EVALUATION OF SMALL MOLECULE INHIBITORS OF HER2, PI3K, mTOR AND Bcl-2 FOR THEIR RADIOMODULATORY EFFECTS IN HUMAN BREAST CANCER CELL LINES

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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: 4 April 2016

Abstract

Breast cancer remains the most commonly diagnosed cancer in women. It is responsible for 32% of all cancers and 15% of all cancer-related deaths in females. Patients with triple-negative breast cancers (TNBC) constitute about one-fifth of all breast cancer patients. TNBC is an aggressive and heterogeneous disease entity in comparison with other types of breast cancer and, therefore, tends to be resistant to existing treatment regimens, such as, targeted and hormone therapies.

Although cancer treatment has evolved from being invasive and highly toxic to being more specific with reduced normal tissue toxicity, intrinsic tumour resistance still limits the benefit of therapy with radiation, drugs, and antibodies. To address this important clinical challenge, attempts have been made to better understand the molecular determinants of treatment resistance. This resistance can be attributed to the heterogeneity in the distribution of potential target antigens in a given tumour cell population, which leads to the inability to effectively target all cells with toxic levels of a particular therapeutic agent.

There is evidence to suggest that proliferative pathways of triple-negative tumours are still poorly understood, which could be the reason for the observed treatment resistance. Targeted treatment modalities that are singly effective for triple-negative breast cancer are lacking, partly due to paucity of relevant targets as they are devoid of the human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), and oestrogen receptor (ER). Novel treatment approaches are, therefore, needed to overcome the challenges in the treatment of triple-negative breast cancers if treatment outcomes are to be improved. Concomitant targeting of cell signalling

entities other than HER2, PR and ER may sensitise triple-negative tumours to radiotherapy.

In this study, inhibition of HER2, phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), and the pro-survival gene (Bcl-2) with small molecule inhibitors, TAK-165 (against HER2), NVP-BEZ235 (against PI3K and mTOR), and ABT-263 (against Bcl-2), singly or as cocktails, resulted in significant radiosensitisation of human breast cell lines with features similar to those of triple-negative cancers. This radiosensitisation was seen at 2 and 6 Gy, indicating that a therapeutic benefit could be derived in conventional as well as stereotactic radiotherapy. A moderate to strong synergism was also demonstrated for NVP-BEZ235/TAK-165 and NVP-BEZ235/ABT-263 cocktails. The strongest synergy was seen in the latter cocktail.

In conclusion, inhibition of PI3K, mTOR and Bcl-2 could potentially be effective in the treatment of triple-negative breast cancer. The therapeutic benefit can be improved, if the target inhibition is followed by radiotherapy.

Opsomming

Borskanker bly steeds die mees gediagnoseerde kanker en is die oorsaak van 32% van alle kankers en 15% van kankersterftes in vrouens. Pasiënte met drievoudige-negatiewe-borskanker (TNBC) bedra een-vyfde van alle borskankerpasiënte. Dit is ‘n aggressiewe en heterogene siekte in vergelyking met ander tipes borskanker, en blyk weerstandig te wees teen geteikende en hormoonterapieë.

Alhoewel kankerbehandeling vanaf ‘n ingrypende en hoogs toksiese terapie verander het na ‘n behandelingstrategie wat hoogs spesifiek met ’n laer toksisiteit is, word die sukses van kombinasie terapie met bestraling, teenliggaampies en middels weens inherente tumorweerstand ondermyn. Om hierdie belangrike kliniese uitdaging te oorbrug, is strategieë dus nodig om die onderliggende molekulêre meganismes van behandelingsweerstand te verstaan, en dan uit te skakel. Hierdie weerstand is die gevolg van die heterogeniteit in die verspreiding van potensiële antigene in ‘n tumorselpopulasie, wat veroorsaak dat nie al die selle geteiken word met ‘n toksiese dosis van die terapeutiese middel nie.

Daar is aanduidings dat gebrekkige begrip en benadering van die proliferasiebane van drievoudige tumore dalk die fundamentele rede vir die waargenome behandelingsweerstand kan wees. Verder is daar ‘n gebrek aan geteikende modaliteitsterapieë wat doeltreffend is vir die behandeling van drievoudige borskanker, as gevolg van die lae uitdrukking van relevante teikens soos menslike epidermale groeifaktorreseptor-2 (HER2), progesteroonreseptor (PR) en estrogeenreseptor (ER). Nuwe behandelingstrategieë is dus nodig om die uitdagings met die behandeling van drievoudige-negatiewe-borskanker te bekamp en om resultate in pasiënte dus aansienlik te verbeter. ‘n Alternatiewe benadering sou kon

wees om ander selseinoordragentiteite as HER2, PR en ER te teiken en die tumore daardeur moontlik meer kwesbaar vir bestraling te maak.

In hierdie studie het inhibisie van HER2, fosforinositied-3-kinase (PI3K), soogdierteiken vir rapamisien (mTOR), en die oorlewensgeen (Bcl-2) met klein molekuulinhibitore, TAK-165 (teen HER2), NVP-BEZ235 (teen PI3K en mTOR), en ABT-263 (teen Bcl-2), afsonderlik of in kombinasie, bewys dat betekenisvolle stralingsensitiwiteit in alternatiewe mensborssellyne, anders as die TNBC maar met dieselfde eienskappe, verkry kon word. Die stralingsensitiwiteit is merkbaar met beide 2 en 6 Gy, wat aandui dat beide gewone en stereotaktiese stralingsterapie ‘n terapeutiese voordeel inhou. ‘n Matige tot sterk sinergisme is ook aangetoon vir NVP-BEZ235/TAK-165- en NVP-BEZ235/ABT-263-kombinasies. Die sterkste sinergie kom in die laasgenoemde mengsel voor.

Opsommend**:** Chemoterapeutiese inhibisie van PI3K, mTOR en Bcl-2 kan potensieel effektief wees vir die behandeling van drievoudige-negatiewe-borskanker en die kliniese uitkoms kan verder verbeter word indien teikeninhibisie vóór bestraling kan plaasvind.

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Dedications

In loving Memory of Laimy Tumeniyeni Shikongo.

21 October 1984 to 19 April 2013

You were always a great achiever

"Silently, one by one, in the infinite meadows of heaven,

Blossomed the lovely stars, the forget-me-nots of the angels."

— Henry Wadsworth Longfellow

Table of Contents

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List of Figures

Chapter one

Chapter two

Chapter three

List of Tables

Chapter one

Chapter three

List of Abbreviations

Akt	Serine-threonine protein kinase
ATP	Adenosine triphosphate
α	Linear coefficient of cell inactivation
β	Quadratic coefficient of cell inactivation
CI	Combination index
Co	Cobalt
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
ER	Oestrogen receptor
FBS	Foetal bovine serum
Gy	Gray
EGFR	Human Epidermal Growth Factor Receptor 1
HER2 /erbB2	Human Epidermal Growth Factor Receptor 2
IC_{50}	Inhibitor concentration for 50% inhibition
MF_{col}	Radiosensitivity modifying factor by clonogenic survival
MFI	Mean fluorescence intensity
MF_{flow}	Radiosensitivity modifying factor by flow cytometry
mTOR	Mammalian target of rapamycin
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
SF	Surviving fraction
SF_2	Surviving fraction at 2 Gy
SF_6	Surviving fraction at 6 Gy
SSD	Source-to-sample distance
TNBC	Triple-negative breast cancer

Chapter One: Introduction

1.1. Breast Cancer: A Clinical Problem

Breast cancer remains the most commonly diagnosed cancer in women. Statistics from the International Agency for Research on Cancer (IARC) indicate that there were 1 677 000 cases of breast cancer and 577 000 related deaths internationally in 2012 (IARC, 2012). The most recent statistics show that in Africa approximately 29 and 15 per 100 000 persons are diagnosed and die of breast cancer, respectively (Jemal et al., 2011). Furthermore, in economically developing countries like South Africa, this disease is the leading cause of cancer-related deaths in females (Jemal et al., 2011). The incidence and mortality rates of breast cancer in Southern Africa are higher than the continental average rates and are 38 and 19 per 100 000 persons, respectively (Jemal et al., 2011).

The different molecular subtypes of breast cancer exhibit significantly different levels of responses to treatment (van't Veer et al., 2002; Yersal et al., 2012). These subtypes may be divided into several groups, namely: (1) human epidermal growth factor receptor 2 (HER2) positive; (2) oestrogen receptor (ER) and progesterone receptor (PR) positive; (3) ER and PR negative; and (4) triple-negative (HER2, ER and PR negative) (Sørlie et al., 2003). Cancers overexpressing the gene encoding HER2 constitute about 30% of invasive breast cancers (Slamon et al., 1987; Eccles et al., 2001; Iqbal et al., 2014; Perez et al., 2014). The humanised monoclonal antibody, trastuzumab, is approved by the United States Food and Drug

Administration (US FDA) for the treatment of HER2 positive cancers. However, trastuzumab only proves beneficial to breast cancers overexpressing HER2, while breast cancers lacking HER2 expression and low expressers of HER2 do not respond favourably to it.

Oestrogen and progesterone positive breast cancers are reportedly the largest sub-group and constitute 65-75% of the global breast cancer population (Carlson et al., 2006). Seventy-five percent of patients with breast cancers expressing the oestrogen receptor receive endocrine therapy, but as many as 50% of this population fail to respond positively to this treatment as a result of acquired resistance (Clarke et al., 2001; Baumgarten and Frasor, 2012).

Triple-negative breast cancers (TNBC) constitute about one-fifth of all breast cancer patients (Foulkes et al., 2010). This subtype of breast cancer is known to occur more frequently in young Black and Hispanic women than in women of other racial or ethnic groups of comparable age (Millikan et al., 2008). TNBC is an aggressive and heterogeneous disease entity in comparison with other types of breast cancer, and therefore tends to be resistant to existing treatment regimens, such as targeted and hormone therapy (Darrel and Cleere, 2010).

It is evident that certain sub-groups of breast cancer do not benefit from existing therapeutic regimens. To alleviate the breast cancer burden, there is an urgent need to develop novel and effective treatment approaches. Chemotherapeutic drugs have yielded a positive but limited treatment outcome in breast cancer patients with metastatic disease (Hurvitz et al., 2013). Radiotherapy, a common treatment

modality for certain types of breast cancer, has also proven beneficial for post-operative local control, but radioresistance appears to lead to the development of recurrences (Jameel et al., 2004). Similarly, alternative treatment methods such as immunotherapy and the use of radiopharmaceuticals have limited success, partly due to their inability to effectively target all malignant cells with toxic amounts of a single therapeutic agent (Kvinnsland et al., 2001; Akudugu et al., 2011; Akudugu and Howell, 2012a, b).

Also, cellular exposure to ionising radiation is known to activate the epidermal growth factor receptor, EGFR (Dittmann et al., 2005), and induce phosphoinositide 3-kinase (PI3K), serine-threonine protein kinase (Akt) and mammalian target of rapamycin (mTOR) activity downstream of the EGFR signalling pathway (Albert et al., 2006). This signalling promotes cell survival and can lead to treatment resistance. Targeting the HER2 pathway by inhibiting PI3K has also been shown to result in significant radiosensitisation (No et al., 2009). Of significance to targeted therapy, triple-negative breast cancers, which are devoid of HER2 activity, tend to predominantly overexpress EGFR (Siziopikou et al., 2006). Therefore, developing therapeutic approaches that concurrently target EGFR family members and their downstream signalling components might significantly sensitise triple-negative breast cancer cells to radiotherapy and improve treatment outcome.

Another plausible therapeutic approach for cancer would be to target signalling components that regulate the mechanism of apoptosis (programmed cell death) (Belka et al., 2004). Changes in this mechanism do not only cause the formation of cancer (Hellemans et al., 1995), but also leads to resistance to standard anticancer

therapies, namely, radiotherapy and cytotoxic agents (Krajewski et al., 1997). One of the suggested approaches to mitigate treatment resistance is to manipulate cellular expression of B-cell lymphoma-2 (Bcl-2) family members. Bcl-2 is an important anti-apoptotic gene which interacts with its pro-apoptotic counterpart, Bax, to keep the balance between new cells and dying cells. Bcl-2 is usually over-expressed in human cancers (Hellemans et al., 1995; Olopade et al., 1997; Schneider et al., 1997; Pena et al., 1999; Trask et al., 2002). Increased levels of Bcl-2 are associated with resistance to chemotherapy and radiotherapy, leading to poor prognosis (Minn et al., 1995; Reed et al., 1996; Simonian et al., 1997; Gallo et al., 1999). Therefore, inhibiting Bcl-2 presents a promising approach to overcoming resistance to conventional anticancer therapies.

The challenges that are encountered due to acquired resistance to therapy and recurrences, therefore, warrant a continuous search for better treatment options for breast cancer.

1.2. Rationale and Problem Statement

Triple-negative breast cancers continue to be the most challenging subtype to treat, because it is a heterogeneous disease consisting of different subtypes, and cells present with different levels of receptor expression (Cleator et al., 2007; Stagg et al., 2013; Jamdade et al., 2015; Lehmann et al., 2015). Inadequate expression of antigens that could potentially be therapeutic targets in triple-negative breast cancers may be responsible for the apparent resistance to existing treatment regimes,

namely, targeted therapy, hormone therapy and radiotherapy. Furthermore, dissimilar to ER, PR and HER2 amplified breast cancers, the lack of high frequency oncogenic driver mutations in TNBC also contribute to limited molecular targeted treatments for this disease (Lehmann et al., 2015). To address these challenges, attempts have been made to better understand the molecular determinants of treatment resistance (Cleator et al., 2007; Weigelt et al., 2015).

This resistance can be attributed to the heterogeneity in the distribution of target antigen expression in a given cell population, which leads to the inability to effectively target all cells with toxic levels of therapeutic agents (Akudugu et al., 2011, Akudugu and Howell, 2012a, b). There is also evidence to suggest that proliferative pathways of triple-negative tumours are still poorly understood, which could be the reason for the observed treatment resistance (Cleator et al., 2007; Ma et al., 2015).

What is lacking, however, is the ability to effectively target components of the cell survival pathways to achieve increased tumour response to therapy. This study sought to target HER2, PI3K, mTOR and Bcl-2 with specific inhibitors, in an attempt to: (1) radiosensitise human breast cancer cells with different expression levels of HER2, ER and PR; and (2) identify potential therapeutic targets for triple-negative breast cancer.

1.3. Hypothesis

Targeting HER2, downstream signalling components of the EGFR family members, and Bcl-2 with specific inhibitors, singly or in combination, can significantly sensitise triple-negative breast cancer cells to ionising radiation.

1.4. Aims and Objectives

The aim of the study was to inhibit potential therapeutic targets in order to radiosensitise breast cancer cells that exhibit low expression levels of HER2, ER and PR. For this, the following specific objectives were pursued:

- To determine intrinsic cellular radiosensitivity of three human breast cell lines following exposure to ^{60}Co γ-rays or X-rays.
- To determine the cytotoxicity of specific inhibitors of HER2, PI3K, mTOR and Bcl-2 in each cell line.
- To determine the radiomodulatory effects of the aforementioned inhibitors, singly or in combination, in each cell line.
- To test whether flow cytometry could be used as a tool for rapid screening of potential target inhibitors as radiosensitisers.

1.5. Significance of the Study

Generally, optimisation of approaches for the treatment of metastatic breast cancers remains controversial. Over a decade ago, it was suggested that combination therapy had a place in the treatment of breast cancers (Miles et al., 2002). The

greatest concern with combination therapy is the toxicity of drugs used, as well as, the chosen combination that should meet three criteria;

i. Each component should have single-agent activity with no antagonism.
ii. There should be evidence of synergy between components.
iii. The components should have no overlapping safety profiles.

These three criteria are rarely met, and consequently many combination therapies have failed. In this study, potential inhibitors were combined to radiosensitise breast cancer cells. The inhibitors TAK-285 and ABT-263 (Aertgeerts et al., 2011; Bajwa et al., 2012) have single agent activity and the NVP-BEZ235 inhibitor has dual agent activity (Maira et al., 2008). The study reported here demonstrates that using a cocktail of NVP-BEZ235 in combination with TAK-165 or ABT-263 can significantly radiosensitise breast cell lines, based on clonogenic cell survival. These findings suggest that the cocktail combinations may not only have the potential for effectively targeting triple-negative breast cancer cells, but that there may be no antagonism between inhibitors. A second advantage of using the cocktail combinations is that the combination indices (CI) for the NVP-BEZ235/TAK-165 and NVP-BEZ235/ABT-263 cocktails for all cell lines ranged between 0.20 and 0.74, indicating synergism for each inhibitor combination (Chou 2006).

Although the inhibitors used here, and radiation, have overlapping safety profiles which includes nausea, diarrhoea, vomiting and fatigue, clinical studies report that the side effects of NVP-BEZ235, ABT-263 and TAK-165 are generally well tolerated (Sridhar et al., 2003; Gandhi et al., 2011; Martini et al., 2013). Other more severe side effects, like thrombocytopenia, from ABT-236 administration are dose-

dependent. Systemic toxicity is also a significant concern in the clinic and lower drug doses are desirable. Combination indices estimated for each cocktail in this study show an improvement in synergism when the concentration of the common component NVP-BEZ235 was decreased, suggesting that radiosensitisation may persist even at low inhibitor concentrations and resulting toxicity may be minimal.

In conclusion, this study demonstrates the following:

- Inhibition of HER2, PI3K, mTOR and Bcl-2 in breast cell lines with diminished expression of HER2, ER and PR with specific inhibitors is cytotoxic. Use of such inhibitors could potentially have a therapeutic benefit for triple-negative breast cancer.
- Pre-treatment of apparently normal and malignant human breast cells with specific inhibitors of HER2, PI3K, mTOR and Bcl-2, singly or in combination, sensitises them to low and high doses of ionising radiation. Concomitant treatment of breast cancers with low expression of HER2, ER and PR with such inhibitors and radiotherapy may improve prognosis in triple-negative breast cancer patients.

Findings of this study may also make a significant contribution to the design of novel treatment approaches for breast cancer in particular, and cancer as a whole.

1.6. Delineations

This study used inhibitors of HER2, PI3K, mTOR and Bcl-2 to radiosensitise three human breast cell lines (MCF-7, MDA-MB-231 and MCF-12A) which are low

expressers of HER2, and cover a wide range of ER, PR, EGFR, PI3K and Bcl-2 expression. The expression levels of the selected targets in the three cell lines were not determined prior to inhibition, as these have been reported in several studies (Horwitz et al., 1975; Kandouz et al., 1996; Rusnak et al., 2001; Konecny et al., 2006; Vasudevan et al., 2009; Subik et al., 2010; Carlson et al., 2010; Brosseau et al., 2012).

The research variables determined in this study are intrinsic cellular radiosensitivity, cytotoxicity of specific inhibitors of HER2, PI3K, mTOR and Bcl-2, radiomodulatory effects of inhibitors given either singly or in combination, and treatment-induced expression of the pro-apoptotic gene, *Bax*.

Clonogenic cell survival following irradiation was determined to enable comparison of intrinsic cellular radiosensitivity of the three cell lines. Cytotoxicity of inhibitors was determined, using the colony forming assay to extract the equivalent concentration for 50% cell killing (EC_{50}) for each cell line. From the cytotoxicity data, combination indices were determined and used to decipher the modes by which inhibitors interacted with each other when combined in a cocktail. The EC_{50}-values were used to guide subsequent radiomodulatory experiments, in which radiation modifying factors were derived to determine how a particular inhibitor or inhibitor cocktail impacted cellular radiosensitivity. Flow cytometry was used to test if a rapid measurement of treatment-induced *Bax* expression can potentially replace the expensive and slow colony forming assay as a tool for high throughput screening of candidate target inhibitors.

These research variables were deemed sufficient to prove the research hypothesis. Some of the data presented have been published (Hamunyela et al., 2015, ***copy attached***).

1.7. Limitations

- This work only used three human breast cell lines as they were the only ones available at the time.
- The ^{60}Co γ-irradiator initially used for the study was decommissioned, and cell irradiation was continued with a Faxitron MultiRad 160 X-irradiator.
- The TAK-165 inhibitor was used as a generic inhibitor of HER2 as the approved trastuzumab that is used routinely for therapy is expensive and not readily accessible for basic research.
- Only one cell line (MDA-MB-231) was used to test the feasibility of using flow cytometry as a rapid high throughput tool for evaluating candidate target inhibitors for therapeutic benefit due to financial constraints.
- Inhibitors were not validated at a molecular level using western blotting, due to technical difficulties arising from a number of attempts.
- The radiomodulatory effects of the inhibitors were not assessed for the full cell survival-dose response curves due the prohibitive cost of inhibitors.
- A TAK-165 and ABT-263 combination was not evaluated due to financial constraints.
- Assessment of additional time points for irradiation after inhibitor treatment would also be of significant value in understanding the mode of action of combination therapy. This was not feasible for reasons of cost.

1.8. Background Literature

1.8.1. Types of Breast Cancer

An approach to identify breast tumour subtypes was first described by Sørlie et al. who used molecular characteristics to group tumours into various biological subtypes (Sørlie et al., 2001). The greatest difference between the subtypes was seen in hormone receptor positive and hormone receptor negative tumours. The hormone receptor positive tumours were clustered in two groups which had expression characteristics seen in luminal epithelial mammary cells. The hormone receptor negative tumours were clustered into three molecular types**:** tumours that had characteristic gene expression seen in basal or myoepithelial mammary cells, tumours with an overexpression of HER2 gene, and tumours that presented with expression characteristics related to normal mammary cells (Sørlie et al., 2001).

In all, breast cancers can be classified into seven common subtypes, namely, claudin-low, basal-like, HER2 positive, normal-like breast tumour, triple-negative, luminal A, and luminal B; as well as a luminal C intrinsic subtype which is discerned from the luminal A and B by a unique set of genes with unknown function, a common characteristic they have with the basal-like and HER2 positive tumours (Perou et al., 2000; Sørlie et al., 2001; Kittaneh et al., 2013). Other newer classifications of “luminal-like” subtypes of breast tumours provide useful information on breast cancer biology, but have not yet been applied in clinical practice.

1.8.2. Clinically Common Breast Cancer Subtypes

The most common clinically used molecular characteristics of breast cancer subtypes are summarised in Table 1.1.

Table 1.1: Gene expression profiles of subtypes of breast cancer.

