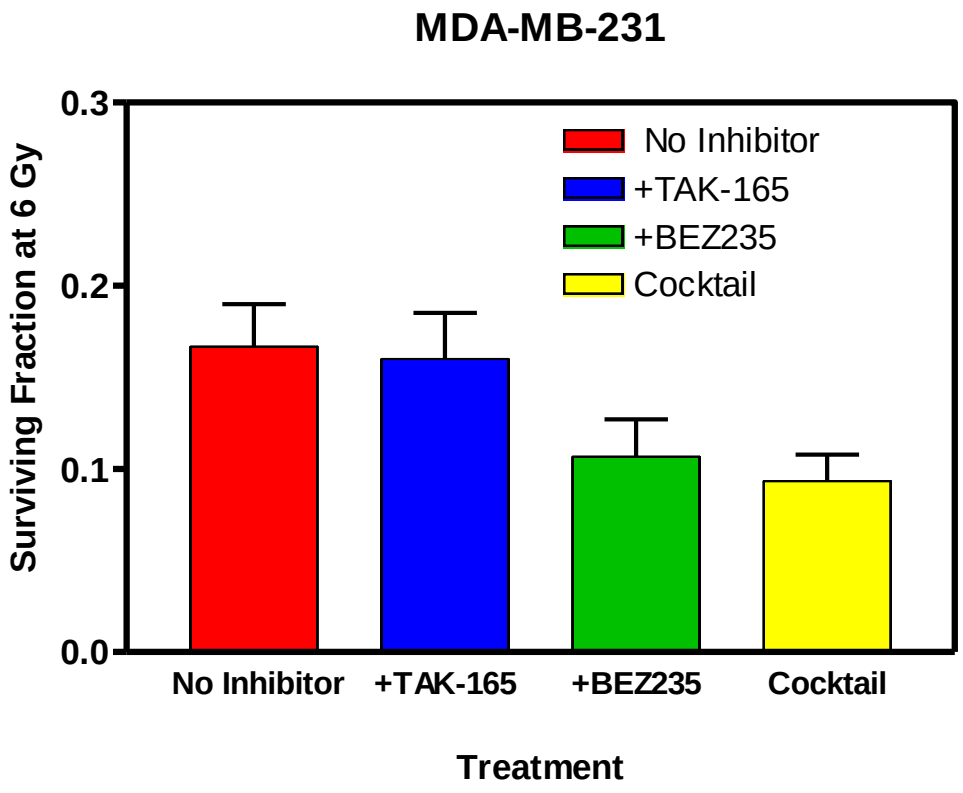

Figure 5: Clonogenic cell survival at 6 Gy of ^{60}Co γ-irradiation for a human breast carcinoma cell line (MDA-MB-231) following single or combined (cocktail) treatment with TAK-165 (HER-2 inhibitor) and BEZ235 (PI3K and mTOR inhibitor).

The surviving fraction at 6 Gy decreased marginally from 0.12 ± 0.04 to 0.08 ± 0.01 (P = 0.4103) when MCF-7 cells were treated with TAK-165 alone (Figure 6). BEZ235 treatment resulted in a similar level of radiosensitisation as obtained for TAK-165, with SF_6 of 0.07 ± 0.02 (P = 0.3791). Treatment of these cells with a cocktail of TAK-165 and BEZ235 did not give rise to a higher level of radiosensitisation than when BEZ235 and TAK-165 were used individually. The SF_6 for cocktail treatment was 0.06 ± 0.03 ($P \geq 0.5081$).

