EVALUATION OF BCL-2 AND PARP-1 AS POTENTIAL THERAPEUTIC TARGETS TO RADIOSENSITISE LUNG CANCER

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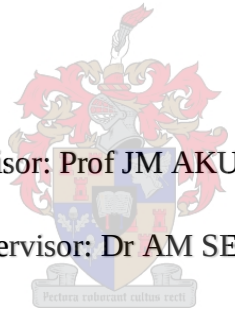

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DECLARATION

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.

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ABSTRACT

Lung cancer remains the most incident malignancy worldwide, representing 13% of all cancers. It is also the leading cause of death in the world, accounting for 18.2% of global cancer-related deaths. The burden of lung cancer in Africa is increasing due to ageing and population growth, increased prevalence of risks factors such as smoking, occupational exposure, infections, lifestyle changes, and environmental pollutants. The efficacy of many therapeutic strategies has been hindered by normal tissue toxicity and treatment resistance. For many cancer patients, radiotherapy has been the chosen therapeutic option to minimise cancer cell spread by shrinking the tumour while ensuring protection of normal tissue. There is evidence that small molecule inhibitors can effectively target cell survival signalling pathways, but cancer cells manage to find molecular escape routes to either repair the damage or evade cell death. Combination therapy appears to be an appropriate approach to address these challenges. Therefore, targeting more than one component of the cell survival signalling pathways could potentially sensitise cancer cells to irradiation and improve the outcome of radiotherapy.

The purpose of this study was to evaluate the role of targeting the anti-apoptotic (B-cell lymphoma 2 (Bcl-2)) pathway and the DNA repair (poly (ADP-ribose) polymerase 1 (PARP-1)) pathway with specific inhibitors in modulating the radiosensitivity of a lung cancer cell line (A549) and an apparently normal lung cell line (L132). For this, Bcl-2 and PARP-1 were inhibited using ABT-737 and ABT-888, respectively.

At a dose of 2 Gy, the typical fractional dose in conventional radiotherapy, combined inhibition of Bcl-2 and PARP-1 or inhibition of Bcl-2 alone resulted in significant radiosensitisation in

only the A549 cells. However, at a larger radiation dose of 6 Gy (a potentially useful fractional dose in hypofractionated radiotherapy), inhibition of Bcl-2 and PARP-1 markedly radiosensitised the apparently normal (L132) and malignant (A549) cell lines, respectively.

These findings suggest that use of Bcl-2 and PARP-1 inhibitors might be beneficial when combined with conventional radiotherapy, but not with hypofractionated radiotherapy when large fractional radiation doses are employed. However, validation of these results with a larger panel of cell lines is warranted.

OPSOMMING

Longkanker bly die wêreldwyd mees maligne maligniteit, wat 13% van alle kankers verteenwoordig. Dit is ook die grootste oorsaak van sterftes ter wêreld, wat verantwoordelik is vir 18,2% van die wêreldwye sterftes aan kanker. Die las van longkanker in Afrika neem toe as gevolg van veroudering en bevolkingsaanwas, verhoogde voorkoms van risikofaktore soos rook, blootstelling aan die werk, infeksies, lewenstylveranderinge en omgewingsbesoedeling. Die doeltreffendheid van baie terapeutiese strategieë word belemmer deur normale weefstoksisiteit en behandelingsweerstand. Vir baie kankerpasiënte was radioterapie die gekose terapeutiese opsie om die verspreiding van kankerselle te verminder deur die gewas te laat krimp, terwyl normale weefsel beskerm word. Daar is bewyse dat kleinmolekule-remmers effektief kan mik op die oorlewingssignale van selle, maar kankerselle slaag daarin om molekulêre ontsnaproetes te vind om die skade te herstel of om die seldood te voorkom. Kombinasie-terapie blyk 'n gepaste benadering te wees om hierdie uitdagings die hoof te bied. Daarom kan die fokus van meer as een komponent van die seinoorlewings seinweë moontlik kankerselle sensitief maak vir bestraling en die uitkoms van bestraling verbeter.