Classification	Immunoprofile	CK5/6 EGFR status	Frequency (%)
Claudin-low	ER-, PR-, HER2-	EGFR+/-	12–14
Luminal A	ER+, PR+/-, HER2-	EGFR -	50–60
Luminal B	ER+, PR+/-, HER2+	EGFR -	10–20
HER2 enriched	ER-, PR-, HER2+	EGFR+/-	10–15
Normal breast type	ER+/-, HER2-	EGFR+	5–10
Basal	ER-, PR-, HER2-	EGFR+	10–20
Triple-negative	ER-, PR-, HER2-	EGFR+	20–25

ER: oestrogen receptor; PR: progesterone receptor; CK5/6 Cytokeratin5/6; HER2: human epidermal growth factor receptor 2; -: negative; +: positive; +/- occasionally positive; -/+: rarely positive.

(Holliday and Speirs, 2011; Eroles et al., 2012; Weigelt et al., 2010)

The luminal-like subtype derives its name from similarities in the expression profile of the normal luminal breast epithelium lining. Luminal A is the most common type of breast cancer. This subtype expresses ER regulated genes and under-expresses HER2 and genes involved in proliferation (Eroles et al., 2012; Kittaneh et al., 2013). Luminal B is characterised by a low ER expression and a moderate HER2

expression, while possessing high expression levels of genes regulating cell proliferation (Kittaneh et al., 2013).

HER2 positive breast cancers are characterised by their expression of the HER2/*neu* gene. These breast cancers mostly present with a low expression of luminal gene clusters (namely, the luminal cytokeratins: CKs, CK7, CK8, CK18 and CK19). As illustrated in Table 1.1, HER2 enriched tumours are ER and PR negative (Eroles et al., 2012; Kittaneh et al., 2013).

The normal-like breast cancer gene expression is identified by the overexpression of the basal epithelial genes and a lower expression of luminal epithelial genes (Perou et al., 2000). The basal-like (BBC) breast cancers are one of the subtypes of breast cancers which derives its name from its expression of genes usually present in the normal myoepithelial cells. Basal-like cancers are commonly identified by their immunohistochemical staining for the expression of cytokeratin 5/6 (CK5/6). Most basal-like cancers do not express ER, PR or HER2; however, a small number do and thus overlap with the triple-negative subtype (Jimenez et al., 2001; Jones et al., 2001; Nielsen et al., 2004; Eroles et al., 2012; Kittaneh et al., 2013).

Triple-negative breast cancers (TNBC) are defined as cancers that do not express hormone receptors (ER and PR) nor overexpress HER2. The features of this group are similar to those of the basal-type (Table 1.1). Consequently, pioneering gene expression and cellular pathway studies that led to development of the different subtypes of breast cancer did not distinguish between the two subtypes (Morris and Carey, 2007; Rakha et al., 2009). Nonetheless, the subtypes are distinguishable in

that while triple-negative cancers do not form a homogeneous cluster in accordance to the relationship between gene expression profiles and response to therapy, their basal-like counterparts do (Bertucci et al., 2008; Rakha et al., 2009). Such heterogeneity in gene expression, as exhibited by triple-negative cancers, can further exacerbate the current treatment challenges. Triple-negative tumours also overexpress epidermal growth factor receptor (EGFR) which is responsible for activating signalling pathways involved in cell survival and carcinogenesis (Cunliffe et al., 2003; Krause and Van Etten., 2005; Pao et al., 2005; Siziopikou et al., 2006). Treatment resistance in TNBC may, therefore, be attributable to EGFR overexpression.

Triple-negative breast cancers also have features of other subtypes of breast cancers. These include the claudin-low breast cancers which do not express normal differentiation markers, hormone receptors, and HER2 (Kittaneh et al., 2013). Also, the triple-negative subtype has features similar to those of the normal breast cancer subtype (Sørlie et al., 2001). Put together, triple-negative breast cancer is the most heterogeneous and complex subtype, and therefore poses a significant treatment challenge. The interrelationships of the various breast cancer subtypes are depicted in the schematic Figure 1.1 below.

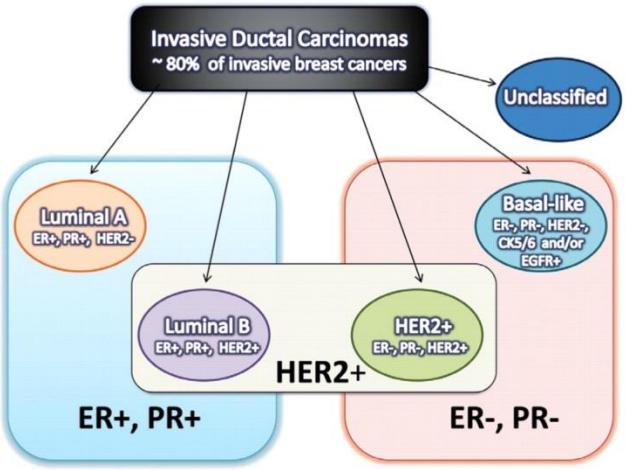

Figure 1.1: Different breast cancer types and their features.

1.8.3. PI3K/Akt/mTOR Pathways in Malignant Transformation

Cell-to-cell communication and how cells interpret such signals into metabolic, survival, proliferative, and death responses has become a central study area (Dent et al., 2003). Domains of the EGFR protein signalling induce receptor homodimerisation or heterodimerisation with members of the ErbB family, namely, HER1, HER2, HER3, and HER4 (Yarden and Sliwkowski, 2001). Dimerisation of ErbB family members further leads to activation of the tyrosine kinase domain, autophosphorylation, and thus activation of various downstream pathways, such as, the PI3K/Akt/mTOR pathway and the Ras/Raf/MAPK pathway (Valabrega et al., 2007; Bender and Nahta, 2008). EGFR signalling either promotes cell proliferation via the Ras-MAPK pathway or inhibits apoptosis (leading to cell survival) through the Akt/mTOR pathway (Yarden and Sliwkowski, 2001). Identification of resistance mechanisms in the PI3K/Akt/mTOR pathway can provide information that may aid in developing new approaches which will lead to improved breast cancer management.

This pathway is the most commonly activated pathway in human breast cancers, and thus presents itself as a potential target for therapy (Liu et al., 2009).

Figure 1.2 shows possible treatment targets in breast cancers. Potential targets include the EGFR family members and components of the MAPK and PI3K/Akt/mTOR pathways.

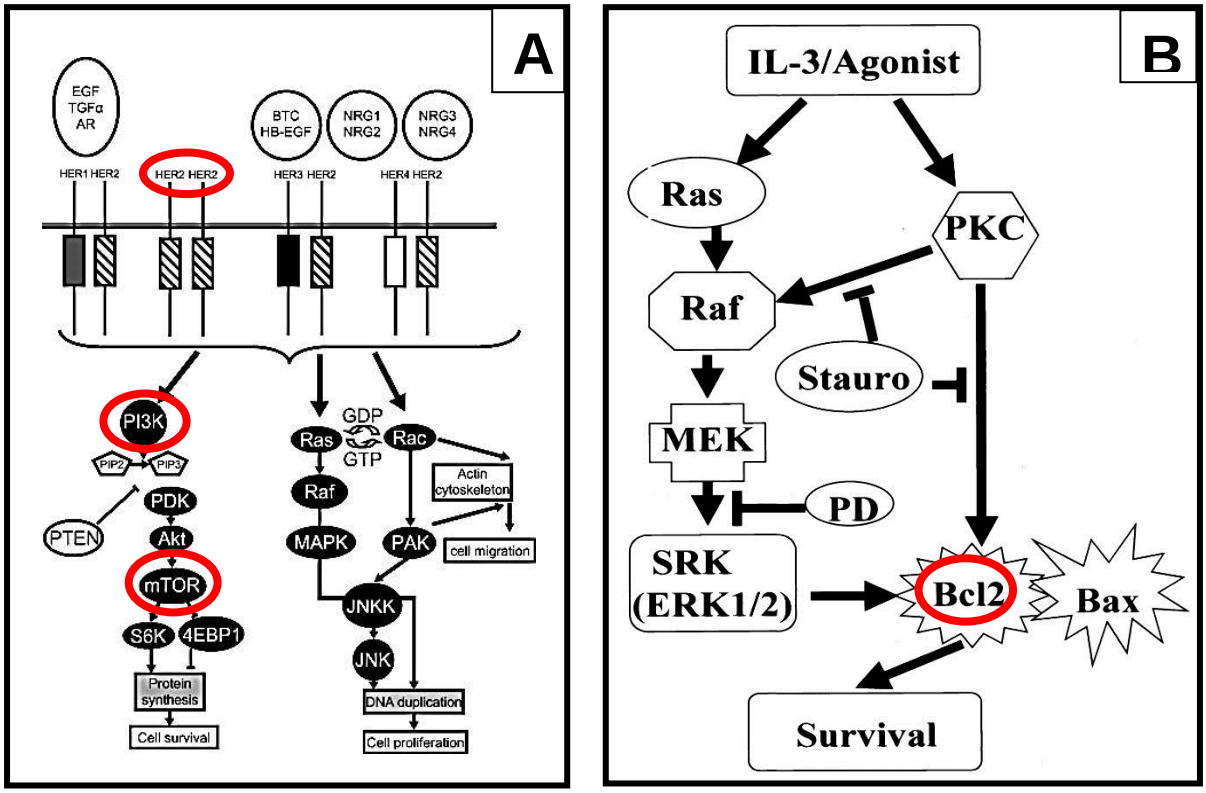

Figure 1.2: Potential therapeutic targets (red ovals) for breast cancer (Cleator et al., 2007; Martin et al., 2013).

The following is a brief summary of the modes by which the targets evaluated in the study (HER2, the components of the PI3K/Akt/mTOR pathway, and Bcl-2) are involved in treatment resistance:

1.8.4. HER2

Trastuzumab has been shown to have no clinical benefit in breast cancers expressing normal or low levels of the HER2 protein (p185HER2), indicating that a certain level of gene amplification is necessary for trastuzumab to be effective (Burstein, 2005; Vicario et al., 2015). In general, a cell population would exhibit a wide variation in the distribution of antigens of interest with sub-populations of cells expressing low, moderate, or high levels of the antigens. Another challenge in this treatment modality is that some HER2 positive cancers express a constitutively active truncated form of the protein (p95HER2). This truncated HER2 does not present with the extracellular domain necessary for trastuzumab binding, thereby, leading to poor treatment response (Pohlmann et al., 2009). This significance of p95HER2 in apparent resistance to trastuzumab therapy cannot be overemphasized. As much as 60% of HER2 positive tumours are known to express p95HER2 (Christianson et al., 1998; Molina et al., 2002; Scaltriti et al., 2015). Lapatinib, another drug approved by the US FDA, appears to be more potent for treating cancers exhibiting high levels of p95HER2 (Scaltriti et al., 2007). Failure to accurately identify the variant of HER2 that is overexpressed (i.e. p185HER2 or p95HER2) can lead to ineffective targeting and treatment resistance. Heterogeneity in the distribution of p185HER2 or p95HER2 in a tumour cell population can result in the inability to effectively target all cells with toxic levels of trastuzumab or lapatinib, respectively.

1.8.5. PI3K and mTOR

The phosphatase and tensin homolog (PTEN) is a tumour suppressor gene, which inhibits PI3K. Loss of functionality of PTEN via self-mutation or transcriptional regulation has been noted in 50% of breast cancers (Pandolfi, 2004). This loss of PTEN activity results in constitutive up-regulation of PI3K/Akt/mTOR phosphorylation and signalling which, in turn, prevents cell death, making tumours resistant to conventional treatment (Pohlmann et al., 2009). Also, PI3K in its mutated form has been implicated in resistance to therapy (Eichhorn et al., 2008). Mutations in PI3K have been shown to significantly activate the PI3K/Akt/mTOR pathway, leading to enhanced cell survival following cancer therapy (Jimenez et al., 1998; Philp et al., 2001).

1.8.6. Bcl-2

Stimuli, such as, DNA damage, hypoxia, high concentrations of cytosolic Ca_2^+, and severe oxidative stress can trigger apoptotic cell death via an intrinsic mitochondrial pathway. This pathway is initiated when the pro-apoptotic Bcl-2 family member, Bax, translocates from the cytoplasm onto the mitochondrial membrane causing the mitochondrion to become very permeable resulting in the release of the pro-apoptotic molecule (Cytochrome C) into the cytoplasm (Danial and Korsmeyer, 2004). The release of Cytochrome C then results in an irreversible cascade of pro-apoptotic processes that are mediated by a panel of caspases, namely, caspases 9, 3, 6 and 7. The anti-apoptotic protein, Bcl-2, blocks this pathway by inhibiting the translocation of Bax onto the outer mitochondrial membrane. An overexpression of

Bcl-2 in a tumour can, therefore, be expected to result in an increased evasion of apoptosis when cells are exposed to therapeutic agents, thereby leading to treatment resistance and poor prognosis (Minn et al., 1995; Reed et al., 1996; Simonian et al., 1997; Gallo et al., 1999; Ong et al., 2001).

1.8.7. Treatment for Breast Cancer

Breast cancer treatment choices are generally based on its tumour-node-metastasis status. Other important factors to consider are lymphovascular spread, histologic grade, hormone receptor status (ER and PR), HER2 expression status, the presence of other pathologies, menopausal state, and age. This section summarises the recommended treatment regimens for different types of breast cancers according to their stage, histology and the expression of ER, PR and HER2. Furthermore, this section focuses on the treatment options relevant to this research study. Here, radiation absorbed doses of 2 and 6 Gy are used. The relevance of using these doses with reference to clinical fractionation radiotherapy regimes is also highlighted. Table 1.2 summarises the treatment options for the different stages of breast cancers (Maughan et al., 2010).

Table 1.2: Treatment options for breast cancer by stage.

Breast Cancer Stage	Therapy*
Stage 0: in situ	• No treatment or consider prophylaxis with tamoxifen; • Breast-conserving surgery (mastectomy and radiotherapy are considered if extensive or multifocal)
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; bisphosphate for pain

**Adapted from Maughan et al.* (Maughan et al., 2010).

Table 1.3 summarises the treatment options for the different subtypes of breast cancer according to their expression of the biomarkers ER, PR and HER2 (Goldhirsch et al., 2011).

Table 1.3: Treatment options for breast cancer subtypes.

Breast Cancer Subtype	Recommended therapy*
Luminal A	Endocrine therapy.
Luminal B	Endocrine therapy, cytotoxic therapy and anti-HER2 therapy.
HER2 enriched	Cytotoxic therapy and anti-HER2 therapy.
Basal-like	Cytotoxic therapy.
Triple-Negative	Cytotoxic therapy.

**Adapted from Goldhirsch et al.* (Goldhirsch et al., 2011).

1.8.8. Treatment Options

1.8.8.1. Radiation Therapy

The use of radiation has been appreciated in the clinical setting as a cancer treatment option since its discovery over a century ago. Sixty percent of solid tumours are treated with radiation, and this emphasises the importance of this therapeutic regimen with 15% of cancer patients receiving radiation alone and 45% treated with a combination of radiation and other modalities (Prasanna et al., 2014).

Although ionising radiation damages the deoxyribonucleic acid (DNA) of normal and malignant cells and can prevent cells from dividing (Jackson and Bartek, 2009) or may induce cell death (Baskar et al., 2012), the rationale for radiation therapy is to deliver high doses to the cancerous cells while keeping doses to healthy cells at a minimum. A differential response to radiotherapy between normal and tumour cells can be harnessed, as tumour cells are highly proliferative and may be more radiosensitive than their normal counterparts.

In the clinical setting, radiation may be given alone with the intention to completely cure a cancer or simply for palliative purposes. Often, radiotherapy may be used to either shrink a tumour prior to surgery (neoadjuvant therapy) or to destroy residual disease after surgery (adjuvant therapy). Different types of cancers have different levels of radiosensitivity. While some early cancers (e.g. cancers of the skin, prostate, lung, cervix, and head-and-neck) are curable with radiation alone, others (e.g. cancers of the breast, rectum, anus, bladder, and endometrium; and locally advanced head-and-neck cancers, lymphomas, central nervous system soft tissue sarcomas, and paediatric tumours) may be cured with radiation in combination with molecular targeted therapies (Baskar et al., 2012).

In general, radiotherapy has been beneficial in the treatment of breast cancer, especially for palliative treatment. Depending on the stage of the disease, radiation therapy may reduce recurrences and improve patient survival. Despite the positive outcomes seen in breast cancer radiotherapy, multimodality treatment approaches involving the combined use of radiotherapy, chemotherapy, hormonal therapy, and molecular targeted therapy are highly desirable if the disease is to be completely eradicated (Eniu et al., 2006). There are numerous clinical trials on the use of

combinations of small molecule inhibitors and chemotherapy drugs that demonstrate acceptable safety profiles and significant therapeutic benefit for breast cancer management (Miller et al., 2005; Burstein et al., 2008; Liljegren et al., 2009; Brufsky et al., 2011; Robert et al., 2011; Gianni et al., 2012; Hurvitz et al., 2013; Pritchard et al., 2013; Luu et al., 2014; Roviello et al., 2016). However, there appears to be a general paucity of similar studies involving the combination of these inhibitors with radiotherapy (Jacob et al., 2014; Murphy et al., 2015). This is likely due to the fact that the concept of targeted therapy is relatively new, and studies combining small molecule inhibitors and radiotherapy are predominantly still in the preclinical phase (Sambade et al., 2010; Li et al., 2012).

Typically, radiotherapy may be given as many fractions at 2 Gy per fraction (conventional radiotherapy) or as fewer fractions at 3-20 Gy per fraction (hypofractionated radiotherapy) (Prasanna et al., 2014). The main advantages of hypofractionated radiotherapy are reduced treatment time and cost. The risk of encountering unintended treatment gaps is also significantly reduced relative to that for conventional radiotherapy. Although there are some concerns regarding normal tissue toxicity from the high fractional doses employed in hypofractionated radiotherapy, improvements in radiation therapy planning and dose delivery have adequately allayed these fears.

Besides causing cell death, DNA damage induced by ionising radiation can cause cells to express a variety of proteins that are crucial for damage repair, cell survival, and proliferation. For instance, radiation-induced damage activates the ataxia telangiectasia mutated (ATM) or ATM- and RAD3-related proteins, which

subsequently induce growth factor receptors to promote proliferation. Irradiation of cancer cells can also increase expression of transforming growth factor α (TGFα) which activates EGFR (Baselga et al., 1996; Levenson et al., 1998). Increased proliferation rates in cancers and poor prognosis have been correlated with elevated expression of EGFR (Putz et al., 1999). Clinically relevant radiation doses of 1-2 Gy are known to activate HER2 and its downstream effectors PI3K, Akt and mTOR (Contessa et al., 2002; Dent et al., 2003; Escriva et al., 2008). The same is true for cellular exposure to much higher doses of radiation (Liang et al., 2003). Cell exposure to ionising radiation also induces PTEN and PI3K activity (Contessa et al., 2002; Escriva et al., 2008). The EGFR, PI3K/Akt/mTOR and Ras/Raf/mitogen activated protein kinase (MAPK) pathways have been shown to mediate cellular radiosensitivity (Valerie et al., 2007). Targeting these molecular signalling components could lend itself as an effective approach for breast cancer treatment.

1.8.8.2. Targeted Therapy

The rationale for targeted therapy is to capitalise on proteins that are differentially overexpressed in cancer cells, relative to their normal counterparts. These proteins usually play a key role in cancer cell proliferation and survival. Interfering with the activity of these protein targets can, therefore, have a specifically adverse effect on cancer cell growth and survival. Traditional chemotherapy targets and kills both cancerous cells and rapidly dividing cells of normal tissues (e.g. hair, bone marrow, and gastrointestinal epithelium). In contrast, targeted therapies are aimed at molecular targets that are associated with carcinogenesis and tend to block tumour proliferation (Gerber, 2008).

Agents used in this therapeutic approach are usually present as small molecules for targets within cells or as relatively large molecules for targets outside the cells, or on the cell membrane (Gerber, 2008). These are usually only effective if a tumour presents with the specific target of interest. The most common cancer proliferation pathways targeted in breast cancer are those of the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGF), and human epidermal growth factor receptor (HER2/neu). These pathways can be blocked by neutralising molecules that bind to receptor sites on cells to prevent dimerisation, stopping receptor signalling within the cell, or interfering with signalling proteins downstream of the pathways (Gerber, 2008).

The use of targeted therapy has changed the outcomes of some cancers in the clinical setting. The tyrosine-kinase inhibitor, imatinib, has had great effects on chronic myeloid leukaemia (Thiele et al., 2004). The monoclonal antibody against B-cell surface protein CD20, rituximab, and another tyrosine-kinase inhibitor, Sunitinib, have improved the outcome of non-Hodgkin's lymphoma and renal cell carcinoma (Bharthuar et al., 2009; Dotan et al., 2010; Vázquez-Alonso et al., 2012). Also, the humanised monoclonal antibody, trastuzumab, has revolutionised the treatment of HER2 positive cancers (Romond et al., 2005). Combining chemotherapy with targeted therapy has also proven beneficial in some instances. The survival rate has been found to increase from 17% to 24% when patients with advanced pancreatic cancer are treated with chemotherapy in combination with the EGFR inhibitor, erlotinib (Moore et al., 2007). In addition to improving treatment prognosis, targeted therapy lends itself as a tolerable therapeutic alternative for patients scheduled for

anticancer therapy. For example, in elderly patients with small-cell lung carcinoma and non-Hodgkin's lymphoma who present with other medical conditions that limit the use of traditional chemotherapy, erlotinib and rituximab are less toxic and more tolerated.

Monoclonal antibodies have led to truly tailored therapy. Trastuzumab, a monoclonal antibody related to EGFR that interferes with the HER2 receptor, has been used with significant success in the treatment of breast cancer. This antibody was formulated after overexpression of human epidermal growth factor receptor 2 (HER2) was identified both as an aggressive disease marker and a treatment target (Burstein, 2005). Cancers overexpressing the gene encoding HER2 constitute 30% of invasive breast cancers (Slamon et al., 1987; Eccles et al., 2001). Biochemically, trastuzumab binds to the extracellular juxtamembrane domain of the full-length HER2. This binding causes a downregulation of HER2 expression, changes downstream signalling and regulatory pathways in the cancer cell cycle, inhibits the formation of new vasculature and leads to cell death (Burstein, 2005). Trastuzumab is, therefore, ineffective in breast cancers that do not overexpress HER2 (Bast et al., 2001; Romond et al., 2005) or those overexpressing the constitutively active truncated form of the protein which does not present with the extracellular domain necessary for trastuzumab binding (Pohlmann et al., 2009). In the latter cases, lapatinib may be used. The involvement of HER2 in many cellular response pathways, as mentioned, makes it an ideal target for effective breast cancer treatment.