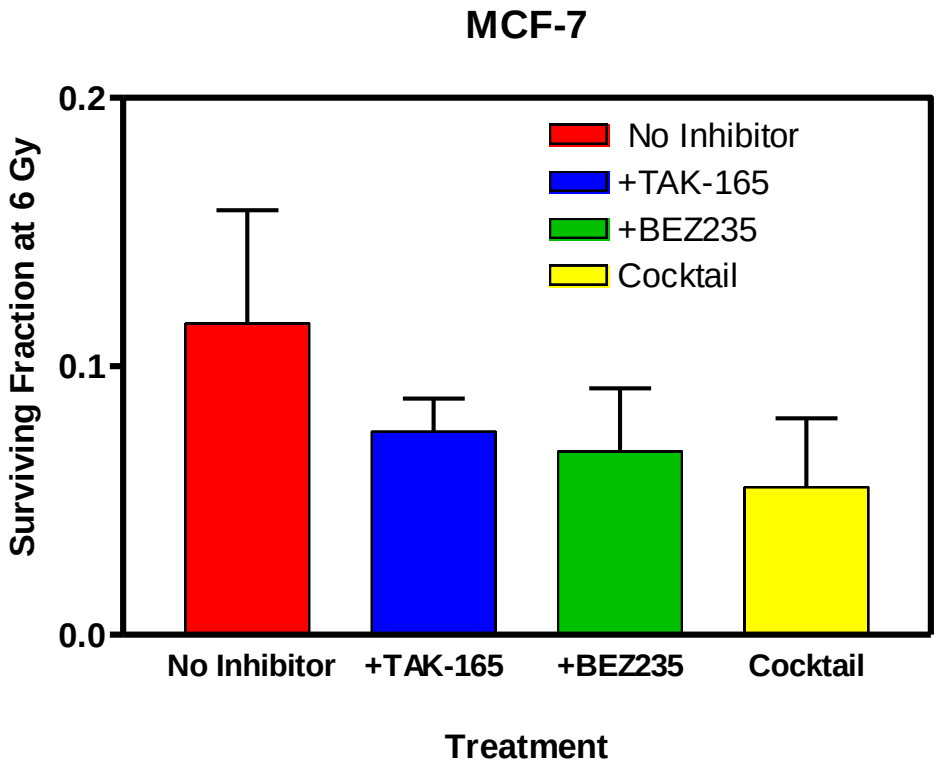

Figure 6: Clonogenic cell survival at 6 Gy of ^{60}Co γ-irradiation for a human breast carcinoma cell line (MCF-7) following single or combined (cocktail) treatment with TAK-165 (HER-2 inhibitor) and BEZ235 (PI3K and mTOR inhibitor).

To further evaluate the effect of inhibitors on radiation-induced cell kill, radiation enhancement factors (REF) were calculated for each cell line and treatment scenario from the data presented in Figures 3 - 6. The radiosensitivity data for the MDA-MB-231 and MCF-7 cell lines are summarised in Table 3 and Table 4, respectively.

Table 3: Radiation enhancement data at 2 and 6 Gy for MDA-MB-231 cells treated with TAK-165 and BEZ235.

Treatment	SF	REF*
2 Gy (no inhibitor)	0.55 ± 0.07	
2 Gy + TAK-165	0.48 ± 0.03	1.15 ± 0.16
2 Gy + BEZ235	0.41 ± 0.08	1.34 ± 0.31
2 Gy + Cocktail	0.27 ± 0.04	2.04 ± 0.40
6 Gy (no inhibitor)	0.17 ± 0.02	
6 Gy + TAK-165	0.16 ± 0.03	1.06 ± 0.23
6 Gy + BEZ235	0.11 ± 0.02	1.55 ± 0.34
6 Gy + Cocktail	0.09 ± 0.02	1.89 ± 0.48

*Error calculated using an appropriate error propagation formula

In Table 3, it is observed that, on average, treatment of MDA cells with BEZ235 results in a higher level of radiation enhancement than TAK-165 at 2 Gy. Cocktail treatment showed a 2-fold radiation enhancement (Equation (2)). At 6 Gy, TAK-165 did not appear to have an effect on the radiosensitivity of MDA cells (Table 3). On the other hand, treatment with BEZ235 led to a higher radiosensitisation than that obtained for 2-Gy irradiation. Cocktail therapy preceding 6 Gy of radiation yielded a similar level of enhancement as in the case of 2 Gy (Table 3).

Table 4: Radiation enhancement data at 2 and 6 Gy for MCF-7 cells treated with TAK-165 and BEZ235.