Die doel van hierdie studie was om die rol van die anti-apoptotiese (B-sel limfoom 2 (Bcl-2)) pad en die DNA herstel (poly (ADP-ribose) polimerase 1 (PARP-1)) pad met spesifieke inhibeerders te evalueer. in die modulering van die radiosensitiwiteit van 'n longkanker-sellyn (A549) en 'n skynbaar normale longsellinie (L132). Hiervoor is Bcl-2 en PARP-1 geïnhibeer deur onderskeidelik ABT-737 en ABT-888 te gebruik.

By 'n dosis van 2 Gy het die tipiese fraksionele dosis in konvensionele radioterapie, gekombineerde inhibisie van Bcl-2 en PARP-1 of inhibisie van Bcl-2 alleen gelei tot beduidende radiosensitisering in slegs die A549-selle. By 'n groter bestralingsdosis van 6 Gy ('n potensieel nuttige fraksionele dosis in hipofraktioneerde radioterapie) het die inhibisie van Bcl-2 en PARP-1 egter die skynbaar normale (L132) en kwaadaardige (A549) onderskeidelik radiosensitiviseer.

Hierdie bevindings dui daarop dat die gebruik van Bcl-2 en PARP-1-remmers voordelig kan wees as dit gekombineer word met konvensionele radioterapie, maar nie met hipofraktionerende radioterapie wanneer groot fraksionele bestralings dosisse gebruik word nie. Validering van hierdie resultate met 'n groter paneel sellyne is egter nodig.

DELINEATIONS AND LIMITATIONS

During this research, biomolecules as inhibitors of Bcl-2 (ABT-737) and PARP-1 (ABT-888) were used to potentially radiosensitise two lung cell lines, A549 (cancer) and L132 (apparently normal). The parameters that were determined using the colony formation assay included the innate cellular radiosensitivity and the cytotoxicity of each inhibitor alone or when combined with irradiation. The inhibitor-irradiation experiments were to assess the radiomodulatory effects of the inhibitors. Radiomodulatory effects (expressed as modifying factors in cell survival) were evaluated in each cell line at inhibitor equivalent concentrations for 50% cell kill (EC_{50}).

The main limitation of this study is that it was performed using only two lung cell lines: the cancer cell line, A549, and the apparently normal cell line, L132. This finding could be strengthened if a larger panel of cell lines were used.

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I would like to devote this master's thesis to the following:

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α	Linear coefficient of cell inactivation
β	Quadratic coefficient of cell inactivation
BAK	Bcl-2 antagonist/killer 1
ATP	Adenosine triphosphate
BAX	Bcl-2-like protein 4
Bcl-2	B-cell lymphoma 2
Bcl-W	B-cell leukemia/lymphoma-w
Bcl-XL	B-cell lymphoma-extra large
BH3	B-cell homology 3
CTK	Cytoplasmic tyrosine kinase
DISC	Death inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DSBs	Double-strand breaks
DSBR	Double-strand break repair
EC_{50}	Equivalent concentration for 50% cell kill
EGFR	Epidermal growth factor receptor
FBS	Foetal bovine serum
LET	Linear energy transfer
MF	Modifying factor
mTOR	Mammalian target for rapamycin
NSCLC	Non-small cell lung cancer
PARP-1	poly (ADP-ribose) polymerase 1
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
RPMI	Roswell Park Memorial Institute
RTK	Receptor tyrosine kinase

SCLC	Small cell lung cancer
SEM	Standard error of the mean
SF_2	Surviving fraction at 2 Gy
SF_6	Surviving fraction at 6 Gy
SSBs	Single-strand breaks
SSBR	Single-strand break repair
TNF	Tumour necrosis factor