Compared to monoclonal antibodies, small molecule inhibitors are less specific and have the ability to bind to multiple targets. These inhibitors tend to compete with

adenosine triphosphate (ATP) to prevent phosphorylation of tyrosine kinases like EGFR, HER2 and VEGF, and subsequent downstream signalling events (Aertgeerts et al., 2011). For instance, lapatinib is a small molecule that inhibits both EGFR and HER2 (Moulder et al., 2001; Tural et al., 2014).

1.8.8.3. Radiosensitisation by Targeted Therapy

There are many cell signalling cascades that govern the response of cells to different stimuli. The PI3K/Akt/mTOR pathway has been demonstrated to have a clear role in cellular response to ionising radiation (Kirshner et al., 2006). Activation of this pathway leads to increased uptake of glucose for metabolism, suppression of apoptosis, and enhanced cell survival (Schlessinger, 2000; LoPiccolo et al., 2008). Some monoclonal antibodies and small molecule inhibitors against components of this pathway are now in use as modulators of radiation response in the clinic. The EGFR-specific antibody, cetuximab, when used in combination with radiotherapy, has been most beneficial in increasing locoregional control and overall survival in patients with head-and-neck cancer (Bonner et al., 2006; 2010). Small molecule inhibitors against proteins of the PI3K/Akt/mTOR pathway and receptor tyrosine kinase inhibitors are also able to interfere with EGFR signalling and lead to tumour radiosensitisation (Bianco et al., 2002; Dutta and Maity, 2007; Feng et al., 2007).

1.8.8.4. Treatment Options for Triple-Negative Breast Cancer

Although a subset of triple-negative breast cancers respond to cytotoxic chemotherapy, the long-term prognosis of the treatment modality varies markedly

(Haffty et al., 2006; Kassam et al., 2009; Foulkes et al., 2010). In the neo-adjuvant setting, patients with triple-negative breast cancer who do not respond to chemotherapy before surgery relapse within two years and have a poor overall survival of 3 years (Dent et al., 2007; Morris and Carey, 2007; Millikan et al., 2008).

Triple-negative breast cancers are the most difficult to treat, and there are limited or no specific treatment options as these cancers are often devoid of significant expression of targets such as HER2, PR, and ER. A few systemic treatment approaches that are currently used for the management of triple-negative cancer are listed in Table 1.4.

Table 1.4: Potential systemic target-specific treatment protocols for triple-negative breast cancer.

Treatment	**Target***
Cytotoxic chemotherapy with agents that cause inter-strand breaks (e.g. platinum-based drugs)	DNA
PARP1 inhibitors	PARP1
Antibodies (e.g. Cetuximab) and small molecule inhibitors (e.g. Gefitinib)	EGFR
c-KIT tyrosine kinase inhibitors (e.g. Imatinib)	c-KIT
Multi-kinase inhibitors (e.g. Lapatinib and Pertuzumab)	EGFR/ERBB2
Second-messenger inhibitors	Ras, Raf, MEK, mTOR, Src, Hsp90

**Adapted from Cleator et al.* (Cleator et al., 2007).

Recurrent and metastatic triple-negative breast cancers tend to be very aggressive with a high proliferation index, invading the visceral and central nervous system (Liedtke et al., 2008; Smid et al., 2008). In such scenarios, the mean survival may be shortened to just 12 months.

Although there is evidence to suggest a benefit for adjuvant radiotherapy in the management of triple-negative breast cancer (Abdulkarim et al., 2011), it has been asserted that this regimen does not significantly enhance locoregional control and reduce disease progression (Haffty et al., 2006; Panoff et al., 2011; Dragun et al., 2011). Identification and validation of specific targets for triple-negative breast cancer therapy are highly desirable in the clinic. To this end, intensifying the search for effective small molecule inhibitors targeting proliferative and survival pathways, as alluded to in Table 1.4, and evaluating how these inhibitors may modulate the effects of radiotherapy, is warranted.

The unique features of the pro-survival PI3K/Akt/mTOR pathway have made its inhibition an attractive option for the development of new strategies for cancer treatment. A new generation of inhibitors that target the PI3K pathways are emerging, and these inhibitors are overcoming initial problems of poor target selectivity, undesired pharmacokinetics, and excessive toxicity. A few of these agents have entered early phase clinical trials. Inhibitors of HER2 and EGFR, such as TAK-165, are long known to radiosensitise breast cancer cells (Liang et al., 2003). TAK-165 has also been shown to exhibit significant anti-tumour effects in xenograft models of kidney, bladder and prostate cancer (Nagasawa et al., 2006). The dual inhibitor of PI3K and mTOR, NVP-BEZ235, has been shown to have anti-

proliferative and cytotoxic activity in a panel of 21 cell lines of different origins (Serra et al., 2008). Radiosensitisation of xenografts established from the breast cancer cell lines, MDA-MB-231 and MCF-7, by NVP-BEZ235 has been demonstrated under normoxic and hypoxic conditions (Kuger et al., 2014). NVP-BEZ235 has also been shown to attenuate DNA double-strand-break repair in glioblastoma cells, resulting in marked radiosensitisation (Mukherjee et al., 2012). Inhibition of Bcl-2 alone or in conjunction with mTOR, in *in vitro* systems or xenograft models, has been shown to radiosensitise cells originating from non-small-cell lung carcinoma and head-and-neck cancer (Tse et al., 2008; Kim et al., 2009; Zerp et al., 2015). Collectively, these reports suggest that informed targeting of components of the PI3K/Akt/mTOR pathway and Bcl-2 might render many cancers more susceptible to radiotherapy.

In this study, it is anticipated that targeting HER2, PI3K, mTOR and Bcl-2 with specific inhibitors might preferentially sensitise cancer cells that are either devoid of or are low expressers of HER2, oestrogen receptor (ER) and progesterone receptor (PR) to radiotherapy. NVP-BEZ235 inhibits PI3K and mTOR leading to the induction of apoptosis in breast tumour cell lines (Kuger et al., 2014). HER2 inhibitor Herceptin has also been shown to be cytotoxic in breast cell lines (Liang et al., 2003). Bcl2/Bcl-XL inhibitors proved to have radiosensitising effects in other cancers (Loriot et al., 2014). Despite these promising findings, normal toxicity is of significant concern as normal cells also express some of the targets of interest. However, sensitisation to radiation treatment by some inhibitors appears to be specific for cancer cells. Inhibition of PI3K and mTOR with NVP-BEZ235 has also been shown to radiosensitise prostate cancer cells, but acts as a radioprotector in normal prostate cells and mouse gut (Potiron et al., 2013; Maleka et al., 2015). Testing these HER2,

PI3K, mTOR and Bcl-2 inhibitors on a panel of malignant and normal cell lines may assist in identifying those with higher levels of specificity for malignants, and could aid in inhibitor selection for improved therapeutic benefit. This should provide potential therapeutic approaches for triple-negative breast cancer.

Chapter Two: Research Methodology

2.1. Chemicals, Specific Inhibitors, Antibodies, and Culture Medium

2.1.1. Reagents for Colony Forming Assay

2.1.1.1. Fixative

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

2.1.1.2. Staining Solution

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

2.1.2. Reagents for Flow Cytometry

2.1.2.1. Stock Buffers

2.1.2.1.1. 10× permeabilisation buffer (eBioscience, California, USA; cat #: 00-8333).

2.1.2.1.2. 1× BD Cytofix (eBioscience, California, USA; cat #: 00-8222).

2.1.2.2. Working Buffers

2.1.2.2.1. Permeabilisation buffer: 10× permeabilisation buffer (eBioscience, California, USA; cat #: 00-8333) diluted 1:10 in distilled water.

2.1.2.2.2. Fixation buffer: 1× BD Cytofix (eBioscience, California, USA; cat #: 00-8222).

2.1.2.3. Fluorochrome-Conjugated Antibody

2.1.2.3.1. Mouse anti-human phycoerythrin-conjugated antibody, Bax antibody (6D149) PE (Santa Cruz Biotechnology, Texas, USA; cat. #: sc-70405).

2.1.3. Specific Inhibitors

2.1.3.1. NVP-BEZ235

As illustrated in Figure 2.1, NVP-BEZ235 ($C_{30}H_{23}N_5O$; M_w = 469.55; Santa Cruz Biotechnology, Texas, USA; cat #: 364429) is a synthetic low molecular mass compound belonging to the class of imidazoquinolines that potentially and reversibly inhibits class 1 PI3K catalytic activity by competing for its ATP-binding sites (Maira, 2008). NVP-BEZ235 also inhibits mTOR catalytic activity, but does not target other protein kinases. It is known to have an inhibitory concentration for 50% inhibition of p110δ activity in breast cancer cell lines: CCL-247, HTB-38 and HTB-20 of ~7 nM (Maira et al., 2008).

For this study, a stock solution of 106 mM NVP-BEZ235 was reconstituted in dimethyl sulfoxide (DMSO) and stored at -20ºC until needed. To assess the influence of inhibitor concentration on cytotoxicity, cells were exposed to final concentrations of 0.6–70 nM of NVP-BEZ235.

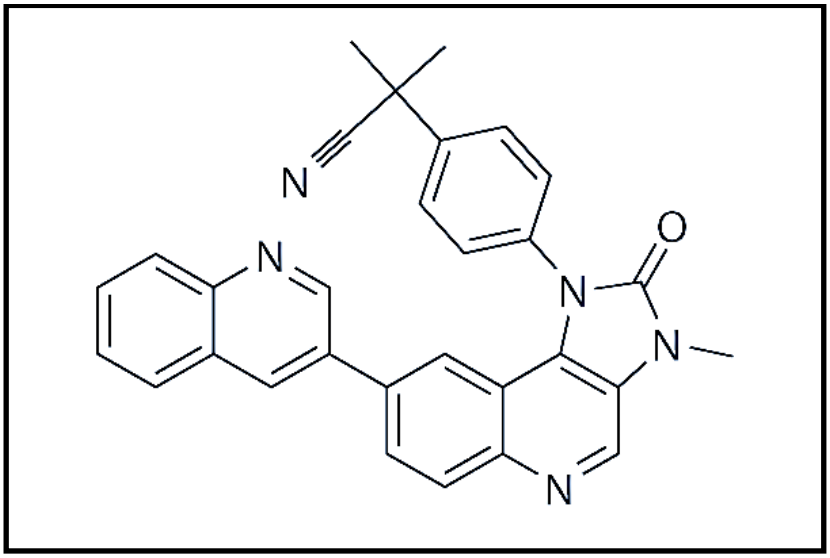

Figure 2.1: Chemical structure of NVP-BEZ235.

2.1.3.2. TAK-165

Figure 2.2 shows the chemical structure of TAK-165 ($C_{25}H_{23}F_3N_4O_2$; M_w = 468.47; TOCRIS Biosciences, UK; cat #: 3599). TAK-165 is a low molecular mass compound that was designed and synthesised by Takeda Pharmaceutical Company, Osaka, Japan. Biochemically, TAK-165 selectively inhibits HER2 and EGFR kinase activities with inhibitory concentrations of 6 and 25 000 nM for 50% inhibition (Nagasawa et al., 2006).

For this study, a stock solution of 21 mM of TAK-165 was prepared in dimethyl sulfoxide (DMSO) and stored at -20ºC until used. To assess the influence of inhibitor concentration on cytotoxicity, cells were exposed to a final concentration of 0.9–135 nM of TAK-165.

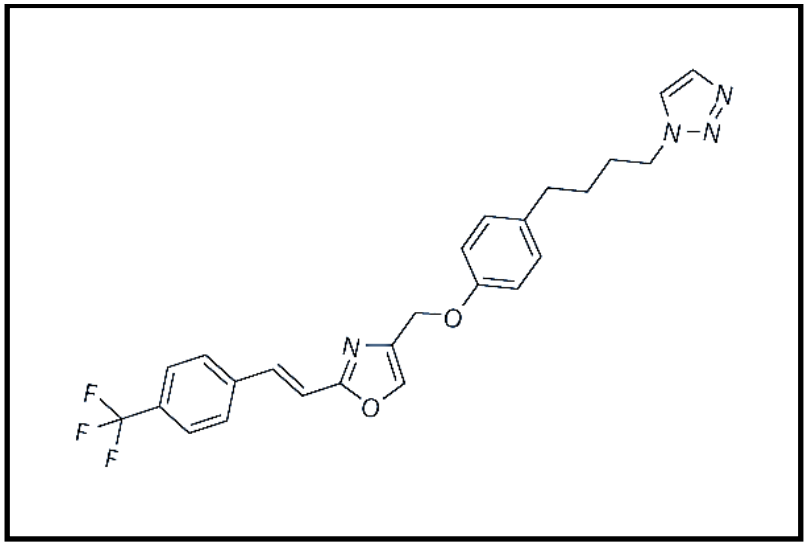

Figure 2.2: Chemical structure of TAK-165.

2.1.3.3. ABT-263

As shown in Figure 2.3, ABT-263 ($C_{47}H_{55}ClF_3N_5O_6S_3$; M_w = 1047.52; gift from the Chemotherapeutic Agents Repository of the Drug Synthesis and Chemistry Branch, National Cancer Institute, USA) is a Bad-like BH3 mimic for Bcl-2, Bcl-xl, and Bcl-w. Biochemically, ABT-263 disrupts Bcl-2/Bcl-xL interactions with pro-death proteins, leading to induced apoptosis within 2 hours after treatment. More so, in human cancer cells, ABT-263 induces Bax translocation, Cytochrome C release, and subsequent apoptosis (Bajwa et al., 2012). The stock solution consisted of 955 µM of ABT-263 in dimethyl sulfoxide (DMSO) and was stored at -20ºC until needed. To assess the influence of inhibitor concentration on cytotoxicity, cells were exposed to final concentrations of 0.03-10 000 nM of ABT-263.

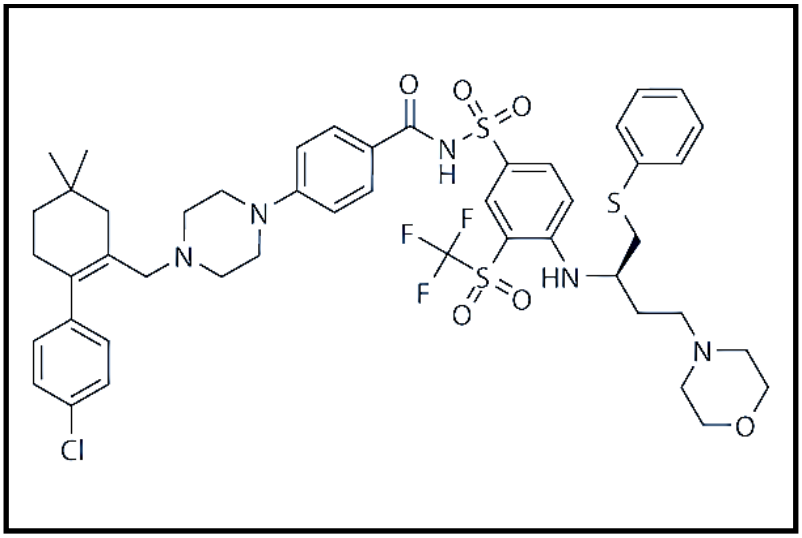

Figure 2.3: Chemical structure of ABT-263.

2.1.4. Cell Culture Medium

2.1.4.1. Dulbecco's Modified Eagle's Medium Nutrient Mixture (F-12 Ham)

Dulbecco's modified Eagle's medium nutrient mixture, F-12 HAM (Sigma-Aldrich, USA; cat #: D8437) is a pre-constituted liquid growth medium, supplemented with 15 mM HEPES, 0.365 gm/L L-glutamine, 1.2 gm/L sodium bicarbonate, 20 ng/ml human epidermal growth factor (Sigma-Aldrich, Germany; cat #: E9644), 0.01 mg/ml bovine insulin (Sigma-Aldrich, Germany; cat #: I5500), and 500 ng/ml hydrocortisone (Sigma-Aldrich, Germany; cat #: H0888). The growth medium was further supplemented with 10% heat-inactivated foetal bovine serum (FBS) (HyClone, UK; cat #: SV30160.30IH), and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Lonza, Belgium; cat #: DE17-602E).

2.1.4.2. Roswell Park Memorial Institute medium (RPMI-1640)

The Roswell Park Memorial Institute medium, RPMI-1640 (Sigma-Aldrich, USA; cat #: R8758), is also a liquid cell culture medium, supplemented with 0.3 gm/L L-glutamine and 2.0 gm/L sodium bicarbonate. The growth medium was further supplemented with 10% heat-inactivated foetal bovine serum (FBS) (HyClone, UK; cat #: SV30160.30IH), and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Lonza, Belgium; cat #: DE17-602E).

2.2. Cell Lines

The human breast cell lines (MDA-MD-231, MCF-7 and MCF-12A) used in this study exhibit diminished expression of HER2, ER or PR, and have been classified as triple-negative elsewhere (Chavez et al., 2010; Leung et al., 2014). The rationale for choosing these cell lines is that they significantly differ in expression of potential target antigens, such as, HER2, ER, PR and EGFR. Although MCF-7 cells are EGFR, HER2, ER and PR positive (Horwitz et al., 1975; Rusnak et al., 2001), their expression of HER2 is low and comparable to that in the MDA-MB-231 cells (Konecny et al., 2006) and MCF-12A cells (Subik et al., 2010). While expression of EGFR in MCF-7 cells is also low, MDA-MB-231 and MCF-12A cells which are known to be ER and PR negative (Subik et al., 2010), express higher levels of EGFR (Konecny et al., 2006; Subik et al., 2010). Furthermore, the MDA-MB-231 and MCF-12A cell lines express wild-type PI3K, whereas the MCF-7 cell line is PI3K mutant (Vasudevan et al., 2009; Carlson et al., 2010).

2.2.1. MDA-MB-231 (passage number: 15-30)

The MDA-MB-231 cell line was established from pleural effusions taken from a 51 year old Caucasian woman with invasive ductal mammary adenocarcinoma, and has been classified as a basal-like breast cancer subtype (Subik et al., 2010). It has an epithelial-like morphology, is adherent, grows as a monolayer, and forms metastatic xenografts in nude mice in an oestrogen-dependent manner. The cells were a gift from Professor S. Prince (University of Cape Town, South Africa), and were routinely cultured in Roswell Park Memorial Institute medium (RPMI-1640).

2.2.2. MCF-7 (passage number: 20-30)

The MCF-7 cell line was established from pleural effusions taken from a 69 year old Caucasian woman with invasive ductal mammary carcinoma, and has been classified as a luminal A breast cancer subtype (Subik et al., 2010). It has an epithelial-like morphology, is adherent, grows as a monolayer, and forms metastatic xenografts in nude mice in an oestrogen-independent manner. The cells were a gift from Professor S. Prince (University of Cape Town, South Africa), and were routinely cultured in Roswell Park Memorial Institute medium (RPMI-1640).

2.2.3. MCF-12A (passage number: 20-35)

The apparently normal immortalised mammary epithelial cell line, MCF-12A, was derived from mammary gland tissue taken from a 60 year old Caucasian woman and was a gift from Professor AM Engelbrecht (Stellenbosch University, South Africa).

The cells were routinely cultured in Dulbecco's modified Eagle's medium/nutrient F-12 HAM.

2.2.4. HeLa Cells (passage number: 43)

The HeLa cell line was derived from cervical cancer tissue taken from a 31 year old African-American woman. The cells were a gift from Prof E. van Helden (Stellenbosch University, South Africa) and were routinely cultured in McCoy's 5A medium (Sigma-Aldrich, South Africa; cat #: M4892).

2.3. Routine Cell Culture and Cryopreservation

Cell cultures were routinely incubated at 37°C in a humidified atmosphere (95% air and 5% CO_2) in SHEL LAB incubators (Sheldon Manufacturing Inc., USA). Cells were grown as monolayers in 75-cm^2 flasks (Greiner Bio-One, Germany; cat #: 658170) and were used for experiments (passages 15–40) upon reaching 70–90% confluence.

For cryogenic storage, cells were trypsinised, centrifuged (4 000 RPM for 5 minutes), and re-suspended in a mixture of 0.9 ml foetal bovine serum and 0.1 ml of dimethyl sulfoxide (DMSO) in cryovials. The vials were stored at -80°C overnight, and then transferred into liquid nitrogen for use at a later stage. To return cells to culture, the frozen cells were re-suspended in the appropriate growth medium and incubated.

2.4. Irradiation of Cell Cultures

Cells grown in 25 cm^2 tissue culture flasks were initially irradiated using ^{60}Co γ-rays at the Tygerberg Academic Hospital. 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 an 8.5 cm thick foamalite slab and a 5 cm thick perspex sheet. The mean dose rate was 0.827 Gy/min (range: 0.782-0.873 Gy/min). Dosimetry was by thermoluminescent dosimetry (TLD chips).

Following the decommissioning of the ^{60}Co γ-irradiator in December 2014, subsequent cell culture irradiation was performed using a Faxitron MultiRad 160 X-irradiator (Faxitron Bioptics, Tucson, AZ, USA) at the Division of Radiobiology (Faculty of Medicine and Health Sciences, Stellenbosch University). Samples were irradiated at an SSD of 65 cm, measured to the base of the experimental flasks, at a dose rate of 1.0 Gy/min. For this, build-up also consisted of 10 ml of medium in the 25 cm^2 culture flasks. In all cases, cell cultures were irradiated at room temperature (22°C).

2.5. Cell Survival Assay and Radiosensitivity

The colony assay was used to measure intrinsic radiation response in all cell lines. Cultures in exponential growth were trypsinised to give single-cell suspensions and

were plated (500-10 000 cells per flask, adjusted for irradiation dose) into 25 cm^2 culture flasks (Greiner Bio-One, Germany; cat #: 690160), and incubated for 3-4 h to allow the cells to attach. Cell cultures were then irradiated to 0-10 Gy with either ^{60}Co γ-rays or X-rays and reincubated. Three flasks were used per dose point. After growing for 7-10 days, depending on the cell line, colonies were fixed, stained, washed in tap water, air-dried, and counted. Three independent experiments were performed for each dose point, and the mean surviving fractions were fitted to the linear-quadratic (LQ) model of the form:

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

to generate survival curves. *S* is the surviving fraction, *α* and *β* are the linear and quadratic cell inactivation constants, respectively, and *D* is the absorbed dose in Gy. Cellular radiosensitivity was expressed in terms of the surviving fraction at 2 Gy (SF_2).

2.6. Target Inhibitor Toxicity Measurements

Single-cell suspensions were plated (1000-4000 cells per flask) into 25 cm^2 culture flasks, and incubated for 3-4 h to allow the cells to attach. To assess the influence of inhibitor concentration on cytotoxicity, cells were exposed to NVP-BEZ235 (0.6-70 nM), TAK-165 (0.9-135 nM) and ABT-263 (0.03-10 000 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 killing (EC_{50}), the surviving fractions (SF) were plotted as a function of

log(inhibitor concentration) and were fitted to a 4-parameter logistic equation of the form:

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

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. Three independent experiments were performed for each cell line and dose point.