Treatment	SF	REF*
2 Gy (no inhibitor)	0.32 ± 0.05	
2 Gy + TAK-165	0.27 ± 0.06	1.19 ± 0.32
2 Gy + BEZ235	0.23 ± 0.04	1.39 ± 0.33
2 Gy + Cocktail	0.21 ± 0.04	1.52 ± 0.37
6 Gy (no inhibitor)	0.12 ± 0.04	
6 Gy + TAK-165	0.08 ± 0.01	1.50 ± 0.53
6 Gy + BEZ235	0.07 ± 0.02	1.71 ± 0.75
6 Gy + Cocktail	0.06 ± 0.03	2.00 ± 1.20.

*Error calculated using an appropriate error propagation formula.

From the radiosensitivity enhancement data, BEZ235 emerged as a better radiosensitiser of MCF-7 cells than TAK-165 (Table 4). A higher sensitisation resulted from cocktail treatment. A similar trend in radiosensitisation was seen at 6 Gy, although the extent of radiation enhancement was in all treatment scenarios higher than those at 2 Gy. A 2-fold radiosensitisation was also obtained when MCF-7 cells were pre-treated with a BEZ235/TAK-165 cocktail.

3.5. Western Blot Analysis

3.5.1 Ponceau Stain

Figure 7 shows an image of a PVDF membrane that has been Ponceau stained after it has been through protein transfer for MDA-MB-231 cells. The Ponceau stain is a general stain and is used to determine if the transfer of proteins has taken place. Bands representing the standard protein ladder and samples are illustrated with arrows to indicate the relative positions of the target proteins**:** mTOR, HER-2 and PI3K. Ponceau staining of the PVDF membrane for the MCF-7 cell line produced a similar image.