CHAPTER 1

1.1. Introduction

The study done by the World Health Organization in 2015 revealed that cancer is the number one cause of death before the age of 70 years in 91 of 172 countries; and ranks third in an additional 22 countries. GLOBOCAN, a consortium that uses data from the International Association of Cancer Registries (IACR) to provide statistics on cancer mortality and prevalence, estimates that lung cancer is the most frequent cancer and leading cause of death among males and the third cause among females (Bray et al., 2018). Lung cancer is also the leading cause of cancer death amongst men in Southern and Northern Africa, as well as being the fourth leading cause of death among women in Southern Africa (Dela Cruz et al., 2011). Data reporting of cancer epidemiology in the African continent has been limited by the lack of reliable registries. The incidence and mortality of lung cancer in Africa are lower compared to other continents due to low prevalence of smoking (10% in men and less than 2% in women) associated with lower life expectancy of the population (Jemal et al., 2012). An average of 10 - 20% of non-smokers reportedly have lung cancer, with a much higher incidence in women than in men (Wakelee et al., 2007). Aside from tobacco smoking as the main cause, other contributory factors associated with the onset of lung cancer may be environmental exposure to radon or different chemicals, radiation, coal smoke as well as indoor emission of burning fuel (Jemal et al., 2010; Loomis et al., 2013). The incidence and mortality rates of this pandemic seem to mirror one another since most patients diagnosed with the disease eventually die from it. A recent report confirmed that lung cancer remains the leading cause of cancer death with

an estimated 1.8 million deaths, on average 18% of the global cancer mortality (Sung et al., 2021).

Conventional cancer treatment options include chemotherapy, radiotherapy, and surgery, depending on the type of lung cancer (small or non-small cell), the size and position of the cancer, the stage, and the patient's overall health. Globally, lung cancer appears to show an annual mortality rate approaching that of breast and prostate cancer combined (Sung et al., 2021); although an increasing number of lung cancer patients, *akin* to their breast and prostate counterparts, receive some form of treatment and the mentioned therapeutic options are often used in combination with each other.

Surgery alone might not yield the required treatment outcome, as the removal of a primary tumour may miss tissue that has been invaded by tumour cells, and radiotherapy is often used to treat the tumour bed. In cases of advanced tumour stages, systemic treatment by means of chemotherapy is employed. These combinations often lead to severe side-effects from which patients may not recover.

Previous studies have been focused on the use of inhibitors to target pathways like those of PI3K/mTOR and EGFR to radiosensitise breast, prostate, and cervical cancer cell lines (Hamunyela et al., 2015; Maleka et al., 2015; 2019; Hamid et al., 2016; Hamid, 2019). These signaling pathways are involved in cancer proliferation and migration, and prevent cancer cell death. The results of these studies are varied and demonstrate no marked radiosensitisation in

cervical cancer cells, high level of toxicity in apparently normal lung cells and breast and prostate cancer cells, and radioprotection in normal prostate cells (Hamunyela et al., 2015; Maleka et al., 2015; Hamid, 2019). There is, therefore, the need to identify new and more effective treatment approaches that could improve prognosis in lung cancer patients. The current study aimed to evaluate small molecule inhibitors (ABT737 and ABT888) of key survival signalling pathways (Bcl-2 and PARP-1) for their capacity to preferentially enhance the effectiveness of ionising radiation in cancer cells.

1.2. Rationale and Problem Statement

Lung cancer is a regular form of malignancy accounting for thousands of deaths in Western and Eastern countries (Ferlay et al., 2007; 2010; 2013). At the beginning of the 20th century, lung cancer was seen as a rare disease but rose dramatically a decade later due to an increase in smoking and environmental and genetic factors. Continuous exposure to these factors may lead to genetic mutations which disrupt protein synthesis. The main genes responsible for the development of lung cancer are B-cell lymphoma-2 (*Bcl-2*) and tumour protein (EC :2.7. 1.37) (*p53*) for small cell lung cancer (SCLC), and epidermal growth factor receptor (EGFR), ataxia-telangiectasia mutant (*ATM*), Kirsten rat sarcoma virus (*KRAS*), and cyclin-dependent kinase inhibitor 2A (*p16*) for non-small cell lung cancer (NSCLC) (Cagle et al., 2013; Lindeman et al., 2013; 2018). Mutation of these genes leads to the formation of unusual proteins causing abnormal molecular or biological processes that results in the development of cancer cells (Hassanpour and Dehghani, 2017).

Cancer treatment aims to destroy cancer cells without killing the normal cells. The preferred treatments are surgery, chemotherapy, and radiotherapy which can be used either alone or in combination with other therapies (Wild-Bode et al., 2001; Zappa and Moussa, 2016; Huang et al., 2017)

Radiotherapy is commonly used for all types of lung cancer. However, patients often experience significant levels of treatment-related side-effects, and most do not respond

favorably (Torres et al., 2015). To partially address these clinical challenges, it is necessary to identify biomolecules that would preferentially sensitise cancer cells to the effects of radiation. This could form the basis for the development of novel clinical strategies that may be vital in improving the outcome of lung cancer therapy.