2.7. Determination of Radiosensitivity Modification by Target Inhibitors

To investigate the influence of inhibitor exposure on radiosensitivity, attached cells were treated with 30 nM of TAK-165 (~1.0–1.5 times the predetermined EC_{50} for the cell lines), 17 nM of NVP-BEZ235 (~3.4–4.1 times the predetermined EC_{50} for the cell lines) and 97 nM ABT-263 (~0.4–1.0×EC_{50} predetermined for the cell lines), or a cocktail of both inhibitors at the same concentrations, and irradiated immediately with 2 or 6 Gy, typical doses per fraction in conventional and hypofractionated radiotherapy, respectively. The use of relatively higher concentrations of NVP-BEZ235 was to ensure adequate inhibition of the dual targets (PI3K and mTOR), as these would be expected to present a larger number of binding sites. For each experiment, sets of cell culture flasks given inhibitors alone (singly and in combination) and unirradiated flasks without inhibitors served as controls for cultures irradiated with and without inhibitors, respectively. 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 killing. The interaction between inhibitors and irradiation at 2 and 6 Gy was expressed as a modifying factor (MF_{col}), which is given by the ratio of surviving fractions (*SF*) in the absence and presence of inhibitors as follows:

$$MF_{col} = \frac{SF(2\ Gy)}{SF([inhibitor]+2\ Gy)} \quad or \quad MF_{col} = \frac{SF(6\ Gy)}{SF([inhibitor]+6\ Gy)} \qquad (3).$$

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

2.8. Flow Cytometric Analysis

The MDA-MB-231 cells were used to test whether flow cytometry could be used as a surrogate for the clonogenic cell survival assay for rapid screening of potential target inhibitors as radiosensitisers. This was achieved by determining treatment-induced changes in the levels of the pro-apoptotic protein, *Bax*. Cell cultures at approximately 80% confluency were treated with 17 nM of NVP-BEZ235, exposed to 2 Gy of X-rays, and then incubated for 24 hours. The growth medium was decanted and the cell cultures were trypsinised and reconstituted in growth medium to give single-cell suspensions. The cells were counted, pelleted and re-suspended in foetal bovine serum. Aliquots of 0.9 ml containing 1 000 000 cells each were transferred into flow cytometry tubes, and centrifuged at 4 000 RPM for 5 minutes. Cell pellets were re-

suspended in 100 µl of IC fixation buffer (eBiosciences, CA, USA; cat #: 00-8222) and 100 µl permeabilisation buffer (eBiosciences, CA, USA; cat #: 00-8333) for 20 minutes at 4°C (protected from light). The fixed and permeabilised cells were further centrifuged at 500×g for 5 minutes and the supernatant discarded. The cells were then re-suspended in 500 µl of 10% dimethyl sulfoxide (DMSO) in foetal bovine serum (FBS) and stored at -80°C until used.

For intracellular staining, tubes containing frozen cells were retrieved from -80°C, thawed at 37°C, centrifuged at 500×g for 5 minutes and the supernatant discarded. The cells were then washed twice in PBS (centrifuged two times at 500×g for 5 min) to remove the freezing medium, and fixed and permeabilised again. This re-fixing and re-permeabilisation are required prior to staining as the process of permeabilisation is reversible over prolonged cryopreservation. This was then followed by washing the cells two times with 120 µl of permeabilisation buffer. The washed cells in each tube were then resuspended in 500 µl of antibody staining solution (2% FBS in PBS) and 5 µl of a mouse anti-human phycoerythrin-conjugated antibody, Bax antibody (6D149) PE (Santa Cruz Biotechnology, Texas, USA; cat. #: sc-70405). Samples were then incubated for at least 30 minutes at room temperature in the dark before flow cytometric analysis.

Flow cytometry data were acquired using a BD FACSCalibur equipped with CellQuest PROTM software (Becton Dickinson, San Jose, CA, USA) and 2 Lasers (488 and 633 nm), and a capacity to simultaneously detect up to four fluorescent probes. Typically, 10 000 events per sample were collected and analysed using FlowJo v.X.0.7 (TreeStar Software, Inc. Ashland, OR, USA). Pro-apoptotic Bax

activity was expressed in terms of the mean fluorescence intensity (*MFI*) of Bax antibody (6D149) PE. The *MFI*-value was then used to derive a radiation modifying factor according to the equation:

$$MF_{flow} = \frac{MFI_{(inhibitor + 2\ Gy)} - MFI_{(inhibitor + 0\ Gy)}}{MFI_{(2\ Gy)} - MFI_{(0\ Gy)}} \quad (4),$$

and compared with that obtained using the colony forming assay (MF_{col}).

2.9. Data Analysis

Statistical analyses were performed using the GraphPad Prism (GraphPad Software, San Diego, CA, USA.) computer program. For associations, linear regression analyses were used. Standard equations were used to fit nonlinear relationships. Data were calculated as the means (± 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.

Chapter Three: Results

3.1. Calibration of Faxitron MultiRad 160 X-irradiator

Human cervical carcinoma cells (HeLa) were used to validate the equivalent biological effectiveness of the decommissioned ^{60}Co γ-irradiator and the newly installed Faxitron MultiRad 160 X-irradiator, and to allow direct use of all data generated in this study. For this, clonogenic cell survival of HeLa cells was determined concurrently following irradiation with the Faxitron MultiRad 160 X-irradiator and the decommissioned ^{60}Co γ-irradiator. The data in Figure 3.1 show that the cell survival curves for the γ- and X-irradiation are congruent, indicating that the biological effectiveness of the 160 kVp X-rays from the Faxitron MultiRad 160 X-irradiator does not differ significantly from that of the ^{60}Co γ-irradiator. It is not surprising that the 160 kVp X-rays and ^{60}Co γ-rays emerged equally effective in cell killing. Although the linear energy transfers of 100 – 250 kVp X-rays range between ~5 and 2 keV/µm, respectively, and are orders of magnitude higher than that of ^{60}Co γ-rays (~0.3 keV/µm), the relative biological effectiveness of these radiation types in many biological systems do not differ by greater than 10% (Spadinger and Palcic 1992; Hering 1986; Nikjoo and Lindborg 2010).

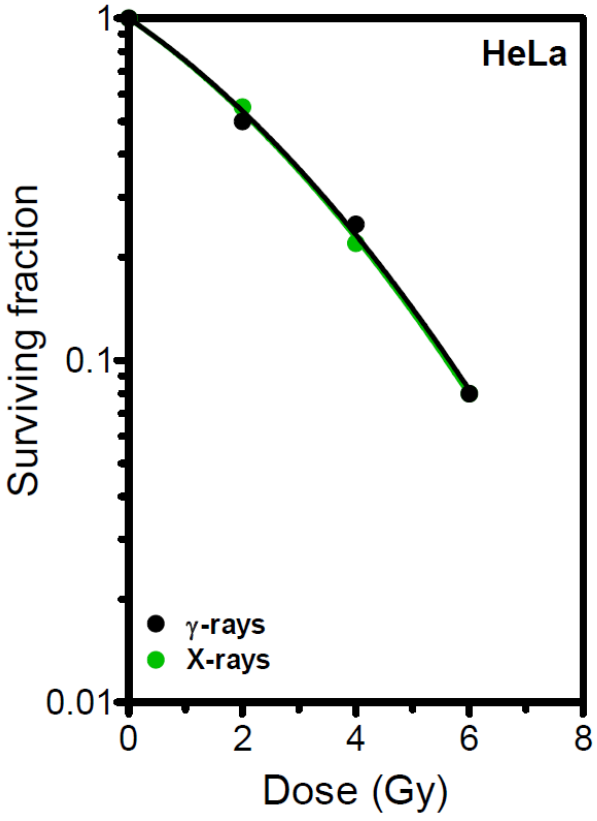

Figure 3.1: The clonogenic cell survival curves for human cervical carcinoma cells when irradiated with ^{60}Co γ-rays (black) and Faxitron MultiRad 160 X-rays (green). Each point represents data derived from a single experiment consisting of a triplicate of cell culture flasks.

3.2. Intrinsic Radiosensitivity of MDA-MB-231, MCF-7, and MCF-12A Cells

Figure 3.2 shows clonogenic cell survival data for three human breast cell lines (MDA-MB-231, MCF-7 and MCF-12A). Intrinsic cellular radiosensitivity expressed in terms of the surviving fraction at 2 Gy, SF_2. SF_2-values were obtained by fitting the mean surviving data to the linear-quadratic model. From the cell survival data in Figure 3.2, the SF_2-values for the MDA-MB-231 (PI3K wild-type), MCF-7 (PI3K mutant) and MCF-12A (PI3K wild-type) cell lines were 0.59 ± 0.07, 0.23 ± 0.01, and

0.60 ± 0.07, respectively. The MDA-MB-231 and MCF-12A cell lines show a similar radiation response at 2 Gy and are deemed radioresistant, whereas the MCF-7 cell line is deemed radiosensitive. The radiobiological features of the 3 cell lines are summarised in Table 3.1. In general, the cell survival curves did not exhibit prominent shoulders, consistent with the almost non-existent β-component of cell kill. Therefore, the intrinsic cellular radiosensitivity and the surviving fraction at 6 Gy were directly correlated with the α-component of cell kill, with the most radiosensitive MCF-7 cell line emerging with the largest α-coefficient.

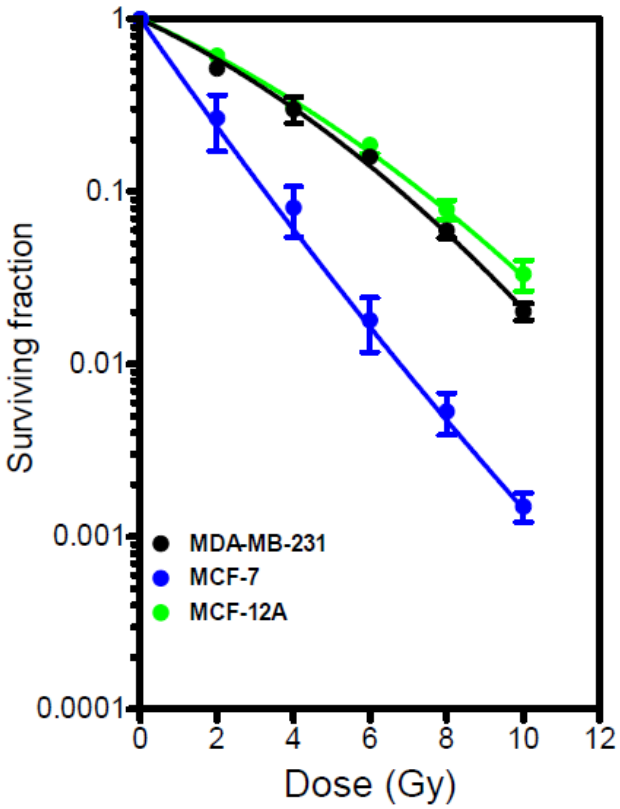

Figure 3.2: Clonogenic cell survival curves for 3 human breast cell lines after ^{60}Co γ-irradiation. Symbols represent the mean surviving fraction ± SEM from three independent experiments. Standard errors are not transformed into a logarithmic scale. Survival curves were obtained by fitting experimental data to the linear-quadratic model.

Table 3.1: Summary of radiobiological parameters for 3 human breast cell lines (MDA-MB-231, MCF-7 and MCF-12A). SF_2 and SF_6 denote the surviving fractions at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. Data are presented as the mean ± SEM from 3 independent experiments.

Cell line	SF_2	SF_6	α (Gy^{-1})	β (Gy^{-2})
MDA-MB-231	0.57 ± 0.02	0.128 ± 0.027	0.24 ± 0.03	0.015 ± 0.028
MCF-7	0.23 ± 0.01	0.096 ± 0.024	0.73 ± 0.09	0.008 ± 0.010
MCF-12A	0.63 ± 0.05	0.178 ± 0.009	0.23 ± 0.03	0.012 ± 0.004

3.3. Cytotoxicity of TAK-165, NVP-BEZ235 and ABT-263

Treatment of cells with inhibitors induced a concentration-dependent cell kill (Figure 3.3). At cell survival rates ranging from 20-90%, NVP-BEZ235 was clearly more potent in cell killing than TAK-165 and ABT-263 in all cell lines. The equivalent concentrations of NVP-BEZ235, TAK-165 and ABT-263 for 50% cell survival for the MDA-MB-231 cell line were found to be 4.25 ± 0.23 nM (95% CI: 3.78–4.76 nM), 28.36 ± 4.65 nM (95% CI: 21.65–37.14 nM) and 96.80 ± 9.53 nM (95% CI: 79.2–118.3 nM), respectively. The corresponding EC_{50}-values for the MCF-7 cell line emerged as 4.15 ± 0.34 nM (95% CI: 3.48–4.95 nM), 24.87 ± 2.26 nM (95% CI: 20.42–30.30 nM) and 232.2 ± 39.4 nM (95% CI: 163.9–328.9 nM). The EC_{50}-values of NVP-BEZ235, TAK-165 and ABT-263 for the MCF-12A cell line emerged as 5.01 ± 0.81 nM (95% CI: 3.46–7.23 nM), 20.26 ± 3.84 nM (95% CI: 13.49–30.42 nM) and 112.9 ± 8.8 nM (95% CI: 96.02–132.70 nM), respectively. To facilitate comparison, the inhibitor toxicity data are summarised in Table 3.2.

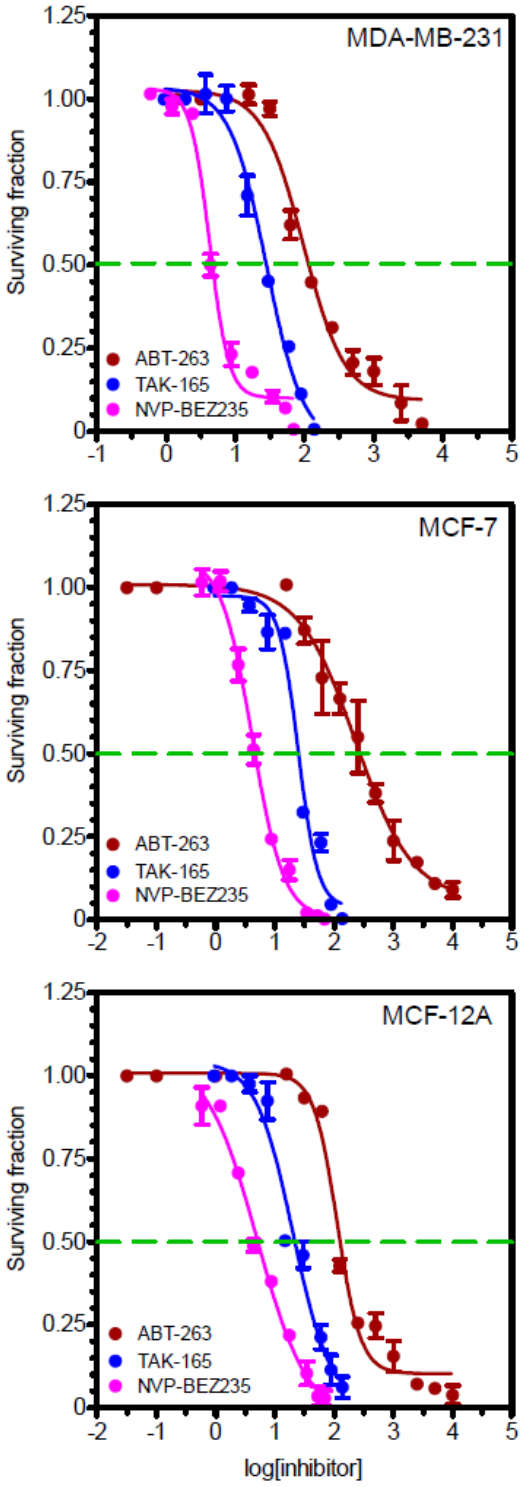

Figure 3.3: Cytotoxicity curves for HER2 inhibitor (TAK-165: 0.9-135 nM), PI3K and mTOR inhibitor (NVP-BEZ235: 0.6-70 nM), and Bcl-2 inhibitor (ABT-263: 0.03-10 000 nM) for 3 human breast cell lines (MDA-MB-231, MCF-7 and MCF-12A). Curves were obtained by plotting cell survival as a function of log(inhibitor concentration). Cell survival was determined by the colony assay, and data were fitted to a 4-parameter logistic equation. Data points are means ± SEM of 3 independent experiments.

Table 3.2: Summary of cytotoxicity data for 3 human breast cell lines (MDA-MB-231, MCF-7 and MCF-12A) treated with HER2 inhibitor (TAK-165), PI3K and mTOR inhibitor (NVP-BEZ235), and Bcl-2 inhibitor (ABT-263). EC_{50} denotes the equivalent concentration for 50% cell survival. *T* and *B* are the maximum and minimum of the concentration-response curve, respectively (Figure 3.3). *HS* is the steepest slope of the curve.

Cell line	Treatment	EC_{50} (nM)	*T*	*B*	*HS*
MDA-MB-231	TAK-165	28.36±4.65	1.03±0.03	0.00±0.07	-1.62±0.28
	NVP-BEZ235	4.25±0.23	1.03±0.03	0.10±0.02	-3.14±0.51
	ABT-263	96.80±9.53	1.03±0.02	0.09±0.03	-1.46±0.19
MCF-7	TAK-165	24.87±2.26	0.98±0.03	0.04±0.05	-2.65±0.58
	NVP-BEZ235	4.15±0.34	1.09±0.03	0.00±0.02	-1.64±0.22
	ABT-263	232.2±39.4	1.01±0.02	0.06±0.04	-0.91±0.12
MCF-12A	TAK-165	20.26±3.84	1.04±0.05	0.01±0.09	-1.38±0.36
	NVP-BEZ235	5.01±0.81	1.06±0.09	0.00±0.06	-1.01±0.22
	ABT-263	112.9±8.8	1.01±0.02	0.10±0.02	-2.20±0.33

3.4. Modulation of Radiosensitivity by TAK-165 and NVP-BEZ235 at 2 Gy

To evaluate the impact of cellular exposure to inhibitors of HER2, PI3K and mTOR on radiosensitivity at a relatively low radiation absorbed dose per fraction, based on clonogenic cell survival, cell cultures were irradiated to 2 Gy immediately after administering TAK-165 and NVP-BEZ235 singly or in combination. As shown in Figure 3.4, pre-treatment with the HER2 inhibitor (TAK-165) alone led to a small and insignificant radiosensitisation in all cell lines, with SF_2 decreasing from 0.57 ± 0.02 to 0.51 ± 0.03, 0.23 ± 0.01 to 0.22 ± 0.02, and 0.63 ± 0.05 to 0.55 ± 0.01 for the MDA-MB-231, MCF-7 and MCF-12A cells, respectively. This correspondingly translated to about 11, 4 and 13% increase in radiotoxicity (Table 3.3). On the other hand, inhibiting PI3K and mTOR activity with NVP-BEZ235 significantly radiosensitised MDA-MB-231 and MCF-7 cells, but not the apparently normal MCF-12A cells. The resulting SF_2-values were 0.32 ± 0.04, 0.14 ± 0.02 and 0.48 ± 0.03 for the MDA-MB-231, MCF-7 and MCF-12A cell lines, respectively. The corresponding modifying factors were 1.78 ± 0.23**,** 1.64 ± 0.25 and 1.31 ± 0.13 (Table 3.3). In all cases, NVP-BEZ235 was more radiosensitising than TAK-165. Concomitant treatment with TAK-165 and NVP-BEZ235, significantly enhanced radiosensitivity in all cell lines, yielding ~4-, ~3-, and ~8-fold reduction in cell survival in the MDA-MB-231, MCF-7 and MCF-12A cell lines, respectively.

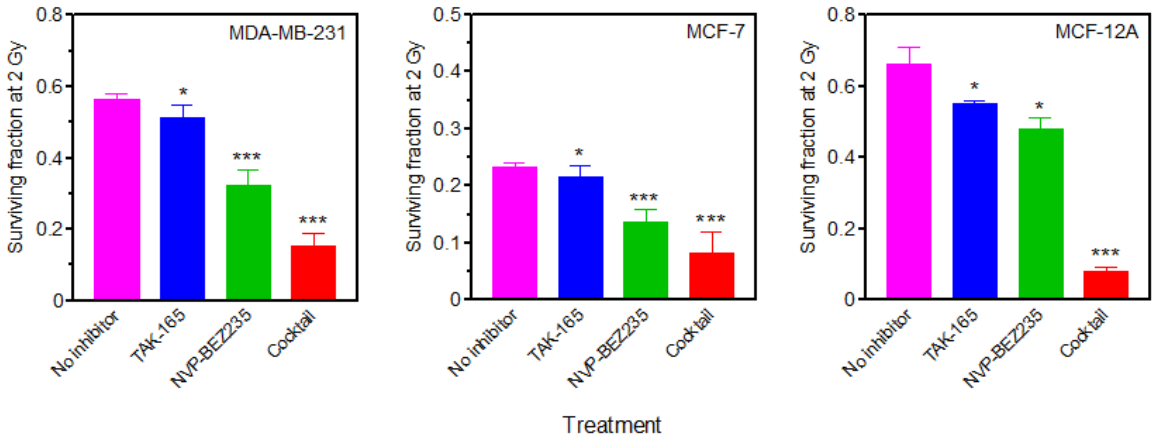

Figure 3.4: Clonogenic cell survival at 2 Gy (SF_2) for 3 human breast cell lines after irradiation with X-rays: MDA-MB-231, MCF-7, and MCF-12A cells were irradiated without or in the presence of TAK-165 (HER2 inhibitor: 0.9-135 nM) and NVP-BEZ235 (dual inhibitor of PI3K and mTOR: 0.6-70 nM), either administered singly or in combination. Bars represent the mean surviving fraction ± SEM from three independent experiments. In comparison with SF_2 without inhibitors: *$P > 0.05$; ***$P < 0.005$.

Table 3.3: Summary of radiosensitivity and dose modifying data for three human breast cell lines treated with inhibitors TAK-165 (for HER2) and NVP-BEZ235 (for PI3K and mTOR).