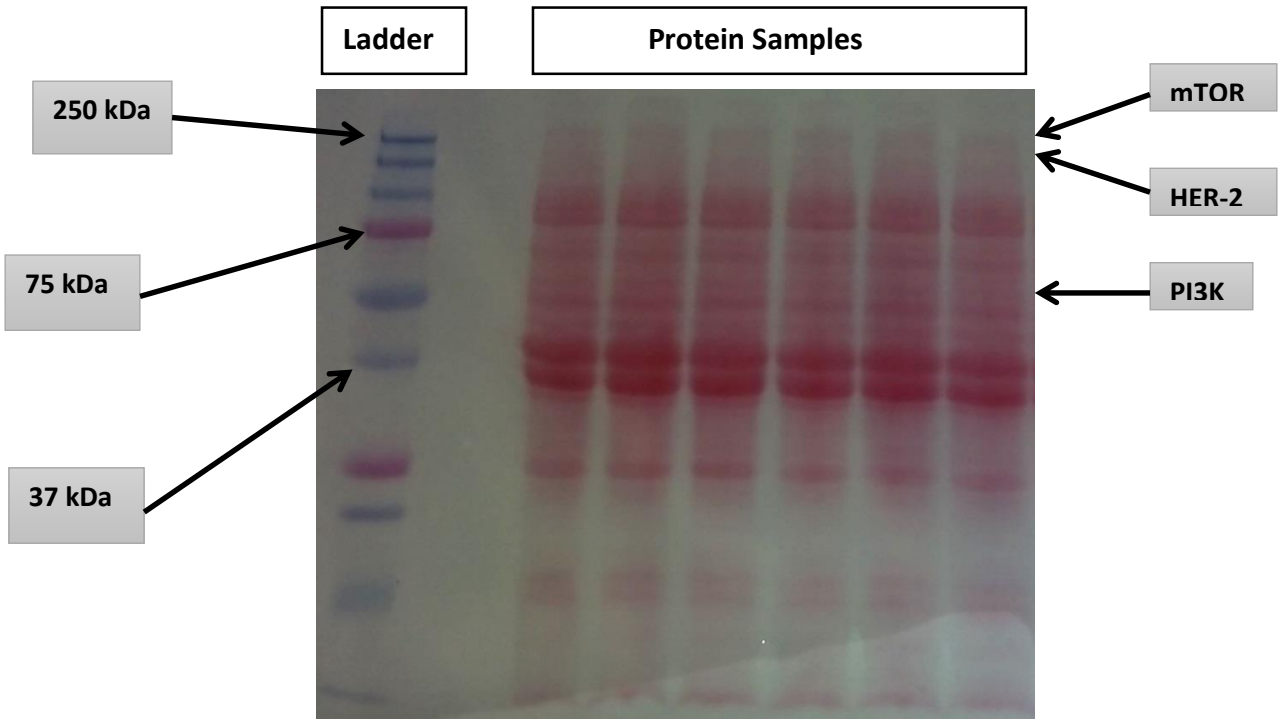

Figure 7: An image of a PVDF membrane that has been Ponceau stained after the transfer of proteins MDA-MB-231 cells.

3.5.2. Immuno-Blots

Figure 8 is an image of a PVDF membrane developed from unirradiated and irradiated MDA-MB-231 cells. Immuno-detection for total protein was used to identify HER-2, PI3K and mTOR. All proteins were expressed. Neither radiation dose nor incubation time of the order of 6 hours had an impact on the expression levels of the target proteins. Immuno-blotting of the MCF-7 membranes were unsuccessful.

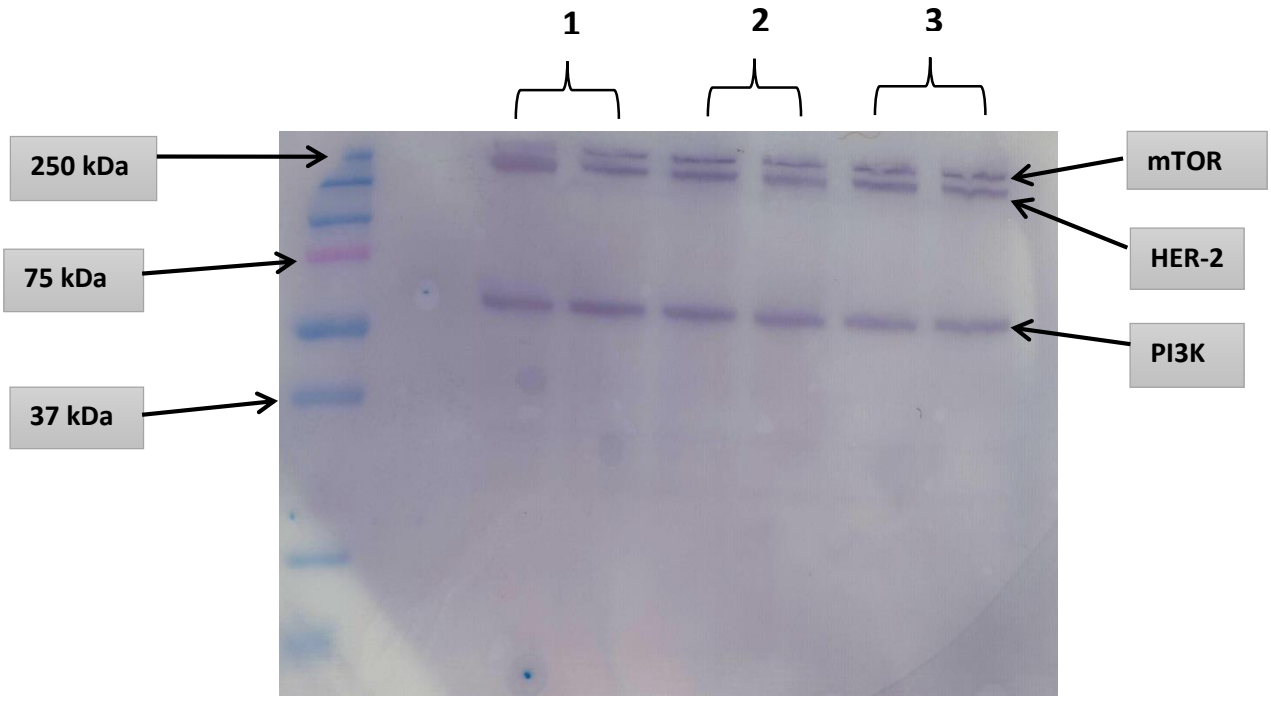

Figure 8: An immuno-blot of total HER-2, PI3K and mTOR expression. Protein samples were loaded in duplicate: Lanes 1 (unirradiated control); Lanes 2 (2 Gy with no incubation); and Lanes 3 (2 Gy with a 6-hour incubation).