Specific biomolecules appear to be a potential alternative to sensitise cancer cells to irradiation while protecting critical tissues. Small molecules, such as ABT-263, ABT-737, and ABT-888, are used in targeted therapy to locate key specific genes and/or proteins involved in cellular pathways that control cell survival. Programmed cell death (apoptosis) has become an attractive molecular process for new cancer therapy (Chen et al., 2005). Similarly, the perturbing DNA repair via inhibition of key components of the PARP-1 pathway shows potential for selectively sensitising cancer cells to radiation insult (Donawho et al., 2007), and could serve as a viable approach to improving radiotherapy outcome.

1.3. Research Question

Does the use of biological inhibitors of pro-survival signalling pathways preferentially enhance the radiosensitivity of cancer cells *in vitro*?

1.4. Hypothesis

It is postulated that *in vitro* treatment of cancer cells with mammalian proteins B-cell lymphoma 2 (Bcl-2) and poly (ADP-ribose) polymerase 1 (PARP-1) signalling pathway inhibitors, singly or in combination, can potentially increase their radiosensitivity.

1.5. Aims and Objectives

The purpose of this study was to assess the effect of targeting Bcl-2 and PARP-1, using small molecule inhibitors, on the radiosensitivity of normal and cancer cells of the human lung *in vitro*. The objectives towards achieving the goal are as follows:

1. To determine the intrinsic radiosensitivity of two lung cell lines (1 normal; 1 cancer).
2. To assess the effect of the Bcl-2 and PARP-1 inhibitors, administered singly or in combination at EC_{50}, on the radiosensitivity of the cancer and normal cells.

1.6. Literature Review

1.6.1. Cancer

Cancer is a somatic disease characterised by uncontrolled cell proliferation and disruption of programmed cell death. Hanahan and Weinberg published a review attempting to organise the immense complexity of cancer biology into six major hallmarks (Hanahan and Weinberg, 2000). This study explained that cancer cells possess the ability to grow independently using different signalling pathways. A key characteristic of cancer cells is their ability to disrupt cell death mechanisms and replicate continuously (Hanahan and Weinberg, 2000; 2011); and cancers were suggested to display the following characteristics: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. The lack of specific growth signals and non-specific receptors justifies the complexity of the control of tumour proliferation and allows these cells to be independent from the external factors.

The vascularisation of tumour and, thus, enhanced nutrient supply contributes to the initiation, autonomy, and development of new cancer blood vessels (Hanahan and Weinberg, 2000; 2011). Tumour vascularisation is an attractive target for therapy since the blood vessels are very important for tumour growth by supplying oxygen and other nutrients, and vascular

disrupting agents have gained popularity as antitumour drugs (Hinnen and Eskens, 2007; Chen et al., 2012; Lei et al., 2018).

Radiotherapy is an essential component in the treatment of cancers for both curative and palliative intervention, with as many as 40% cured by radiotherapy alone, compared to 11% cured by chemotherapy alone (Atun et al., 2015). Despite the great contributions of radiotherapy towards cancer management, major clinical limitations are still observed during or after treatment. Tumour resistance to ionising radiation, the protection or damage of critical tissues, the determination of tumour control dose or toxicity dose are clinical obstacles undermining the effectiveness of radiotherapy (Buchholz, 2009). Efforts were initially focused on conventional therapies to tackle these challenges, but chemotherapy involves very toxic drugs that affect both cancer and normal cells by causing infections and immune suppression. Surgery is invasive and characterised with a high morbidity rate; and radiotherapy is cytotoxic, destroys normal cells and may lead to radioresistance.

It is important, then, to investigate novel therapies that target different proteins to tackle the aforementioned clinical challenges. The objective of this study, therefore, was to use targeted therapeutic agents to inhibit survival signalling markers, such as Bcl-2 and PARP-1, in two human lung cell lines (normal: L132; cancer: A549) in combination with radiotherapy, to possibly plot a way forward in the treatment of lung cancer.