Cell line	Treatment	SF_2*	MF_2#
MDA-MB-231	2 Gy	0.57 ± 0.02	
	2 Gy + TAK-165	0.51 ± 0.03	1.12 ± 0.07
	2 Gy + NVP-BEZ235	0.32 ± 0.04	1.78 ± 0.23
	2 Gy + NVP-BEZ235 + TAK-165	0.15 ± 0.03	3.80 ± 0.77
MCF-7	2 Gy	0.23 ± 0.01	
	2 Gy + TAK-165	0.22 ± 0.02	1.04 ± 0.11
	2 Gy + NVP-BEZ235	0.14 ± 0.02	1.64 ± 0.25
	2 Gy + NVP-BEZ235 + TAK-165	0.08 ± 0.04	2.88 ± 1.44
MCF-12A	2 Gy	0.63 ± 0.05	
	2 Gy + TAK-165	0.55 ± 0.01	1.15 ± 0.09
	2 Gy + NVP-BEZ235	0.48 ± 0.03	1.31 ± 0.13
	2 Gy + NVP-BEZ235 + TAK-165	0.08 ± 0.02	7.88 ± 2.07

SF_2 and MF_2 denote the surviving fraction and radiation modifying factor at 2 Gy, respectively. *Mean ± SEM. #Mean ± error: errors were calculated using appropriate error propagation formulae.

3.5. Modulation of Radiosensitivity by TAK-165 and NVP-BEZ235 at 6 Gy

To investigate whether radiosensitisation of breast carcinoma cell lines exist at higher fractional doses, as may be encountered in stereotactic radiotherapy, the effects of blocking the activities of HER2, PI3K and mTOR, and immediately irradiating cell cultures to 6 Gy was assessed. The cell survival data are presented in Figure 3.5 for the three cell lines. Pre-treatment of MDA-MB-231 cells with the HER2 inhibitor (TAK-165) appeared to increase radioresistance, whilst treatment with either the dual inhibitor of PI3K and mTOR (NVP-BEZ235) alone or in combination with TAK-165 resulted in a slight reduction in radiosensitivity ($P > 0.38$). On the other hand, MCF-12A cells were significantly radiosensitised when pre-treated with TAK-165 and NVP-BEZ235, either singly or as a cocktail, giving radiation modifying factors of 2.34 ± 0.39, 2.62 ± 0.93 and 3.24 ± 1.54, respectively. While HER2 inhibition had no effect on the radiosensitivity of the MCF-7 cell line, inhibition of PI3K and mTOR yielded a 12-fold radiosensitisation (Figure 3.5 and Table 3.4). Pre-treatment with the inhibitor cocktail resulted in a modifying factor of only 2.34 ± 0.68.

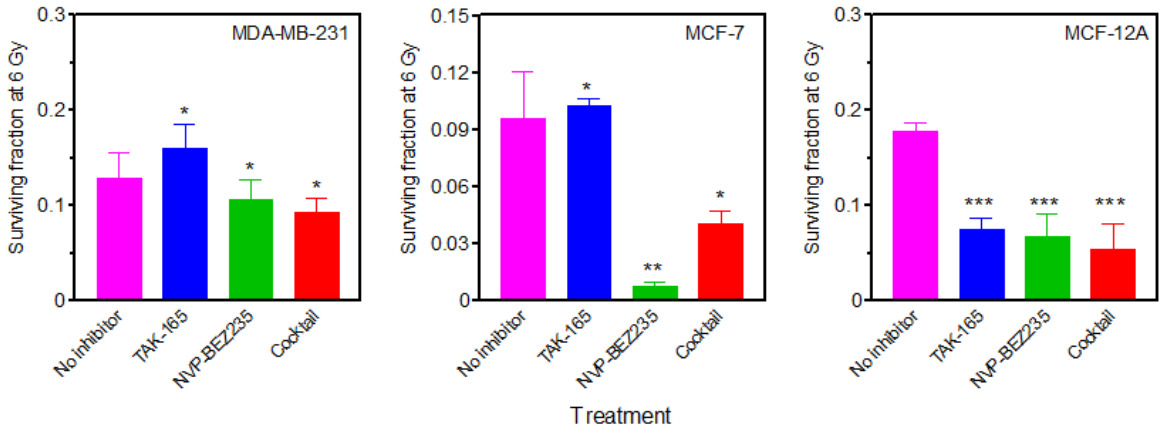

Figure 3.5: Clonogenic cell survival at 6 Gy (SF_6) for 3 human breast cell lines after irradiation with X-rays: MDA-MB-231, MCF-7, and MCF-12A cells were irradiated without or in the presence of TAK-165 (HER2 inhibitor: 0.9-135 nM) and NVP-BEZ235 (dual inhibitor of PI3K and mTOR: 0.6-70 nM), either administered singly or in combination. Bars represent the mean surviving fraction ± SEM from three independent experiments. In comparison with SF_6 without inhibitors: *$P > 0.05$; **$0.005 \leq P \leq 0.05$; ***$P < 0.005$.

Table 3.4: Summary of radiosensitivity and dose modifying data for three human breast cell lines treated with inhibitors TAK-165 (for HER2) and NVP-BEZ235 (for PI3K and mTOR).

Cell line	Treatment	SF_6*	MF_6[#]
MDA-MB-231	6 Gy	0.128 ± 0.027	
	6 Gy + TAK-165	0.160 ± 0.025	0.80 ± 0.21
	6 Gy + NVP-BEZ235	0.107 ± 0.020	1.20 ± 0.34
	6 Gy + NVP-BEZ235 + TAK-165	0.093 ± 0.015	1.38 ± 0.37
MCF-7	6 Gy	0.096 ± 0.024	
	6 Gy + TAK-165	0.102 ± 0.004	0.94 ± 0.24
	6 Gy + NVP-BEZ235	0.008 ± 0.002	12.00 ± 4.24
	6 Gy + NVP-BEZ235 + TAK-165	0.041 ± 0.006	2.34 ± 0.68
MCF-12A	6 Gy	0.178 ± 0.009	
	6 Gy + TAK-165	0.076 ± 0.012	2.34 ± 0.39
	6 Gy + NVP-BEZ235	0.068 ± 0.024	2.62 ± 0.93
	6 Gy + NVP-BEZ235 + TAK-165	0.055 ± 0.026	3.24 ± 1.54

SF_6 and MF_6 denote the surviving fraction and radiation modifying factor at 6 Gy, respectively. *Mean ± SEM. [#]Mean ± error: errors were calculated using appropriate error propagation formulae.

3.6. Modulation of Radiosensitivity by NVP-BEZ235 and ABT-263 at 2 Gy

Pre-treatment of MDA-MB-231 cells with Bcl-2 inhibitor, ABT-263, alone or a cocktail of ABT-263 and NVP-BEZ235 resulted in significant radiosensitisation, with the latter yielding the highest effect (Figure 3.6). The corresponding radiation modifying factors emerged as 1.96 ± 0.15 and 4.39 ± 1.69 (Table 3.5). In this cell line, ABT-263 was marginally more radiosensitising than NVP-BEZ235. Exposure of MCF-7 cells with ABT-263 did not affect their radiosensitivity (Figure 3.6). This was also reflected in the observation that addition of ABT-263 had no added benefit in the radiosensitisation seen with NVP-BEZ235 alone. For the MCF-12A cell line, inhibition of Bcl-2 alone (with ABT-263) was more radiosensitising than when both PI3K and mTOR were inhibited (with NVP-BEZ235). The corresponding radiation modifying factors were 1.85 ± 0.22 and 1.31 ± 0.13 (P = 0.06). Pre-treatment of these cells with both inhibitors yielded a 7-fold radiosensitisation, similar to that obtained when MCF-12A cells were pre-treated with a cocktail of TAK-165 and NVP-BEZ235 (Tables 3.3 and 3.5).

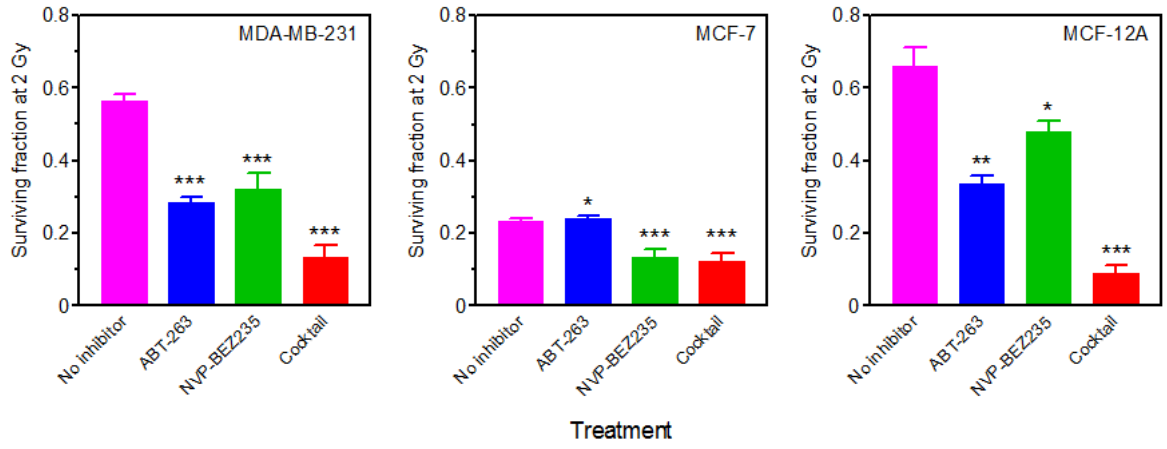

Figure 3.6: Clonogenic cell survival at 2 Gy (SF_2) for 3 human breast cell lines after irradiation with X-rays: MDA-MB-231, MCF-7, and MCF-12A cells were irradiated without or in the presence of ABT-263 (Bcl-2 inhibitor: 0.03-10 000 nM) and NVP-BEZ235 (dual inhibitor of PI3K and mTOR: 0.6-70 nM), either administered singly or in combination. Bars represent the mean surviving fraction ± SEM from three independent experiments. In comparison with SF_2 without inhibitors: *$P > 0.05$; **$0.005 \leq P \leq 0.05$; ***$P < 0.005$.

Table 3.5: Summary of radiosensitivity and dose modifying data for three human breast cell lines treated with inhibitors ABT-263 (for Bcl-2) and NVP-BEZ235 (for PI3K and mTOR).

Cell line	Treatment	SF_2*	MF_2[#]
MDA-MB-231	2 Gy	0.57 ± 0.02	
	2 Gy + ABT-263	0.29 ± 0.02	1.96 ± 0.15
	2 Gy + NVP-BEZ235	0.32 ± 0.04	1.78 ± 0.23
	2 Gy + NVP-BEZ235 + ABT-263	0.13 ± 0.05	4.39 ± 1.69
MCF-7	2 Gy	0.23 ± 0.01	
	2 Gy + ABT-263	0.24 ± 0.01	0.96 ± 0.06
	2 Gy + NVP-BEZ235	0.14 ± 0.02	1.64 ± 0.25
	2 Gy + NVP-BEZ235 + ABT-263	0.12 ± 0.02	1.92 ± 0.33
MCF-12A	2 Gy	0.63 ± 0.05	
	2 Gy + ABT-263	0.34 ± 0.03	1.85 ± 0.22
	2 Gy + NVP-BEZ235	0.48 ± 0.03	1.31 ± 0.13
	2 Gy + NVP-BEZ235 + ABT-263	0.09 ± 0.02	7.00 ± 1.65

SF_2 and MF_2 denote the surviving fraction and radiation modifying factor at 2 Gy, respectively. *Mean ± SEM. [#]Mean ± error: errors were calculated using appropriate error propagation formulae.

3.7. Modulation of Radiosensitivity by ABT-263 and NVP-BEZ235 at 6 Gy

To assess whether blocking the activities of PI3K, mTOR and Bcl-2, with specific inhibitors results in changes in cellular radiosensitivity at relatively large fractional radiation absorbed doses, cell cultures were treated with NVP-BEZ235 (dual inhibitor of PI3K and mTOR), ABT-263 (inhibitor of Bcl-2), or a combination of both inhibitors, and immediately irradiated to 6 Gy. In MDA-MB-231, MCF-7 and MCF-12A cell lines, inhibition of Bcl-2 with ABT-263 alone led to a significant radiosensitisation (Figure 3.7). The radiation modifying factors for the cell lines were similar and emerged as 4.57 ± 1.02, 4.34 ± 0.57 and 4.57 ± 1.16, respectively (Table 3.6). While NVP-BEZ235 significantly radiosensitised MCF-12A (MF_6 = 2.62 ± 0.93) and MCF-7 (MF_6 = 12.00 ± 4.24) cells, its radiomodulatory effect on MDA-MB-231 cells was minimal (Figure 3.7). Pre-treatment of cell cultures with a cocktail of ABT-263 and NVP-BEZ235 yielded a very large radiosensitisation of ~14-fold in the MCF-12A and MDA-MB-231 cell lines, while a 2-fold radiosensitisation was seen in the MCF-7 cell line (Figure 3.7 and Table 3.6).

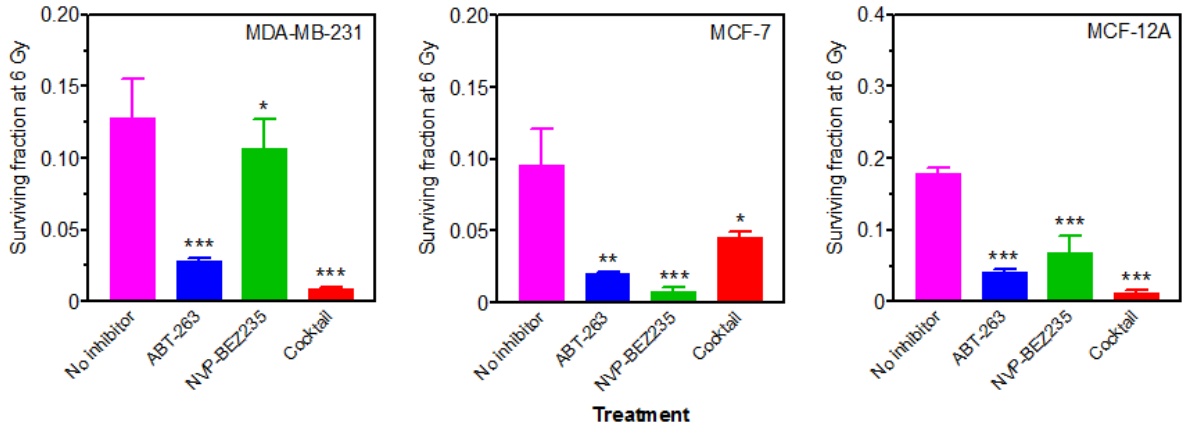

Figure 3.7: Clonogenic cell survival at 6 Gy (SF_6) for 3 human breast cell lines after irradiation with X-rays: MDA-MB-231, MCF-7, and MCF-12A cells were irradiated without or in the presence of ABT-263 (Bcl-2 inhibitor: 0.03-10 000 nM) and NVP-BEZ235 (dual inhibitor of PI3K and mTOR: 0.6-70 nM), either administered singly or in combination. Bars represent the mean surviving fraction ± SEM from three independent experiments. In comparison with SF_6 without inhibitors: *P > 0.05; **0.005 ≤ P ≤ 0.05; ***P < 0.005.

Table 3.6: Summary of radiosensitivity and dose modifying data for three human breast cell lines treated with inhibitors ABT-263 (for Bcl-2) and NVP-BEZ235 (for PI3K and mTOR).

Cell line	Treatment	SF_6*	MF_6#
MDA-MB-231	6 Gy	0.128 ± 0.027	
	6 Gy + ABT-263	0.028 ± 0.002	4.57 ± 1.02
	6 Gy + NVP-BEZ235	0.107 ± 0.020	1.20 ± 0.34
	6 Gy + NVP-BEZ235 + ABT-263	0.009 ± 0.001	14.22 ± 3.39
MCF-7	6 Gy	0.096 ± 0.024	
	6 Gy + ABT-263	0.021 ± 0.001	4.57 ± 1.16
	6 Gy + NVP-BEZ235	0.008 ± 0.002	12.00 ± 4.24
	6 Gy + NVP-BEZ235 + ABT-263	0.045 ± 0.004	2.13 ± 0.57
MCF-12A	6 Gy	0.178 ± 0.009	
	6 Gy + ABT-263	0.041 ± 0.005	4.34 ± 0.57
	6 Gy + NVP-BEZ235	0.068 ± 0.024	2.62 ± 0.93
	6 Gy + NVP-BEZ235 + ABT-263	0.013 ± 0.005	13.69 ± 5.31

SF_6 and MF_6 denote the surviving fraction and radiation modifying factor at 6 Gy, respectively. *Mean ± SEM. #Mean ± error: errors were calculated using appropriate error propagation formulae.

3.8. Summary of Radiomodulation by Specific Inhibitors

To facilitate comparison, radiation modifying factors were plotted against inhibitor pre-treatment for the MDA-MB-231, MCF-7 and MCF-12A cells (Figure 3.8). When cells were pre-treated with cocktails of TAK-165 and NVP-BEZ235 or ABT-263 and NVP-BEZ235, followed by a 2-Gy irradiation, modifying factors of ~4.0–8.0 were observed in the MCF-12A and MDA-MB-231 cell lines, which are low expressers of HER2, ER negative, PR negative, and PI3K wild-type (Figure 3.8A). This indicates that these cocktails might be beneficial in the management of triple-negative breast cancers. MCF-7 cells which are also low expressers of HER2, but ER positive, PR positive, and PI3K mutated exhibited corresponding radiation modifying factors of ~2.0–3.0 for these treatments. Interestingly, a modifying factor of 12.0 was obtained in MCF-7 cells when pre-treated with only NVP-BEZ235 and irradiated to 6 Gy (Figure 3.8B). While both inhibitor cocktails yielded only about 2-fold radiosensitisation in MCF-7 cells at the higher radiation absorbed dose, large modifying factors emerged in the MDA-MB-231 and MCF-12A cells when treated with the NVP-BEZ235/ABT-263 cocktail. This suggests that inhibition of PI3K and mTOR, or in conjunction with inhibition of Bcl-2 may serve as a means to enhance the response of cancers to large fractions of radiation dose.

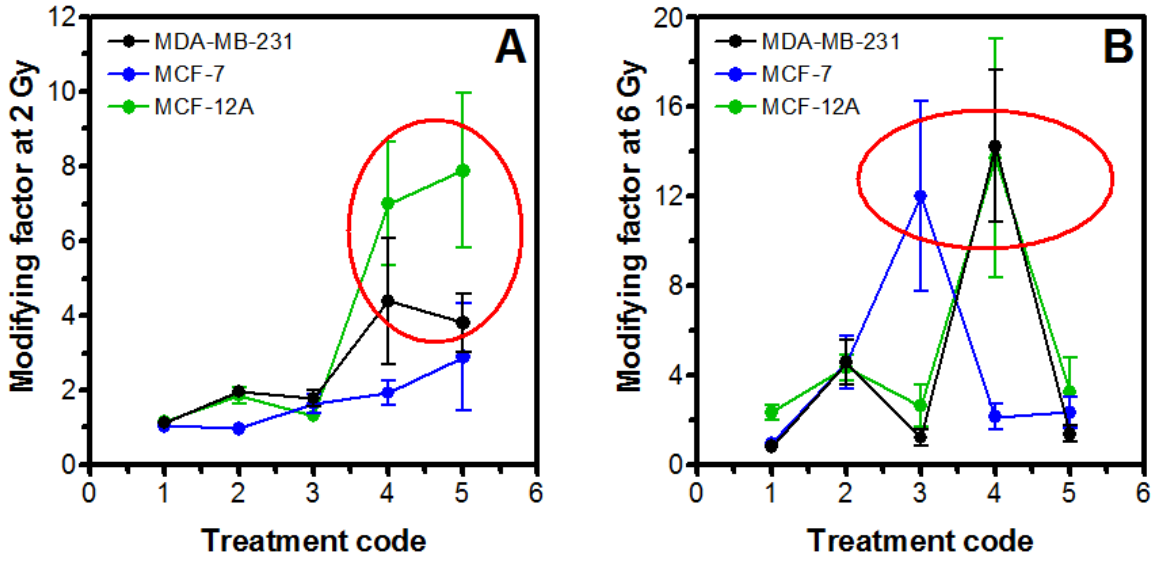

Figure 3.8: Summary of radiomodulation by specific inhibitors in three human breast cell lines (MDA-MB-231, MCF-7 and MCF-12A) on the basis of radiation modifying factor derived from clonogenic cell survival. Treatment codes are denoted as follows: 1 (TAK-165); 2 (ABT-263); 3 (NVP-BEZ235); 4 (ABT-263 + NVP-BEZ235); and 5 (TAK-165 + NVP-BEZ235). Red ovals indicate modifying factors of >4.0 (A) and >12.0 (B).

3.9. Inhibitor Interaction

The data in Figure 3.9 represent median-effect plots for MDA-MB-231, MCF-7 and MCF-12A cells, treated with NVP-BEZ235, TAK-165 and ABT-263. Inhibitor toxicity 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, and the coefficient m is an indicator of the shape of the inhibitor concentration-effect relationship, D_m is the median-effect concentration of inhibitor, and D is the concentration of inhibitor (Chou 2006). Although differences in the shapes of inhibitor concentration-effect curves as presented in Figure 3.3 may not be visually obvious, doubling the inhibitor concentration should lead to less than 50% increase in effect if the response curve follows a hyperbolic shape. The corresponding increase in the case of a flat-sigmoidal response is much less than for the hyperbolic response. On the other hand, if doubling the inhibitor concentration gives more than a 2-fold increase in effect, the response curve is classified as sigmoidal. Mathematically, the shape parameter m = 1, >1, and <1 for hyperbolic, sigmoidal, and flat-sigmoidal inhibitor concentration-effect curves, respectively. Table 3.7 summarises the fitted parameters for each inhibitor in each cell line. For NVP-BEZ235 and TAK-165 treatment, m–values ranged from 1.40 ± 0.12 to 2.18 ± 0.21 indicating sigmoidal inhibitor concentration-effect curves. As such, the corresponding D_m–values were relatively low and emerged as 4.90 ± 0.18 to 25.36 ± 0.72 nM. On the other hand, ABT-263 yielded flat-sigmoidal or hyperbolic concentration-effect curves with m–values ranging from 0.75 ± 0.10 to 1.10 ± 0.10. This is reflected in the correspondingly large D_m–values of 172.59 ± 1.58 to 308.51 ± 1.41 nM. In all cases, the correlation coefficients were high and greater than or equal to 0.93, signifying a

strong conformity of the data to the mass-action law (Chou 2006). For inhibitors with sigmoidal concentration-effect curves, D_m–values were comparable with EC_{50}-values (Table 3.2). In contrast, they were found to be significantly larger than the corresponding EC_{50}-values following ABT-263 treatment which gave either flat-sigmoidal or hyperbolic concentration-effect curves.