CHAPTER 4

4. Discussion

4.1. Cellular Resistance to Radiotherapy

Radiotherapy has become part of standard care in both curative and palliative breast cancer treatment (Katz et al., 2009; Pearce et al., 2001). However, inherent or acquired radioresistance is thought to be the reason why many tumours do not respond favourably to radiotherapy (Jung et al., 2011; Li et al., 2012; Liang et al., 2003). It has been shown that the treatment of breast cancer cells with 2 - 6 Gy of radiation results in the elimination of radiosensitive sub-populations of cells, leaving the radioresistant cells to repopulate (Pearce et al., 2001). It is assumed that the surviving and repopulating carcinoma cells are capable of providing molecular protection against the cytotoxic effects of radiation therapy.

This research aimed to investigate radio-enhancement for a better therapeutic outcome. Knowledge of the differential radiosensitivity of breast tumours could provide a basis for adjuvant radiotherapy approaches, and help optimise breast cancer management. To address this need, two breast carcinoma cell lines (MDA-MB-231 and MCF-7) with different intrinsic radiosensitivities were used in this study.

Figure 2 shows the cell survival curves for the MDA-MB-231 and MCF-7 cell lines. Although the two cell lines appeared to exhibit similar radiation response at low doses (SF_2 of 0.64 and 0.58 for MDA-MB-231 and MCF-7, respectively), their radiosensitivities differ significantly at higher doses. While the survival curve of the

radiosensitive MCF-7 cell line has a prominent shoulder (smaller α/β ratio of 2.33 Gy), that for its more radioresistant MDA-MB-231 counterpart shows virtually no shoulder (large α/β ratio of ~21 Gy). The α/β ratio is the dose at which the linear and quadratic components of cell killing are equal. This relative radiosensitivity between these cell lines is consistent that reported elsewhere (Albert et al., 2008). The phosphatase and tensin homolog (PTEN) which impedes PI3K/mTOR pathway activity to sensitise cells to cytotoxic insult is wildly expressed in both cell lines (Carlson et al., 2010; Lue et al., 2007). Therefore, the observed differences in radiosensitivity, as illustrated in Figure 2, cannot be attributed to disparities in PTEN activity. While the more radioresistant MDA-MB-231 cell line is known to express wild-type PI3K, the radiosensitive MCF-7 cells are PI3K mutant (Vasudevan et al., 2009; Carlson et al., 2010). A reduction in PI3K/mTOR signalling in MCF-7 cells due to the PI3K mutation can compromise their ability to recover from radiation-induced damage.

4.2. Simultaneous Inhibition of HER-2 and PI3K Pathways Enhance Radiosensitivity of Breast Cancer Cells

There is evidence to show that inhibition of proteins involved in the PI3K/Akt/mTOR pathway can enhance cancer cell radiosensitisation (Awada et al., 2012; Rexer et al., 2014). Therefore, targeting of survival proteins, like PI3K, Akt, and mTOR, could improve HER-2 targeted treatment response in subtypes of breast cancer that show low or no expression of HER-2. Targeted therapy has improved overall patient survival, but its efficacy has been limited by inherent or acquired cellular resistance

(Higgins and Baselga, 2011; Hurvitz et al., 2013). Successes in targeted therapy using single agents are marginal, and it has been argued that treating with multiple agents could lead to better patient responses (Normanno et al., 2009).