1.6.2. Lung Cancer

The lungs are the most important organs of respiration, located on either side of mediastinum, with functions to transport oxygen in the body and release carbon dioxide from the body. In 2020, new cases of lung cancer totalled 235 760 (119 100 in men and 116 660 in women), and deaths from lung cancer totalled 131 880 (69 410 in men and 62 470 in women). Lung cancer is subdivided into two types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (Sung et al., 2021). NSCLC represents more than 80% of lung cancers, whilst SCLC covers less than 20%. NSCLC is composed of adenocarcinoma, squamous cell cancer, and large cell carcinoma. Lung cancer has the characteristics of being heterogeneous, highly aggressive, and is often clinically detected very late (Lemjabbar-Alaoui et al., 2015). These factors lead to poor patient response to treatment.

1.6.3. Conventional Cancer Therapies

1.6.3.1. Surgery

The use of surgery to treat cancer consists of the removal of the tumour and surrounding tissues during an operation. Lung cancer surgery is complex with serious consequences and requires a highly experienced surgeon. Curative cancer surgery has long been considered as sole therapeutic modality to remove localised tumours, but several factors such as tumour stage, difficulties in accurate pre- and intraoperative staging, risks of residual disease and recurrence,

lymphogenic metastatic and surgical complications selectively influence and affect the success of surgery (Kappas and Roukos, 2002). According to the American Cancer Society, surgery remains the first treatment of choice for patients with early stage locoregional tumours, but not preferred for distant metastasis. Surgery has been reported to cause local and/or systemic inflammation, promote cancer signalling pathways, and induce postoperative cancer recurrence (Hiller et al., 2018). Nevertheless, 60% of patients are treated with surgery, offsetting the postoperative deleterious effects of alternative novel therapies that combine surgery with radiotherapy, targeted therapy or chemotherapy (Hiller et al., 2018).

1.6.3.2. Chemotherapy

Chemotherapy makes use of drugs to block cell proliferation (cell mitosis) or to inhibit DNA repair, and its use usually depends on the stage/type of cancer, overall health of the patient, and the location of the cancer (Rajman et al., 2018). It is considered a systemic treatment, affecting the entire body, and aims to reduce the total number of cancer cells in the body, lower the likelihood of cancer spreading, and, in special cases, shrink the tumour size to allow a more effective therapy to be applied concomitantly. The benefits of chemotherapy in the relief of cancer symptoms, minimisation of cancer recurrence, and increase in the survival rate of patients, aside, chemotherapeutics have shortcomings in the damage of normal tissues due to cumulative cytotoxicity caused by prolonged drug treatment and in the management or control of metastatic tumours in both SCLC and NSCLC. Because SCLC is rarely localised at diagnosis, surgery plays little role in its treatment and most patients with SCLC receive

chemotherapy while 18% only of patients with NSCLC undergo surgery and 68% of NSCLC are treated either with chemotherapy or radiotherapy (Bunn Jr and Kelly, 1998; Zarogoulidis et al., 2013; Horn et al., 2018). Although quality of life may be improved the side-effects of the chemotherapeutic agents have led researchers to investigate alternative treatment modalities, such as combined therapeutic methods, to address these risks and further improve survival (Kubota et al., 1994; Horn et al., 2018).

1.6.3.3. Radiotherapy

Radiotherapy is the treatment of cancer using beams of high energy rays, such as X-rays. The form of energy used in cancer therapy is known as ionising radiation. This form of energy is produced from natural or artificial sources and has high enough energy to break chemical bonds and cause damage to living tissue. Radiation may affect healthy cells as well as cancer cells. Specific side-effects depend on factors such as the area receiving treatment, the tumour cells, the person's overall health, and the radiation dose/type.

The two main types of radiations are: (1) sparsely ionising radiation (or low linear energy transfer (LET) radiation), for example, X-rays or gamma rays; and (2) densely ionising radiation (or high LET radiation), such as neutrons or alpha particles. Low LET particles act on biological tissue via an "indirect effect". This process is observed when sparsely ionising radiation passes through water and creates free radicals which then interact with the DNA of

cells. When high LET ionising radiation causes direct injury in biological tissue, a “direct effect” is said to have occurred.