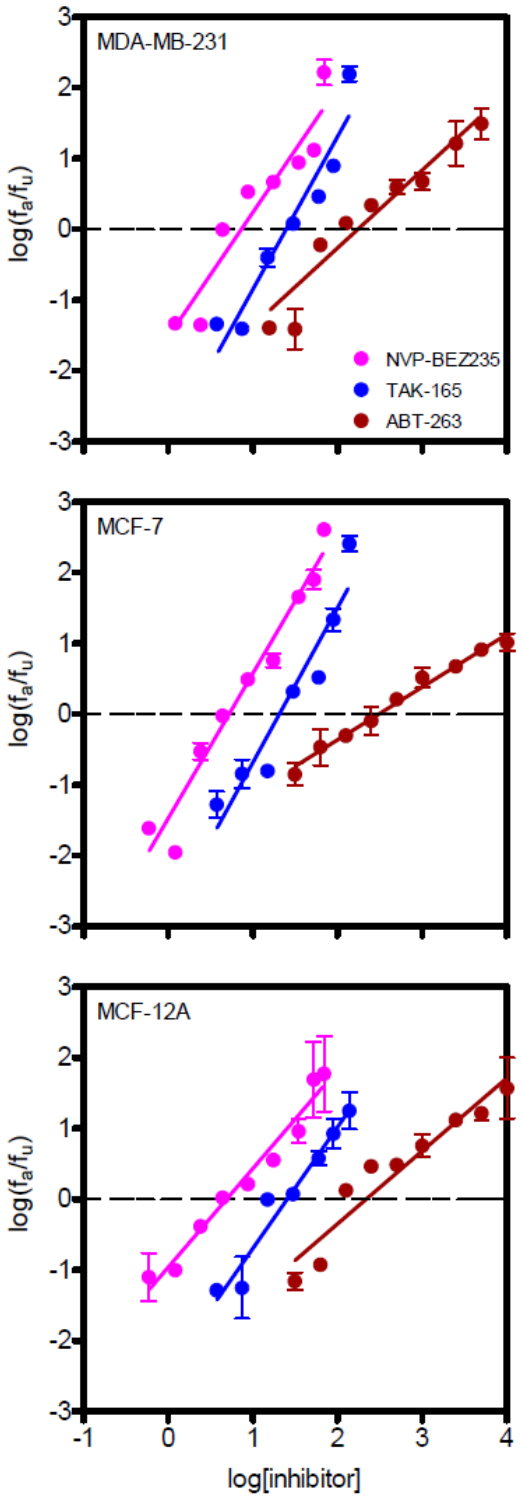

Figure 3.9: Median-effect plots for 3 human breast cell lines, treated with NVP-BEZ235, TAK-165 and ABT-263, from toxicity data presented in Figure 3.3. Transformed data were fitted to the function: $log(f_a/f_u) = m \times log(D) - m \times log(D_m)$, where f_a and f_u are the affected and unaffected fractions of cells, respectively, and the coefficient m is an indicator of the shape of the inhibitor concentration-effect relationship (m = 1, >1, and <1 indicate hyperbolic, sigmoidal, and flat-sigmoidal inhibitor concentration-effect curves, respectively), D_m is the median-effect concentration of inhibitor, and D is the concentration of inhibitor (Chou 2006). Horizontal dotted lines are the median-effect axes.

Table 3.7: Summary of parameters of median-effect plots for HER2 inhibitor (TAK-165), PI3K and mTOR inhibitor (NVP-BEZ235), and Bcl-2 inhibitor (ABT-263) in 3 human breast cell lines (MDA-MB-231, MCF-7 and MCF-12A).

Cell line	Treatment	*m*	D_m (nM)	Shape of concentration-effect curve
MDA-MB-231	TAK-165	2.16 ± 0.23	24.70 ± 0.73	sigmoidal
	NVP-BEZ235	1.76 ± 0.16	7.29 ± 0.27	sigmoidal
	ABT-263	1.10 ± 0.10	172.59 ± 1.58	hyperbolic
MCF-7	TAK-165	2.18 ± 0.21	20.50 ± 0.60	sigmoidal
	NVP-BEZ235	2.05 ± 0.11	5.21 ± 0.13	sigmoidal
	ABT-263	0.75 ± 0.05	308.51 ± 1.41	flat-sigmoidal
MCF-12A	TAK-165	1.71 ± 0.18	25.36 ± 0.73	sigmoidal
	NVP-BEZ235	1.40 ± 0.12	4.90 ± 0.18	sigmoidal
	ABT-263	1.03 ± 0.09	218.36 ± 1.82	hyperbolic

To interrogate any potential mode of interaction between NVP-BEZ235 and TAK-165 or NVP-BEZ235 and ABT-263, combination indices (CI) were estimated for each cocktail and the fitted parameters listed in Tables 3.2 and 3.7 according to the equation:

$$CI = \frac{D_1}{\left\{D_{m1}\times\left(\frac{f_{a1}}{1-f_{a1}}\right)^{\frac{1}{m_1}}\right\}} + \frac{D_2}{\left\{D_{m2}\times\left(\frac{f_{a2}}{1-f_{a2}}\right)^{\frac{1}{m_2}}\right\}} \quad (5),$$

where D_1 is the concentration of NVP-BEZ235 and D_2 is the concentration of TAK-165 or ABT-263. m_1 and m_2 are the respective shape parameters. The corresponding median-effect concentrations are denoted as D_{m1} and D_{m2}, f_{a1} and f_{a2} and given as: (1 – *SF*, as defined in equation (2)). For each cocktail and in all cell lines, CI-values were significantly less than 1.0 and ranged from 0.20 – 0.74 (Table 3.8), indicating synergism for each inhibitor combination at the concentrations used.

Table 3.8: Summary of combination indices for PI3K and mTOR inhibitor, NVP-BEZ235 (Agent 1), when used at different concentrations (D_1, 0.5D_1 and 0.25D_1) with HER2 inhibitor (TAK-165) or Bcl-2 inhibitor (ABT-263) in 3 human breast cell lines (MDA-MB-231, MCF-7 and MCF-12A). TAK-165 and ABT-263 are denoted as Agent 2 and are at concentrations of 30 and 97 nM, respectively. D_1 = 17 nM.

Cell line	Agent 2	at D_1	at 0.5D_1	at 0.25D_1
MDA-MB-231	TAK-165	0.69	0.37	0.19
	ABT-263	0.74	0.41	0.24
MCF-7	TAK-165	0.61	0.52	0.48
	ABT-263	0.28	0.20	0.15
MCF-12A	TAK-165	0.22	0.19	0.17
	ABT-263	0.20	0.18	0.16

3.10. Flow Cytometry as a Potential Surrogate for the Colony Assay

To test whether flow cytometry could be employed as a rapid screening tool for identifying target inhibitors as potential radiosensitisers, MDA-MB-231 cells pre-treated with NVP-BEZ235 and irradiated to 2 Gy were analysed using flow cytometry techniques. Gated cell populations as shown in Figure 3.10A were analysed for their incorporation of anti-Bax (6D149) PE (Figure 3.10B). A single experiment was performed. The mean fluorescence intensities emerged as 1711 and 1510 for the unirradiated cultures in the absence and presence of NVP-BEZ235, respectively. The corresponding values for the irradiated cell cultures were found to be 2131 and 2468. Based on these fluorescence intensities and Equation (4), a radiation modifying factor of 2.28 was derived.

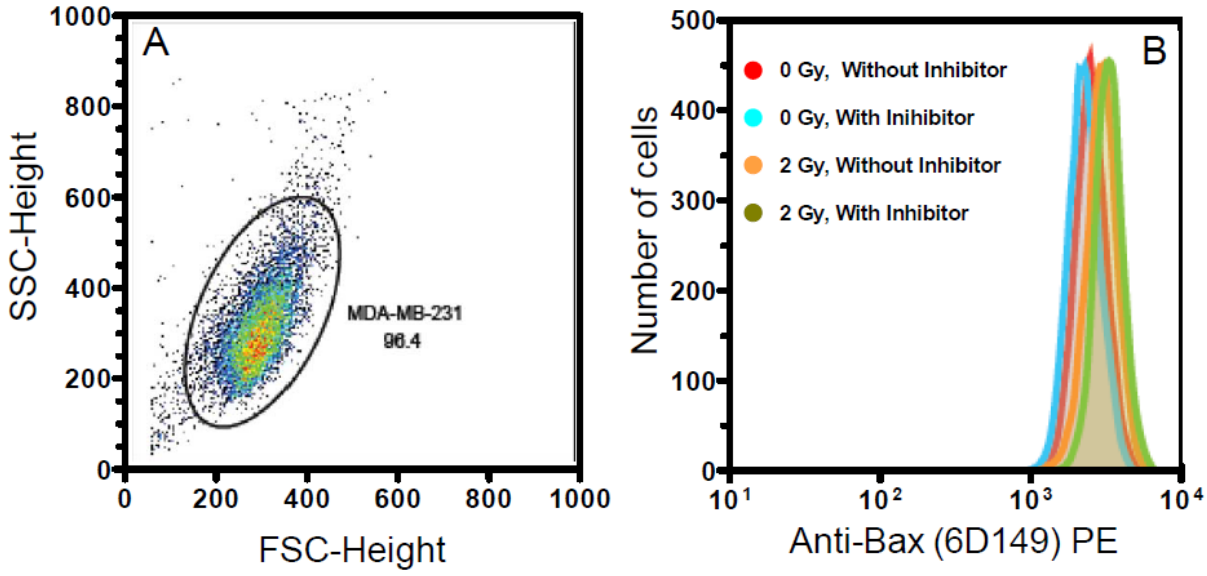

Figure 3.10: Flow cytometry data: (A) Gated representative dot-plot for MDA-MB-231 cells treated with NVP-BEZ235 and subsequently irradiated to 2 Gy of X-rays; (B) Distribution of cellular incorporation of anti-Bax (6D149) PE by MDA-MB-231 cells, shown as flow cytometry–generated histograms of cellular fluorescence intensity.

Chapter four: Discussion

4.1. Inherent Radiosensitivity

Figure 3.2 shows the cell survival curves for the MCF-7, MDA-MB-231 and MCF-12A cell lines used in this study. The MDA-MB-231 and the MCF-12A exhibit similar radiation responses at low doses, but marginally differ in radiosensitivity at higher doses. On the other hand, the MCF-7 cell line emerged as radiosensitive. The observed differences in radiosensitivity cannot be attributed to disparities in phosphatase and tensin homolog (PTEN) which impedes PI3K/mTOR pathway activity, thereby sensitising cells to cytotoxic insult. PTEN is wildly expressed in all cell lines used here (Carlson et al., 2010). While the more radioresistant MDA-MB-231 and MCF-12A cell lines express wild-type PI3K, the radiosensitive MCF-7 cells are PI3K mutant (Carlson et al., 2010; Vasudevan et al., 2009). This may explain the relatively high radiosensitivity in the latter cell line. 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. Other genes that play an important role in radiosensitivity are the ataxia-telangiectasia mutated (ATM) and tumour suppressor gene (p53). The significantly higher radiosensitivity seen in the MCF-7 cells relative to their MDA-MB-231 and MCF-12A counterparts cannot also be attributed to disparities in their ATM and p53 status. While ATM is not mutated in any of the cell lines (Vořechovský et al., 1996; Kenny et al., 2007; Karimi-Busheri et al., 2010), the MDA-MB-231 cell carries a mutated p53 (Kenny et al., 2007; Haldar et al., 1994; O'Connor et al., 1997; Liu et al., 2001). p53 wild-type cell lines tend to be the most radioresistant, with p53 dysfunctional cell lines emerging as the most radiosensitive,

while those with mutated p53 exhibit intermediate radiosensitivity (Michie et al., 2003; Serafin et al., 2003; Akudugu et al., 2004a,b). Therefore, the marked radiosensitivity exhibited by the MCF-7 cells is most likely mediated by its mutated PI3K or other effectors downstream of the ATM/p53 pathway.

4.2. Cytotoxicity of Inhibitors of HER2, PI3K, mTOR and Bcl-2

On the basis of clonogenic cell survival, it is demonstrated for MDA-MB-231, MCF-7 and MCF-12A cells that cytotoxic effects of TAK-165 (HER2 inhibitor), NVP-BEZ235 (PI3K and mTOR inhibitor), and ABT-263 (Bcl-2 inhibitor) are concentration-dependent (Figure 3.3). For NVP-BEZ235 treatment, EC_{50}-values of 4.15–5.01 nM were obtained for all cell lines (Table 3.2) and are comparable with PI3K/mTOR inhibition data reported elsewhere for MDA-MB-231 and the HER2 amplified breast cancer cell lines BT474 and MDA-MB-175-VII (Vasudevan et al., 2009; Carlson et al., 2010). In contrast, significantly higher NVP-BEZ235 concentrations for 50% growth inhibition (IC_{50}) ranging from 6–93 nM have emerged for many other breast cancer cell lines, with HER2 amplified cell lines tending to be more sensitive (Brachmann et al., 2009; Carlson et al., 2010). Interestingly, it was demonstrated that doses of NVP-BEZ235 for 50% cell killing (LD_{50}) as high as ~87 and >20 000 nM were reported for MDA-MB-231 and MCF-7 cells, respectively (Brachmann et al., 2009). The disparity in toxicity noted here can be explained by the fact that cell growth and metabolic assays, as employed by Brachmann and colleagues, which extend over relatively short periods, often tend to overestimate cell survival following cytotoxic treatment. Although cell growth and metabolic assays can give general cytotoxicity trends, they are snapshots of cellular demise and may not adequately

reflect residual cellular reproductive integrity as measured by the colony forming assay (Weisenthal et al., 1983); and the resulting LD_{50}-values can be unrealistically high. The similarity in NVP-BEZ235 toxicity in MDA-MB-231, MCF-7 and MCF-12A cells seems to suggest that NVP-BEZ235-induced cell death cannot be attributed to ER-mediation of PI3K activity, as MCF-7 cells are known to exhibit ER-dependent PI3K activity while MDA-MB-231 do not (Pozo-Guisado et al., 2002). MCF-12A cells, as their MDA-MB-231 counterparts, are ER negative (Subik et al., 2010), and should also not be expected to show an ER-mediated PI3K activity.

TAK-165 was generally less potent than NVP-BEZ235, with an EC_{50}–value of ~20–28 nM (Figure 3.3 and Table 3.2). All cell lines minimally express HER2 (Rusnak et al., 2001; Konecny et al., 2006), and the extensive concentration-dependent TAK-165 induced cytotoxicity observed here cannot be solely attributed to HER2 alone. This finding is likely due to targeting of residual HER2, as well as other critical cellular factors. TAK-165 is a potent inhibitor of EGFR and the cell division cycle protein 2 homolog (Cdc2), which play a crucial role in cell-cycle progression. Perturbation of their activity with TAK-165 can lead to cellular demise during cell division. However, the narrow range of toxicity seen here cannot be explained by differences in Cdc2 and EGFR activity. Cdc2 activity in MDA-MB-231 is intrinsically higher than that in MCF-7 (Pozo-Guisado et al., 2002), indicating a stronger dependence of the former cell line on Cdc2 activity for cell cycle progression. Inhibiting Cdc2 and the residual HER2 activity with TAK-165 should, therefore, be expected to be significantly more toxic in MDA-MB-231 cells than in their MCF-7 counterparts. Also, the MDA-MB-231 and MCF-12A cell lines are higher expressers of EGFR than the MCF-7 cell line (Konecny et al., 2006; Subik et al.,

2010). Therefore, larger disparities in cytotoxicities would have emerged had EGFR been the critical target of TAK-165. It is also worth noting that the TAK-165 concentrations used in the current study (0.9 – 135 nM) are much lower than those that are typically required to significantly suppress Cdc2 and EGFR activity (Nagasawa et al., 2006; Production Technical Information). The IC_{50} concentrations of TAK-165 for Cdc2 and EGFR are 200 and >25 000 nM, respectively. At such high concentrations, TAK-165 has been shown to be ~4-fold more inhibitory than demonstrated here in a variety of cancer cell lines of bladder, kidney and prostate origin in which HER2 expression ranged from weak to high (Nagasawa et al., 2006).

ABT-263 emerged as the least toxic inhibitor, with EC_{50}-values ranging from ~97 to 232 nM (Figure 3.3 and Table 3.2). These findings are, however, at variance with the significantly higher EC_{50}–value of ~1 µM reported elsewhere for a similar panel cell lines (Brosseau et al., 2012). The relatively high EC_{50}-value observed by Brosseau and colleagues is likely due to the use of a cell growth assay for the assessment of cell viability (Brosseau et al., 2012). Cell viability assays often overestimate clonogenic cell survival, as not all metabolically active cells are capable of forming viable colonies in the longer term (Weisenthal et al., 1983). The rank order of EC_{50}-values as listed in Table 3.2 is: MCF-7 > MCF-12A > MDA-MB-231, and correlates with Bcl-2 expression of the cell lines. Bcl-2 expression in the MCF-7 cell line is higher than that in the MDA-MB-231 cell line, and may be as high as 4.5-fold (Kandouz et al., 1996; Brosseau et al., 2012). It is, therefore, not surprising that a higher concentration of ABT-263 is required for a 50% cell killing in the MCF-7 cell line compared with that for the MDA-MB-231 cell line (Table 3.2).

4.3. Radiomodulation by TAK-165 and NVP-BEZ235

In this presentation, it is demonstrated that pre-treatment of all cell lines with TAK-165 yielded only 4–15% radiosensitisation at 2 Gy (Figure 3.4 and Table 3.3). This is not unexpected, as the cell lines express very low levels of HER2 (Konecny et al., 2006; Subik et al., 2010). The role of HER2 perturbation in radiosensitivity modulation should, therefore, be minimal. This is consistent with data reported elsewhere indicating that trastuzumab (a potent HER2 inhibitor) had little or no effect on radiation-induced cell death in breast cancer cell lines that show low to no expression of HER2 (Liang et al., 2003). However, when cells were pre-treated with the dual inhibitor of PI3K and mTOR, moderate radiosensitisation was seen with the least radiomodulatory effect emerging in the MCF-12A cell line (Table 3.3). These data cannot be explained in terms of PI3K status, as NVP-BEZ235 induced radiosensitisation was also noted in the PI3K mutant MCF-7 cell line. The marked disparity between the less than 2-fold radiosensitisation observed here and the ~4-fold radiosensitisation reported elsewhere (Kuger et al., 2014), might be due to differences in experiment design. The NVP-BEZ235 concentration of 100 nM used by Kuger and colleagues was ~6 times that used in the present study (Kuger et al., 2014), and corresponds to NVP-BEZ235 doses at which cell survival levels should become very low (as depicted in Figure 3.3) 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). Also, delayed trypsinisation and re-plating of cells after drug and radiation treatment can significantly modify the extent to which the drugs modulate radiosensitivity.

With the exception of the MCF-12A cell line, pre-treatment of cell cultures with TAK-165 did not yield a measurable radiosensitisation at 6 Gy (Figure 3.5 and Table 3.4). The radiosensitisation of MCF-12A cells at a higher radiation dose cannot be explained by differences in HER2 expression as it is the MCF-7 cell line that exhibits marginally higher expression of this protein (Horwitz et al., 1975; Rusnak et al., 2001), and radiosensitisation resulting from HER2 inhibition would have been higher in the latter cell line. No added benefit was apparent when MCF-12A cells were exposed to a TAK-165/NVP-BEZ235 cocktail, suggesting that the effect of targeting HER2 and PI3K/mTOR either singly or concurrently in tumours that are low expressers of HER2 may be minimal. Neither pre-treatment of MDA-MB-231 cells with NVP-BEZ235 nor a cocktail of TAK-165 and NVP-BEZ235 resulted in a significant radiosensitisation. Although MDA-MB-231 and MCF-12A cells are PI3K wild-type, they are known to be ER and PR negative (Subik et al., 2010). This indicates that tumours with signalling features similar to those of these cell lines might not benefit from adjuvant hypofractionated radiotherapy with inhibitors of HER2, PI3K and mTOR. However, inhibition of PI3K and mTOR in the PI3K-mutated MCF-7 cell line resulted in a higher than 8-fold radiosensitisation at 6 Gy (Figure 3.5 and Table 3.4). The high level of radiosensitisation observed here is at variance with the relatively low sensitisation noted when another dual inhibitor of PI3K and mTOR (PI-103) was used in the same cell line elsewhere (No et al., 2009). This is likely due to the fact that NVP-BEZ235 appears to be a better radiosensitiser than PI-103. When umbilical venous endothelial cells and bladder and laryngeal cancer cells were pre-treated with the two inhibitors, the former consistently showed higher levels of radiosensitisation at 6 Gy, similar to those observed here (Prevo et al., 2008; Fokas et al., 2012). The marked radiosensitisation demonstrated for inhibition of PI3K and

mTOR in the MCF-7 cell line, and elsewhere (Prevo et al., 2008; Fokas et al., 2012; Kuger et al., 2014) cannot be attributed to differences in PI3K status. Using two PI3K wild-type colon cancer cell lines and their mutant derivatives, Prevo and colleagues demonstrated no significant difference in radiosensitisation by the PI3K and mTOR inhibitor, PI-103 (Prevo et al., 2008). However, a predominantly common feature of the MCF-7 cell line and those used elsewhere (Prevo et al., 2008; Fokas et al., 2012) is that they are oestrogen receptor (ER) positive (Horwitz et al., 1975; Shen et al., 2006; Toth et al., 2008). The high levels of radiosensitisation seen in these cell lines following NVP-BEZ235 treatment may be attributed to inhibition of an enhanced ER-mediation of PI3K activity. ER-dependent activation of PI3K has been demonstrated in MCF-7 cells (Pozo-Guisado et al., 2004). Upregulation of PI3K activity would be expected to render cells more susceptible to radiation-induced death when such activity is inhibited. These data strongly suggest that inhibition of PI3K and mTOR in tumours that are devoid of HER2, PR and ER activity (e.g. triple-negative breast cancers), as well as in those that are devoid of only HER2, might sensitise them to radiotherapy regimens that employ fractional doses of the order of 6 Gy. Furthermore, the results do not demonstrate a benefit for concomitant inhibition of residual HER2. HER2 targeting would, therefore, not be necessary and systemic toxicity that otherwise arises from such treatment could potentially be avoided.