This study explored whether the dual inhibitor of PI3K and mTOR, BEZ235, and TAK-165 which is an HER-2 inhibitor, could sensitise human breast cancer cells to ionising radiation. Furthermore, the study assessed whether a cocktail of the two inhibitors would yield a greater radiosensitisation in cells, in comparison to a single inhibitor. The effects of the inhibitors combined with radiation were assessed at a cellular level, and clonogenic survival was measured. At the molecular level expression of HER-2, PI3K and mTOR was evaluated using Western blot analysis.

Targeting the HER-2 pathway by inhibiting PI3K has been shown to inactivate the PI3K/Akt/mTOR pathway and significantly radiosensitise cancer cells (No et al., 2009; Riesterer et al., 2004). This is in agreement with the results presented here. Pre-treatment of MDA-MB-231 cells with the dual inhibitor of PI3K and mTOR, BEZ235, reduced the surviving fractions at 2 and 6 Gy, giving average radiation enhancement factors of 1.34 and 1.55, respectively (Figures 3 and 5; Table 1). The sensitisation at both radiation doses was similar. This suggests that radiosensitisation of MDA-MB-231 cells via inhibition of PI3K and mTOR is likely independent of radiation dose, and that BEZ235 could potentially be used as a radiosensitiser in conventional, as well as, hypofractionated radiotherapy. Similar levels of radiation enhancement were seen in the relatively more sensitive MCF-7 cell line (Figures 4 and 6; Table 2). The marked disparity between radiosensitisation observed here and the ~4-fold radiosensitisation reported elsewhere (Kuger et al., 2014), might be due to differences in experimental design. The BEZ235

concentration of 100 nM used by Kuger and colleagues was ~6 times that used in the present study (Kuger et al 2014), and correspond to BEZ235 doses at which very few cells would be expected to survive if the residence time of the drug was over the entire colony forming period. Drug cytotoxicity strongly depends on residence time (Akudugu and Slabbert, 2008). Trypsinisation and plating of cells a day after drug and radiation treatment can also affect extent to which the drug modulates radiosensitivity. In contrast to BEZ235 treatment, exposure of MDA-MB-231 cells to the HER-2 inhibitor TAK-165 had virtually no effect on the radiosensitivity at 2 and 6 Gy (Figures 3 and 5; Table 3). The response of MCF-7 cells at 2 Gy to treatment with the HER-2 TAK-165 was comparable to that of MDA-MB-231 cells (Figures 4 and 6; Table 4). However, TAK-165 treatment yielded an enhancement factor of 1.50 at 6 Gy, indicating that certain breast cancer subtypes may benefit from combining HER-2 targeted therapy with high radiation doses. As expected, the lack or minimal radiosensitisation seen in both cell lines may be attributed to the fact that they do not overexpress HER-2 (Konecny et al., 2006; Subik et al., 2010). Therefore, the role of HER-2 perturbation in radiosensitivity modulation would be minimal. This is consistent with data reported elsewhere indicating that trastuzumab (a potent HER-2 inhibitor) had little or no effect on radiation-induced apoptosis in breast cancer cell lines that show low to no expression of HER-2 (Liang et al 2003).

It is important to focus not only on the upstream target, HER-2, but also on the downstream targets, PI3K and mTOR, since inhibition of only one target upstream or downstream may not fully counteract the resistance of cells to irradiation. Co-targeting HER-2, PI3K and mTOR may, theoretically, maximise the radiosensitivity of cancer cells. The rationale or the desired expectation for this combination therapy

approach is to target sub-populations of tumour cells that may be resistant to one or other cytotoxic agent, and, furthermore, to simultaneously target all cells on a cell-by-cell basis (Akudugu and Howell., 2012a, b; Akudugu et al., 2011; Kvinnsland et al., 2001). Pre-treatment with a cocktail of TAK-165 and BEZ235 seems to be superior at radiosensitising both breast cancer cell lines than when cells were exposed to each agent individually (Tables 3 and 4). A 2-fold and dose-independent radiosensitisation emerged, suggesting that concomitant inhibition of HER-2, PI3K and mTOR in breast cancer radiotherapy modalities that employ low to high fractional doses might be beneficial. However, it would be desirable to demonstrate that the cocktail is non-toxic in normal tissue.