Of specific note is the marked radiosensitisation observed in the MCF-12A cell line at 2 Gy when cells were pre-treated with the inhibitor cocktail (Table 3.3). It is not likely that the inhibitory activity of the TAK-165/NVP-BEZ235 is directed towards ER and PR, as suggested elsewhere (Pozo-Guisado et al., 2002; 2004), since radiosensitisation was found to be higher in the ER-negative and PR-negative cell

lines (MDA-MB-231 and MCF-12A) than the ER and PR overexpressing MCF-7 cell line (Konecny et al., 2006; Subik et al., 2010). These findings seem to suggest that the activity of the TAK-165/NVP-BEZ235 cocktail may be specific for other targets, such as EGFR, which is often overexpressed by cells with compromised activity of HER2, ER and PR (Siziopikou and Cobleigh, 2007). In fact, the rank order of EGFR expression in the three cell lines is: MCF-7 < MDA-MB-231 < MCF-12A (Konecny et al., 2006; Subik et al., 2010), and is strongly correlated with the extent of radiation dose modification (Table 3.3). A similar trend is apparent at 6 Gy, although the level of radiosensitisation was much lower (Table 3.4). Therefore, EGFR is a likely target for a TAK-165/NVP-BEZ235 cocktail adjuvant therapy at low to moderate radiation absorbed doses. This notion can be supported by the observation that simultaneous inhibition of EGFR and PI3K can lead to significant radiosensitisation of breast cancer cells (Li et al., 2012). In spite of the promising observations, concomitant use of TAK-165 and NVP-BEZ235, either singly or as a cocktail, and radiation could potentially elevate normal tissue toxicity and requires further evaluation.

4.4. Radiomodulation by NVP-BEZ235 and ABT-263

While inhibition of Bcl-2 with ABT-263 had no effect on the radiosensitivity of the MCF-7 cell line at 2 Gy, moderate radiosensitisation was seen in the MDA-MB-231 and MCF-12A cells (Figure 3.6 and Table 3.5). These data are consistent with other studies demonstrating that Bcl-2 inhibitors are potent in Herceptin resistant breast cells (Crawford and Nahta, 2011), and can radiosensitise small-cell lung carcinomas (Loriot et al., 2014). The absence of a radiomodulatory effect in the MCF-7 cell line

might be due to the use of a suboptimal concentration of ~0.4 × EC_{50}, given that these cells express as high as 4.5-fold Bcl-2 in comparison with the MDA-MB-231 cells (Kandouz et al., 1996; Brosseau et al., 2012).

As demonstrated for the TAK-165/NVP-BEZ235 cocktail, a strong correlation is also evident between radiosensitisation by the ABT-263/NVP-BEZ235 cocktail and EGFR expression. The rank order of radiation modifying factors at 2 Gy is: MCF-12A > MDA-MB-231 > MCF-7 (Table 3.5), and is consistent with the order of EGFR expression (Konecny et al., 2006; Subik et al., 2010). As indicated earlier, the low radiosensitisation seen in the MCF-7 cells may be attributable to an inadequate inhibition of Bcl-2 (concentration of ~0.4 × EC_{50} used).

At 6 Gy, however, no difference exists in the radiomodulatory effect of ABT-263, with all cell lines showing a ~4-fold radiosensitisation (Figure 3.7 and Table 3.6). This cannot be explained by the marked differences in Bcl-2 expression (Kandouz et al., 1996; Brosseau et al., 2012), and seems to suggest that the extent of Bcl-2 expression may play an important role in ABT-263 induced radiosensitisation only at low radiation doses. The large modifying factors seen in the MDA-MB-231 and MCF-12A cell lines when treated with an ABT-263/NVP-BEZ235 cocktail can be attributed to the interrelationship between Bcl-2 and PI3K activities. There is evidence to suggest that inhibition of PI3K blocks Bcl-2 expression (Jin et al., 2004). A high level of synergy has also been demonstrated for concomitant inhibition of PI3K and Bcl-2 (Rahmani et al., 2012). Therefore, it can be expected that the concurrent inhibition of PI3K and Bcl-2 would lead to significant radiosensitisation even if suboptimal concentrations of the Bcl-2 inhibitor are used. It is also worth noting that the rank

order in radiosensitisation observed at 2 Gy for the ABT-263/NVP-BEZ235 cocktail appeared to be retained at 6 Gy, although the corresponding levels of radiomodulation were much higher (Tables 3.5 and 3.6). This further supports the suggestion that the activity of this inhibitor cocktail may be mediated by EGFR. It may be recalled that EGFR expression is low in the ER- and PR-positive MCF-7 cell line (Horwitz et al., 1975; Rusnak et al., 2001), and the ER- and PR-negative cell lines (MDA-MB-231 and MCF-12A) are high expressers of EGFR (Konecny et al., 2006; Subik., et al., 2010). An interesting observation is that both NVP-BEZ235/TAK-165 and NVP-BEZ235/ABT-263 cocktails yielded significantly lower levels of radiosensitisation in the MCF-7 cells at 6 Gy than when cells were pre-treated with NVP-BEZ235 and ABT-263 alone (Tables 3.4 and 3.6). This does not corroborate the synergy demonstrated by the components of these cocktails in Table 3.8. However, this finding is attributed to the fact that radiosensitisers can act as antagonists against each other, and their net radiosensitising effects as cocktails often fall below the sum of the individual effects (Millar et al., 1981). It is possible that in the MCF-7 cell system, the presence of either TAK-165 or ABT-263 negates the high radiosensitising effect of NVP-BEZ235. Nonetheless, the data presented here suggest that concomitantly targeting Bcl-2, PI3K and mTOR may be clinically beneficial in some cancers. Further evaluation of these targets in a broader panel of cell lines is desirable.

4.5. Inhibitor Interaction

The combination indices (CI) for the NVP-BEZ235/TAK-165 and NVP-BEZ235/ABT-263 cocktails for all cell lines ranged between 0.20 and 0.74, indicating synergism for each inhibitor combination (Chou 2006). This might explain the more than additive enhancement of radiosensitivity seen in all cell lines. The rank order of synergism for both cocktails in the cell lines is: MCF-12A > MCF-7 > MDA-MB-231, suggesting that the unique cellular features might play a role in inhibitor cocktail cytotoxicity. According to the classification by Chou (Chou 2006), a strong synergism (CI range: 0.1–0.3) emerged in the MCF-12A cell line for both inhibitor cocktails and in the MCF-7 cell line for the NVP-BEZ235/ABT-263 cocktail (Table 3.8, at D_1). This suggests that treatment of tumours with characteristics akin to those of MCF-12A (e.g. triple-negative tumours) with either cocktail may be beneficial. As systemic toxicity is a significant concern in the clinic and lower drug doses are desirable, combination indices were further estimated for each cocktail at reduced NVP-BEZ235 concentrations keeping those of TAK-165 and ABT-263 constant. The CI-values listed in Table 3.8 show an improvement in synergism in all cases. It is, therefore, likely that the radiosensitisation demonstrated here might persist even at low inhibitor concentrations and should be investigated further.

4.6. Flow Cytometry as a Potential Rapid Screening Tool

The clonogenic cell survival assay, although widely accepted as the gold standard for cytotoxicity studies, can be expensive and laborious. In most cases, data may

become available only after 1-2 weeks. Besides, it is difficult and almost impossible to successfully generate colonies from clinically relevant biological material, such as, biopsies. This is one of the biggest challenges encountered in attempting to progress towards personalised cancer management. To partially address this issue, high throughput assays for diagnosing diseases, predicting treatment outcome, and testing the efficacy of new therapeutic agents (e.g. drugs and small molecule inhibitors) needs to be developed. Although flow cytometry has been evaluated for this purpose over the past few decades, most of the efforts have been directed towards haematopoietic malignancies (Ross et al., 1989; Siena et al., 1991; Edwards et al., 2001; Suzuki 2004; Krutzik and Nolan 2006; Krutzik et al., 2008; Bendall et al., 2011; Terwijn et al., 2013; Paiva et al., 2014; Bulian et al., 2014). As such, biological samples have predominantly been from peripheral blood or suspension cultures which are usually not amenable to the clonogenic cell survival assay. High throughput techniques enabling such predictions for solid tumours are not well developed. As a preliminary effort, flow cytometry was used to test whether the radiomodulatory effect of the dual inhibitor of PI3K and mTOR (NVP-BEZ235), as determined via the colony forming assay, can be predicted more rapidly. Radiation modifying factors of 1.78 and 2.28 were obtained for the clonogenic and flow cytometric assays, respectively. The closeness of these factors demonstrates that the concept of potentially replacing the colony forming assay with flow cytometry for screening therapeutic agents of relevance to solid tumours might be viable. This finding may be coincidental, but if validated with a large panel of cell lines the proposed approach may be amenable to clinical samples like tumour biopsies, and can significantly assist in patient staging for therapy.

Chapter five: Conclusions

This study demonstrates that inhibition of PI3K and mTOR, HER2, and Bcl-2, using the respective inhibitors NVP-BEZ235, TAK-165 and ABT-263, either singly or when NVP-BEZ235 is combined with TAK-165 or ABT-263, can significantly radiosensitise human breast cancer cell lines with differing HER2, ER, PR, and EGFR expression levels at 2 Gy. On the basis of clonogenic cell survival, radiosensitisation is more prominent in ER- and PR-negative cells expressing higher levels of EGFR. These findings suggest that a cocktail of NVP-BEZ235 and TAK-165 or NVP-BEZ235 and ABT-263 may have the potential for more effectively targeting triple-negative breast cancer cells.

At 6 Gy, moderate to high radiosensitisation was demonstrated for most inhibitor combinations. The only treatments that did not result in a significant radiosensitisation were: TAK-165 (in MDA-MB-231 and MCF-12A cells), NVP-BEZ235 (in MDA-MB-231 cells) and NVP-BEZ235/TAK-165 cocktail (in MDA-MB-231 cells). In general, these findings show that inhibition of residual HER2, PI3K, mTOR, and Bcl-2, singly or in combination, can enhance the effectiveness of stereotactic radiotherapy of cancers that are devoid of HER2, ER and PR expression.

The radiation modifying effect of inhibitor treatment as measured by flow cytometric assessment of *Bax* expression was comparable with that obtained using the colony forming assay. This suggests that, with some level of validation, flow cytometry can

be used as a rapid and high throughput assay for evaluating clinical samples, such as blood and tumour biopsies, for pretreatment patient staging.

With regards to the NVP-BEZ235/TAK-165 and NVP-BEZ235/ABT-263 cocktails, it was demonstrated that synergism exists for each combination. The MCF-12A cell line which mimics triple-negative cancers more closely showed the strongest synergism for both cocktails. This finding suggests that either combination of inhibitors could potentially be beneficial in the management of triple-negative breast cancer. An interesting observation is that the strong synergy is maintained even at reduced concentration of NVP-BEZ235, indicating that these cocktails could be used at a minimised risk of systemic toxicity.

Future Avenues

Triple-negative breast cancers (TNBC) pose a major challenge to clinicians. This is due to the heterogeneous nature of TNBC and the absence of well-defined molecular targets. This is further exacerbated by the fact that triple-negative breast cancers consist of six subtypes: two basal-like, an immunomodulatory, a mesenchymal, a mesenchymal stem–like, and a luminal androgen receptor. The findings of this study show that a cocktail of specific inhibitors may be an approach in the treatment of TNBC. The results further indicate that radiosensitisation by the different inhibitors, either singly or in combination, varies significantly among the cell lines. The disparities between these cell lines may be due to their different expression of targeted proteins. Using a panel of TNBC cell lines, Lehmann and co-workers pharmacologically targeted prominent signalling pathways and found that sensitivity to targeted therapies was correlated to gene expression signatures of the different TNBC subtypes (Lehmann et al., 2011). They also found that the mesenchymal stem-like (MSL) subtype is enriched in gene expression profiles for the epithelial-mesenchymal transition and growth factor pathways. The MSL subtype is also rich in genes associated with stem cells, such as, the Bcl-2. MSL cells responded favourably to NVP-BEZ235 and Dasatinib (an abl/src inhibitor) (Lehmann et al., 2011). In the study reported here, the MDA-MB-231 cell line is mesenchymal stem-like and the corresponding data for NVP-BEZ235 and ABT-263 combinations are encouraging. So are the data for the MCF-7 and MCF-12A cell lines.

In light of the above, further research avenues involving a larger panel of cell lines representing the different subtypes of TNBC, as well as normal breast cell models, and covering a wider range of gene expression profiles are warranted. These could guide cocktail selection for specific targeted therapies, as well as, assist in the identification of agents that may selectively radiosensitise tumours of each TNBC subtype. In addition to validating the current *in vitro* findings in a larger panel of cell lines, the research should be extended to a preclinical level using an *in vivo* mouse xenograft model. This could be achieved by use of state-of-the-art small animal irradiation platforms with capacity for volumetric assessment and bioluminescence imaging as described elsewhere (Song et al., 2010; Verhaegen et al., 2011; Butterworth et al., 2015).

Specifically, the following should be considered:

- Inhibitor validation at a molecular level using western blotting.
- Assessment of radiomodulatory effects of the inhibitors for full cell survival-dose response curves.
- Evaluation of the impact of a TAK-165 and ABT-263 combination on radiosensitivity.
- Assessment of additional time points for irradiation after inhibitor treatment would also be of significant value in understanding the mode of action of combination therapy.
- Small animal studies, using state-of-the-art irradiation and imaging facilities, as described elsewhere (Song et al., 2010; Verhaegen et al., 2011; Butterworth et al., 2015), would significantly strengthen future studies.

- Assessment of the impact of dose fractionation on radiomodulatory effects of inhibitors, as radiotherapy is seldom given in a single fraction.
- Measurement of DNA damage foci by γH2AX immunofluorescence assay would also assist in understanding the mechanisms underlying the radiomodulatory effect of inhibitors.
- Validation of the finding that flow cytometry might replace the clonogenic cell survival as a rapid high throughput tool for evaluating candidate target inhibitors for therapeutic benefit in a large panel of cell lines, using a Bland-Altman type analysis (Giavarina 2015), would be useful.

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Research Article

A Cocktail of Specific Inhibitors of HER-2, PI3K, and mTOR Radiosensitises Human Breast Cancer Cells

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Abstract

Intrinsic tumour radioresistance limits the benefit of radiotherapy. Targeted treatment modalities that are singly effective for triple-negative breast cancer are lacking, partly due to paucity of relevant targets as they are devoid of the human epidermal growth factor receptor 2 (HER-2), progesterone receptor (PR), and oestrogen receptor (ER); or to resistance to single-target therapies as a consequence of cellular heterogeneity. Concomitant targeting of cell signaling entities other than HER-2, PR and ER may sensitise triple-negative tumours to radiotherapy. In this study, we investigated the effect of an HER-2 inhibitor (TAK-165) and a dual inhibitor of phosphoinositide 3-kinase (PI3K) and mammalian target for rapamycin (mTOR) (NVP-BEZ235) in three human breast cancer cell lines. The potential of simultaneous inhibition of HER-2, PI3K and mTOR with a cocktail of the specific inhibitors TAK-165 and NVP-BEZ235, to radiosensitise human breast cancer cells *in vitro* was examined using the colony forming assay. Combined inhibition of HER-2, PI3K, and mTOR resulted in significant radiosensitisation in all cell lines, independent of HER-2, ER, or PR status. Radiosensitisation was more prominent in ER- and PR-negative cells expressing higher levels of epidermal growth factor receptor (EGFR). These data suggest that a cocktail of TAK-165 and NVP-BEZ235 could potentially be effective in the treatment of triple-negative breast cancer.

Keywords: Triple-negative, breast cancer, targeted therapy, radiosensitisation.

Introduction

Many subtypes of breast cancer exist and exhibit unique characteristics. Different, as well as, specific treatment modalities would be required to benefit all subgroups. Cancers overexpressing the gene encoding the human epidermal growth factor receptor 2 (HER-2) constitute 30% of invasive breast cancers [1]. Approaches for targeting HER-2 are important in the treatment of breast cancers overexpressing HER-2. Although trastuzumab has been approved by the United States Food and Drug Administration (US FDA) for the treatment of HER-2 positive cancers, a significant level

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of resistance to trastuzumab treatment is apparent [2]. Dysregulation of downstream components of the HER-2 signaling pathway have been suggested to be responsible for the observed resistance [2]. Such resistance may also be partly attributable to the heterogeneity in the distribution of target antigen expression in a given cell population, which can lead to the inability to effectively target all cells with toxic levels of therapeutic agents [3]. Another challenge is that some HER-2 positive cancers express a constitutively active truncated form of the protein (p95 HER-2) which does not possess the extracellular domain required for trastuzumab binding [4], and HER-2 targeted treatment may fail.

Also, about one-fifth of women with breast cancer constitutes the subgroup of patients with triple-negative breast cancer [5]. This subtype of breast cancer is known to occur more frequently in young Black and Hispanic women than in young women of other racial or ethnic groups [6]. Triple-negative breast cancers lack expression of HER-2, progesterone receptor (PR), and oestrogen receptor (ER), all of which are promising candidates for targeted therapy. Therefore, patients with triple-negative tumours cannot be treated with endocrine based therapy or therapies targeting HER-2, and treatment outcome in these individuals is relatively poor [7]. To improve the management of triple-negative breast cancer, effective targeting of malignant cells devoid of ER, PR, and HER-2 expression is warranted.

An alternative treatment modality for breast cancer is radiotherapy. Cellular exposure to ionising radiation is known to activate the epidermal growth factor receptor, EGFR [8], and induce phosphoinositide 3-kinase (PI3K), serine-threonine protein kinase (Akt) and mammalian target for rapamycin (mTOR) activity downstream of the EGFR signaling pathway [9]. This signaling promotes cell survival and can lead to treatment resistance. Targeting the HER-2 pathway by inhibiting PI3K has also been shown to result in significant radiosensitisation [10]. Of significance to targeted therapy, triple-negative breast cancers, which are devoid of HER-2 activity, tend to predominantly overexpress EGFR [11]. Therefore, developing therapeutic approaches that concomitantly target EGFR family members and their downstream signaling components might significantly sensitise triple-negative breast cancer cells to radiotherapy and improve treatment outcome.

In the present work, studies were conducted to determine if inhibition of HER-2, PI3K and mTOR can radiosensitise human breast cancer cells. To achieve this, MDA-MB-231, MCF-7 and MCF-12A cells were treated with TAK-165 (an HER-2 inhibitor) and NVP-BEZ235 (a dual inhibitor of PI3K and mTOR) and concomitantly irradiated, and radiation-induced cell death was measured using the colony forming assay. The dependence of radiosensitisation on HER-2, ER and PR expression status and the implications of inhibiting the HER-2/PI3K/mTOR pathway in irradiated breast cancer cell lines are also discussed.

Materials and Methods

Cell lines and culture maintenance

The MDA-MB-231 and MCF-7 human breast tumour cells were derived from pleural effusions taken from women with invasive ductal adenocarcinoma and carcinoma, respectively, and were a gift from Prof S Prince (University of Cape Town, South Africa). They respectively form metastatic xenografts in nude mice in an oestrogen-independent and dependent manner. The immortalised mammary epithelial cell line, MCF-12A, was a gift from Prof AM Engelbrecht (University of Stellenbosch, South Africa). The rationale for choosing these cell lines is that they significantly differ in expression of potential target antigens, such as, HER-2, ER, PR and EGFR. Although MCF-7 cells are EGFR, HER-2, ER and PR positive [12,13], their expression of HER-2 is low and comparable to that in the MDA-MB-231 cells [14] and MCF-12A cells [15]. While expression of EGFR in MCF-7 cells is also low, MDA-MB-231 and MCF-12A cells which are known to be ER and PR negative [15], express higher levels of EGFR [14,15]. Furthermore, the MDA-MB-231 and MCF-12A cell lines express wild-type PI3K, whereas the MCF-7 cell line is PI3K mutant [16,17]. The MDA-MB-231 and MCF-7 cell lines were routinely cultured in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma-Aldrich, USA). MCF-12A cells were cultured in Dulbecco's Modified Eagle medium (DMEM) nutrient mixture F-12 Ham (Sigma-Aldrich, USA), supplemented

with 20 ng/ml human epidermal growth factor (Sigma-Aldrich, Germany, cat # E9644), 0.01 mg/ml bovine insulin (Sigma-Aldrich, Germany, cat # I5500), and 500 ng/ml hydrocortisone (Sigma-Aldrich, Germany, cat # H0888). All growth media were further supplemented with 10% heat-inactivated foetal bovine serum (FBS) (HyClone, UK), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Lonza, Belgium). Cell cultures were routinely incubated at 37°C in a humidified atmosphere (95% air and 5% CO_2). Cells were grown as monolayers in 75-cm^2 flasks (Greiner Bio-One, Germany, cat # 658170) and were used for experiments (passages 18-39) upon reaching 80-90% confluence.

Target inhibitors

TAK-165 (Tocris Bioscience, UK, cat # 3599) is a specific inhibitor of HER-2. NVP-BEZ235 is a dual inhibitor of PI3K and mTOR (Santa Cruz Biotechnology, TX, USA, cat # 364429). Stock solutions of TAK-165 (21 mM) and NVP-BEZ235 (106 mM) were prepared in dimethyl sulfoxide and stored at 4°C and -20°C, respectively, until used.

Cell survival assay and radiosensitivity

The colony assay was used to measure intrinsic radiation response in all cell lines. Cultures in exponential growth were trypsinised to give single-cell suspensions and were plated (500-10000 cells per flask, adjusted for irradiation dose) into 25 cm^2 culture flasks (Greiner Bio-One, Germany, cat # 690160), and incubated for 3-4 h to allow the cells to attach. Cell cultures were then irradiated to 0-10 Gy with ^{60}Co γ-rays and reincubated. The mean dose rate used in this investigation was 0.83 Gy/min (range: 0.78-0.87 Gy/min). Cultures were irradiated at room temperature (22°C). After growing for 7-10 days, depending on the cell line, colonies were fixed in glacial acetic acid:methanol:water (1:1:8, v/v/v), stained with 0.01% amido black in fixative, washed in tap water, air-dried, and were counted. Three independent experiments were performed for each dose point, and the mean surviving fractions were fitted to the linear-quadratic (LQ) model to generate survival curves. Cellular radiosensitivity was expressed in terms of the surviving fraction at 2 Gy (SF_2).

Target inhibitor toxicity measurements

Single-cell suspensions were plated (1000-4000 cells per flask) into 25 cm^2 culture flasks, and incubated for 3-4 h to allow the cells to attach. To assess the influence of inhibitor concentration on cytotoxicity, cells were exposed to TAK-165 (3.7-137.4 nM) and NVP-BEZ235 (0.6-69.2 nM) and incubated for 7-10 days for colony formation. The colonies were fixed in glacial acetic acid:methanol:water (1:1:8, by volume), stained with 0.01% amido black in fixative, 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 were fitted to a 4-parameter logistic equation of the form:

$$SF = B + \frac{T-B}{\{1-10^{[(logEC_{50}-X)HS}\}} \quad (1),$$

where *B* and *T* are the minimum and maximum of the sigmoidal curve, respectively, X is the log(inhibitor concentration), and *HS* is the steepest slope of the curve. Three independent experiments were performed for each cell line and dose point.

Determination of radiosensitivity modification by TAK-165 and NVP-BEZ235

To investigate the influence of inhibitor exposure on radiosensitivity, attached cells were treated with 30 nM of TAK-165 (~EC_{50} predetermined for MDA-MB-231 and MCF-7 cell lines) and 17 nM of NVP-BEZ235 (~4 × EC_{50} for MDA-MB-231 and MCF-7 cell lines), or a cocktail of both inhibitors at the same concentrations, and irradiated immediately with 2 Gy, the typical dose per fraction in conventional radiotherapy, using ^{60}Co γ-rays. The use of a relatively high NVP-BEZ235 concentration was to ensure adequate inhibition of the dual targets, as these would be expected to present a larger number of binding sites. For each experiment, sets of cell culture flasks given inhibitors alone (singly and in combination) and unirradiated flasks without inhibitors served as controls for cultures irradiated with and without inhibitors, respectively. Inhibitor-treated cell cultures were used as controls for those receiving inhibitors and irradiation to allow for interexperimental variations

in inhibitor toxicity, as exposures to predetermined concentrations do not always yield the expected cell kill. The interaction between inhibitors and γ-irradiation (2 Gy) was expressed as a modifying factor (MF), which is given by the ratio of surviving fractions (SF) in the absence and presence of inhibitors as follows:

$$MF = \frac{SF\,(2\ Gy)}{SF\,([inhibitor]+2\ Gy)} \qquad (2).$$

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

Data analysis

Statistical analyses were performed using the GraphPad Prism (GraphPad Software, San Diego, CA, USA.) computer program. Standard equations were used to fit nonlinear relationships. Data were calculated as the means (± SE) 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 indicates a statistically significant difference between the data sets.

Results

Intrinsic radiosensitivity of MDA-MB-231, MCF-7, and MCF-12A cells

Cellular radiosensitivity expressed in terms of the SF_2 was determined from clonogenic cell survival data (Figure 1). SF_2-values were obtained by fitting the mean surviving data to the linear-quadratic model. From the cell survival data in Table 1, the intrinsic SF_2-values for the MDA-MB-231 (PI3K wild-type), MCF-7 (PI3K mutant) and MCF-12A (PI3K wild-type) emerged as 0.59 ± 0.07, 0.23 ± 0.01, and 0.60 ± 0.07, respectively. The MDA-MB-231 and MCF-12A cell lines show similar radiation response at 2 Gy and are deemed radioresistant, whereas the MCF-7 cell line is deemed radiosensitive.

Cytotoxicity of TAK-165 and NVP-BEZ235

Treatment of cells with inhibitors alone induced a concentration-dependent cell kill (Figure 2). At cell survival rates ranging from 20-90%, NVP-BEZ235 was clearly more potent than TAK-165 in both MDA-MB-231 and MCF-7 cell lines. The equivalent concentrations of TAK-165 and NVP-BEZ235 for 50% cell survival for the MDA-MB-231 cell line were found to be 4.25 ± 0.23 nM (95% CI: 3.79-4.76 nM) and 27.12±4.65 nM (95% CI: 18.50-39.74 nM), respectively. The corresponding EC_{50}-values for the MCF-7 cell line emerged as 4.15 ± 0.34 nM (95% CI: 3.48-4.95 nM) and 24.88 ± 2.26 nM (95% CI: 20.32-30.45 nM), respectively.

Modulation of radiosensitivity by TAK-165 and NVP-BEZ235

To evaluate the impact of inhibitor exposure on radiosensitivity, based on clonogenic cell survival, cell cultures were irradiated to 2 Gy immediately after administering TAK-165, NVP-BEZ235, or a combination of both inhibitors (Figure 3). In MDA-MB-231 cells (Figure 3A), inhibition of HER-2 with TAK-165 alone led to a small and insignificant radiosensitisation, with SF_2 decreasing from 0.59 ± 0.01 to 0.49 ± 0.05 (*P*=0.13, R^2=0.48). This translated to about 20% increase in radiotoxicity (Table 1). In contrast, treatment of MCF-7 and MCF-12A cells did not appear to affect cellular radiosensitivity at 2 Gy (Figures 3B and 3C and Table 1). Inhibiting PI3K and mTOR activity with NVP-BEZ235 significantly radiosensitised MDA-MB-231 and MCF-7 cells, but not the apparently normal MCF-12A cells (Figure 3 and Table 1). The resulting SF_2-values were 0.32 ± 0.04 (*P*=0.0046, R^2=0.89), 0.14 ± 0.02 (*P*=0.02, R^2=0.78) and 0.46 ± 0.05 (*P*=0.25, R^2=0.57) for the MDA-MB-231, MCF-7 and MCF-12A cell lines, respectively. The corresponding modifying factors were 1.84 ± 0.23, 1.64 ± 0.05 and 1.30 ± 0.21. Concomitant treatment with TAK-165 and NVP-BEZ235, significantly enhanced radiosensitivity in all cell lines ($0.0002 \leq P \leq 0.0183$), yielding ~4-, ~3-, and ~7-fold reduction in cell survival in the MDA-MB-231, MCF-7 and MCF-12A cell lines, respectively (Table 1).

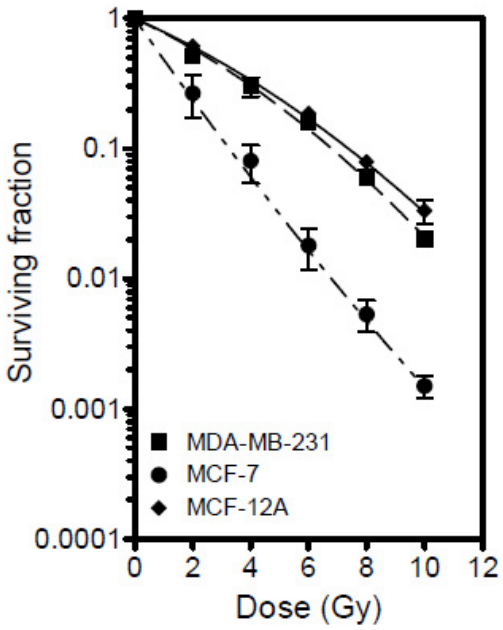

Figure 1: Clonogenic cell survival curves for 3 human breast cell lines after ^{60}Co γ-irradiation. Symbols represent the mean surviving fraction ± SE from three independent experiments. Standard errors are not transformed into a logarithmic scale. Survival curves were obtained by fitting experimental data to the LQ model.

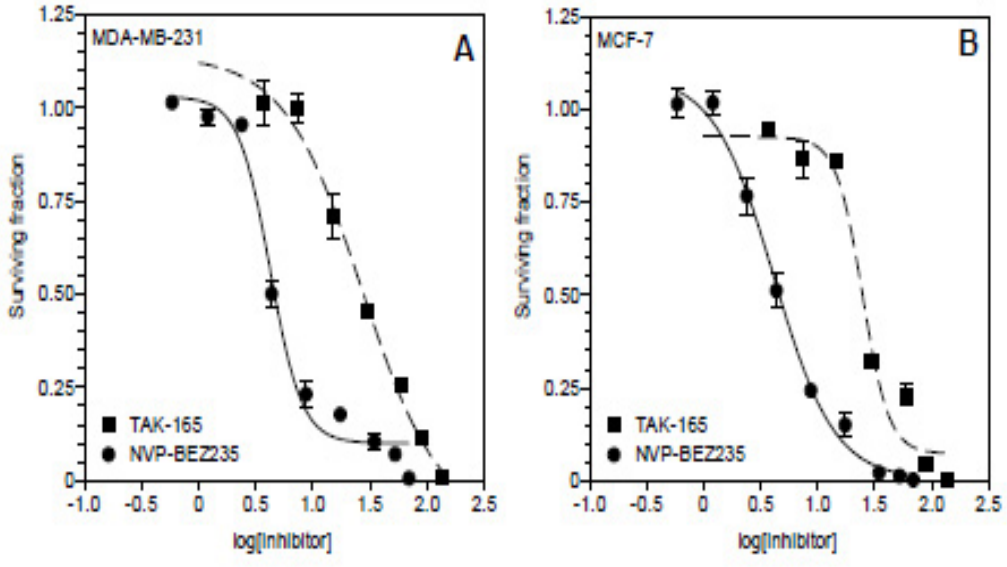

Figure 2: HER-2, PI3K and mTOR inhibitor toxicity in 3 human breast cell lines expressed as cell survival and plotted as a function of log(inhibitor concentration). Cell survival was determined by the colony assay, and data were fitted to a 4-parameter logistic equation. Data points are means±SE of 3 independent experiments.

Discussion

The phosphatase and tensin homolog (PTEN) which impedes PI3K/mTOR pathway activity, thereby sensitising cells to cytotoxic insult, is wildly expressed in all cell lines used here [16]. Therefore, the observed differences in radiosensitivity, as illustrated in Figure 1, cannot be attributed to disparities in PTEN activity. While the more radioresistant MDA-MB-231 and MCF-12A cell lines express wild-type PI3K, the radiosensitive MCF-7 cells are PI3K mutant [16,17]. This may explain the relatively high radiosensitivity in the latter cell line. A reduction in PI3K/mTOR signaling in MCF-7 cells due to the PI3K mutation can compromise their ability to recover from radiation-induced damage.

It is demonstrated for MDA-MB-231 and MCF-7 cells that cytotoxicity of NVP-BEZ235 and TAK-165, based on clonogenic survival, is concentration-dependent (Figure 2). For NVP-BEZ235 treatment, the EC_{50}-values of 4.25 and 4.15 nM obtained for MDA-MB-231 and MCF-7, respectively, are comparable with PI3K/mTOR inhibition data reported elsewhere for MDA-MB-231 and the HER-2 amplified breast cancer cell lines BT474 and MDA-MB-175-VII [16,17]. In contrast, significantly higher NVP-BEZ235 concentrations for 50% growth inhibition (IC_{50}) ranging from 6-93 nM have emerged for many other breast cancer cell lines, with HER-2 amplified cell lines tending to be more sensitive [16,18]. Interestingly, Brachmann et al. [18] demonstrated that doses of NVP-BEZ235 for 50% cell kill (LD_{50}) for MDA-MB-231 and MCF-7 cells can be ~87 and >20 000 nM, respectively. The disparity in toxicity noted here can be explained by the fact that cell growth and metabolic assays which extend over relatively short periods often tend to overestimate cell survival following cytotoxic treatment. Cell growth and metabolic assays are snapshots of cellular demise and may not adequately reflect residual cellular reproductive integrity as measured by the colony forming assay; and the resulting LD_{50}-values can be unrealistically high. The similarity in NVP-BEZ235 toxicity in MDA-MB-231 and MCF-7 cells seems to suggest that NVP-BEZ235-induced cell death cannot be attributed to ER-mediation of PI3K activity, as the latter cell line is known to exhibit ER-dependent PI3K activity while the former does not [19]. Based on clonogenic cell survival, TAK-165 was generally less potent, with an EC_{50} of ~25-27 nM (Figure 2). All cell lines minimally express HER-2 [13,14], and the extensive concentration-dependent TAK-165 induced cytotoxicity observed here cannot be solely attributed to HER-2 alone. This finding is likely due to targeting of residual HER-2, as well as, other critical cellular factors. TAK-165 is a potent inhibitor of EGFR and the cell division cycle protein 2 homolog (Cdc2), which play a crucial role in cell-cycle progression. Perturbation of their activity with TAK-165 can lead to cellular demise during cell division. Cdc2 activity in MDA-MB-231 is intrinsically higher than that in MCF-7 [20], indicating a stronger dependence of the former cell line on Cdc2 activity for cell cycle progression. Inhibiting Cdc2 and the residual HER-2 activity with TAK-165 can, therefore, be expected to be more toxic in MDA-MB-231 cells than in their MCF-7 counterparts. However, TAK-165 concentrations used in the current study are much lower than those that are typically required to significantly suppress Cdc2 and EGFR activity [21]. At such high concentrations, TAK-165 has been shown to be ~4-fold more inhibitory than demonstrated here in a variety of cancer cell lines of bladder, kidney and prostate origin in which HER-2 expression ranged from weak to high [21].

In this presentation, it is demonstrated that treatment of all the three cell lines with TAK-165 yielded only 6-20% radiosensitisation (Figure 3 and Table 1). This is not unexpected, as the cell lines express very low levels of HER-2 [14,15]. The role of HER-2 perturbation in radiosensitivity modulation should, therefore, 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 [22]. However, when cells were treated with the PI3K/mTOR inhibitor, significant radiosensitisation was seen in the MDA-MB-231 and MCF-7 cell lines, but not in the MCF-12A cell line (Figure 3 and Table 1). These data cannot be explained in terms of PI3K status, as NVP-BEZ235 induced radiosensitisation was seen in the MDA-MB-231 (PI3K wild-type) and MCF-7 (PI3K mutant) cell lines, but not in the MCF-12A (PI3K wild-type) cell line. The marked disparity between ~2-fold radiosensitisation observed here and the ~4-fold radiosensitisation reported elsewhere [23],

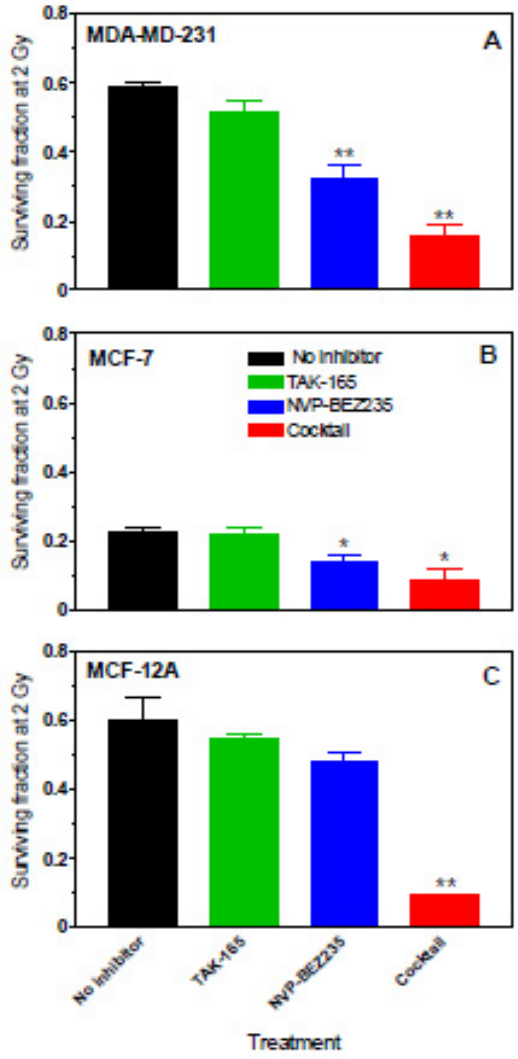

Figure 3: Clonogenic cell survival at 2 Gy (SF_2) for 3 human breast cell lines after ^{60}Co γ-irradiation: (A) MDA-MB-231, (B) MCF-7, and (C) MCF-12A. Cells were irradiated without or in the presence of TAK-165 (HER-2 inhibitor) and NVP-BEZ235 (dual inhibitor of PI3K and mTOR), either administered singly or in combination. Bars represent the mean surviving fraction ± SE from three independent experiments. In comparison with SF_2 without inhibitors: *0.005 < $P \leq 0.02$; **$P \leq 0.005$.

might be due to differences in experiment design. The NVP-BEZ235 concentration of 100 nM used by Kuger and colleagues was ~6 times that used in the present study [23], and corresponds to NVP-BEZ235 doses at which cell survival levels should become very low if the residence time of the drug was over the entire colony forming period (Figure 2). Drug cytotoxicity strongly depends on residence time [24]. Also, delayed trypsinisation and re-plating of cells after drug and radiation treatment can significantly modify the extent to which the drugs modulate radiosensitivity.

Interestingly, although radiosensitisation by TAK-165 was expectedly minimal, a 3- to 6-fold radiosensitisation emerged when cell cultures were pre-treated with a combination of TAK-165 and NVP-BEZ235 (Table 1). To interrogate any potential mode of interaction between the two inhibitors, the data presented in Figure 2 were used to construct median-effect plots, as illustrated in Figure 4, from which combination indices (CI) were

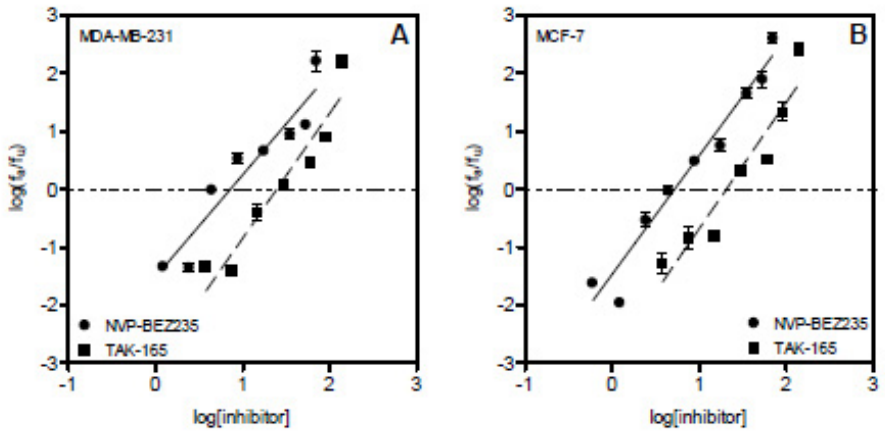

Figure 4: Median-effect plots for 2 human breast cell lines, treated with NVP-BEZ235 (circles and solid lines) and TAK-165 (squares and dashed lines), from toxicity data presented in Figure 2: (A) MDA-MB-231 and (B) MCF-7. Transformed data were fitted to the function: $log(f_a/f_u) = m \times log(D) - m \times log(D_m)$, where f_a and f_u are the affected and unaffected fractions of cells, respectively, and the coefficient m is an indicator of the shape of the inhibitor concentration-effect relationship (m=1, >1, and <1 indicate hyperbolic, sigmoidal, and flat-sigmoidal inhibitor concentration-effect curves, respectively), D_m is the median-effect concentration of inhibitor, and D is the concentration of inhibitor [25]. Horizontal dotted lines are the median-effect axes. For MDA-MB-231, the fitted m and D_m values were 1.78 and 7.28 nM, respectively, for NVP-BEZ235 (r=0.93); and 2.16 and 24.72 nM, respectively, for TAK-165 (r=0.95). The corresponding values for MCF-7 were 2.05 and 5.22 nM for NVP-BEZ235 (r=0.98); and 2.18 and 20.51 nM for TAK-165 (r=0.95). The r-values were high, signifying a strong conformity of the data to the mass-action law [25]. D_m values were marginally lower than corresponding EC_{50}-values.

estimated for the single inhibitor cocktail for the MDA-MB-231 and MCF-7 cell lines [25]. CI-values of 0.72 and 0.73 emerged for the MDA-MB-231 and MCF-7 cell lines, respectively, indicating synergism for TAK-165 and NVP-BEZ235 at the concentrations used here. This might explain the more than additive enhancement of radiosensitivity seen in all cell lines (Table 1). Of specific note is the ~7-fold radiosensitisation observed in the MCF-12A cell line when cells were pre-treated with inhibitor cocktail. It is not likely that the inhibitory activity of the TAK-165/NVP-BEZ235 cocktail is directed towards ER and PR, as radiosensitisation was higher in the ER-negative and PR-negative cell lines (MDA-MB-231 and MCF-12A) than the ER and PR overexpressing MCF-7 cell line [14,15]. The current findings seem to suggest that the activity of the TAK-165/NVP-BEZ235 cocktail is specific for other targets, such as, EGFR which is often overexpressed by cells with compromised activity of HER-2, ER and PR [11]. The rank order of EGFR expression in the three cell lines is: MCF-7<MDA-MB-231<MCF-12A [14,15], and is consistent with the extent of radiation dose modification (Table 1). Therefore, EGFR is a likely target for a TAK-165/NVP-BEZ235 cocktail. The higher radiosensitisation seen in the intrinsically radioresistant immortalised mammary epithelial cell line (MCF-12A), following the cocktail treatment, might be an indication that concomitant use of cocktail and radiation could potentially elevate normal tissue toxicity and requires further evaluation.

In conclusion, this study demonstrates that concomitant inhibition of HER-2, PI3K, and mTOR in human breast cancer cell lines with differing HER-2, ER, PR, and EGFR expression levels results in significant radiosensitisation on the basis of clonogenic cell survival. Radiosensitisation is more prominent in ER- and PR-negative cells expressing higher levels of EGFR. These findings suggest that a cocktail of TAK-165 and NVP-BEZ235 may have the potential for more effectively targeting triple-negative breast cancer cells, and provide the basis for further studies involving a larger panel of cell lines covering a wider range of HER-2, ER, PR, and EGFR expression profiles.

Table 1: Summary of radiosensitivity and dose modifying data for three human breast cell lines treated with inhibitors TAK-165 (for HER-2) and NVP-BEZ235 (for PI3K and mTOR)

Cell line	Treatment	SF_2*	MF_2#
MDA-MB-231	2 Gy	0.59 ± 0.01	
	2 Gy + TAK-165	0.49 ± 0.05	1.20 ± 0.13
	2 Gy + NVP-BEZ235	0.32 ± 0.04	1.84 ± 0.23
	2 Gy + NVP-BEZ235 + TAK-165	0.15 ± 0.03	3.93 ± 0.79
MCF-7	2 Gy	0.23 ± 0.01	
	2 Gy + TAK-165	0.22 ± 0.02	1.06 ± 0.11
	2 Gy + NVP-BEZ235	0.14 ± 0.02	1.64 ± 0.05
	2 Gy + NVP-BEZ235 + TAK-165	0.08 ± 0.04	2.86 ± 1.44
MCF-12A	2 Gy	0.60 ± 0.07	
	2 Gy + TAK-165	0.55 ± 0.01	1.09 ± 0.13
	2 Gy + NVP-BEZ235	0.46 ± 0.05	1.30 ± 0.21
	2 Gy + NVP-BEZ235 + TAK-165	0.09 ± 0.01	6.67 ± 1.07

SF_2 and MF_2 denote the surviving fraction and radiation modifying factor at 2 Gy, respectively. *Mean ± SEM. #Mean ± error: errors were calculated using appropriate error propagation formulae.

Author Disclosures

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