The apparent radiosensitisation observed here notwithstanding, it is worth noting that TAK-165 and BEZ235 were cytotoxic in both cell lines. This suggests that interference with the HER-2 and PI3K/Akt/mTOR pathways without concomitant use of radiotherapy might have a therapeutic benefit. Further evaluation, on the bases of inhibitor concentration and inhibitor residence time, would be needed to ascertain whether the apparent benefit could be maximised with addition of radiation threatment. In additional, studies on the effect of BEZ235 and TAK-165 on normal breast cell lines are desirable for optimisation of these findings.

4.3. Target Expression in Breast Cells

To demonstrate capacity to detect expression of the pathway targets, Western blot analyses were performed on the cell lines. An immuno-blot for total protein shows presence of mTOR, HER-2, and PI3K in MDA-MB-231 (Figure 8). Detection of the

phosphorylated protein would be required to confirm activity status of each target. Although MCF-7 cells are EGFR, HER-2, ER and PR positive (Horwitz et al., 1975; Rusnak et al., 2001), its expression of HER-2 is low and comparable to that in the MDA-MB-231 cells (Konecny et al., 2006). While expression of EGFR in MCF-7 cells is also low, MDA-MB-231 cells which are known to be ER and PR negative (Read et al., 1988), express high levels of EGFR (Konecny et al., 2006; Subik et al., 2010). This indicates that the MDA-MB-231 cell line can potentially be used as a model for triple-negative breast cancer if its residual HER-2 is successfully inhibited. The apparent radiosensitisation induced by BEZ235 and TAK-165 may have significant implications for breast cancer expressing little to no HER-2. The MDA-MB-231 cell line is known to express wild-type PI3K, but PI3K is mutated in MCF-7 cells (Vasudevan et al., 2009; Carlson et al., 2010). Processing of radiation-induced damage in MCF-7 cells can, therefore, involve components other than PI3K. In addition to HER-2, TAK-165 is a potent inhibitor of the cell division cycle protein 2 homolog (Cdc2), which plays a crucial role in cell cycle progression. Perturbation of Cdc2 activity with TAK-165 can lead to cell cycle arrest and subsequent cellular demise during cell division. Cdc2 activity in MDA-MB-231 is intrinsically higher than that in MCF-7 (Pozo-Guisado et al., 2002; Kuo et al., 2006), indicating a stronger dependence of former cell line on Cdc2 activity for cell cycle progression. Therefore, inhibiting residual HER-2 and Cdc2 activity with TAK-165 can be expected to make MDA-MB-231 cells more susceptible to cytotoxic insult than their MCF-7 counterparts. However, this cannot be support by the current findings as there is no clear differential between modulation of radiosensitivity by BEZ235 and TAK-165 in the two cell lines.

4.4. Possible Future Avenues for Research

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

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

3. Studies seeking to determine whether blocking PI3K and mTOR with BEZ235, or HER-2 with TAK-165 leads to radiosensitisation or radiation protection in normal cells would have significant benefit not only in radiotherapy, but also in radiation protection.

CHAPTER 5

5. Conclusion

In the current study, it has been shown that human breast cancer cell lines MDA-MB-231 and MCF-7 have similar radiosensitivity at relatively low doses, with the former being significantly more radioresistant than the latter at much higher doses. Treatment of the cell lines with inhibitors of HER-2, PI3K, and mTOR, singly or in combination, at low and relatively high radiation doses resulted in a measurable but not significant radiosensitisation. Inhibition of PI3K and mTOR appeared to show better potentiation of radiosensitivity, PI3K and mTOR might be potential therapeutic targets in breast cancers devoid of HER-2 expression. Further studies interrogating the roles of PI3K and mTOR in cellular responses to drug and radiation treatment can make a significant contribution towards the formulation of patient-specific therapeutic cocktails that will revolutionise the treatment of breast cancer.

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