DNA is referred to as the blueprint of life and contains all the information required for a cell to grow, develop, survive and reproduce, and represents a critical target of radiation (Vignard et al., 2013). Therefore, it is sensible to ensure that the genetic information coded in DNA can always be recovered, even from a single strand. The preservation of the genetic information, in its original state, is dependent not only on the accuracy of its copying during DNA replication, but also on the timely repair of any damage which may compromise the integrity of the DNA molecule, and/or may change its building blocks, or their arrangement, in any manner.

Ionising radiation may cause damage to single-strand DNA as well as double-strand DNA. Radiotherapy achieves its therapeutic effect by inducing damage and eventual cell death if the damage is not reversed (Baskar et al., 2008). Radiotherapy can cause reversible or repairable damage, called “sublethal damage”, when low doses of ionising radiation are used; but when exposed to high doses, “lethal damage” is induced and can lead to cell death.

Tumour resistance to ionising radiation may result from the ability of cancer cells to withstand radiation insult by using DNA repair mechanisms or evasion of programmed cell death (apoptosis) which may be achieved via escape molecular signalling pathways. Typical examples of DNA repair and pro-survival pathways are those of PARP-1 and Bcl-2, respectively. Radiotherapy is mostly used in conjunction with surgery or with

chemotherapeutic drugs or as a neo-adjuvant with surgery by causing reduction of the tumour size. However, this intervention is limited by tumour radioresistance, systemic tumour progression and local or distant metastasis (Wild-Bode et al., 2001; Huang et al., 2017)

1.6.4. Contemporary Cancer Therapies

1.6.4.1. Targeted Therapy

Targeted therapy is a cancer treatment that uses drugs to locate specific genes and proteins involved in cancer proliferation and survival (Gerber, 2008; Attia et al., 2019). It may impact on the environment helping the cancer growth or it may target cells related to cancer regeneration. Targeted therapy may block or turn off signals involved in the cancer proliferation or spread, may prevent the cancer cells from living longer than normal or may destroy them (Attia et al., 2019). The direct approach targeted therapy focuses on the identification of tumour antigens to alter their signaling pathways either by monoclonal antibodies or by small molecule drugs that interfere with these target proteins. The indirect approaches targets tumour antigens expressed on the cell surface of the cancer cells, using tumour specific monoclonal antibodies or peptide ligands binding to receptors that exist in the tumour cells. Apart from active targeted therapy, a passive targeted mechanism is also observed where tumours are targeted by macromolecules using the enhanced permeability and retention effects associated with hyperpermeable angiogenic tumour vasculature due to the absence of effective tumour lymphatic drainage (Wu et al., 2006; Attia et al., 2019).

1.6.4.1.1. Antibody-Targeted Therapy

The technique to produce monoclonal antibodies (Mabs) was developed in 1975 by Kohler and Milstein and this technology made a great contribution to the treatment of several different malignancies (Harris, 2004). Monoclonal antibodies are man-made proteins that act like human antibodies and have a specific target on a cancer cell where they bind and attack the tumour cell. Some examples of Mabs as targeted therapy are the use of trastuzumab for HER-2-positive breast cancer, cetuximab for EGFR-positive colorectal lymphomas, and rituximab for B-cell surface protein CD20 (Keller et al., 2007).

1.6.4.1.2. Small Molecule Targeted Therapy

The endless knowledge surrounding molecular events that govern the signalling pathways of different types of cancer has led to the development of novel agents. The phosphorylation and dephosphorylation of proteins are important posttranslational modification processes during protein synthesis, playing crucial roles in signal transduction and switching of enzymatic activity (Sacco et al, 2012). Anti-apoptotic behaviour, unrestricted proliferation, and angiogenesis are consequences of aberrant phosphorylation in cancer (Cicenas et al., 2006; Maatta et al., 2006; Troussard et al., 2006). Therefore, small molecule inhibitors of protein kinases such as plasma membrane-associated protein tyrosine kinases have emerged as indispensable in targeted therapy. Some examples of tyrosine kinase inhibitors are imatinib, which inhibits ATP binding to the abi kinases, blocking the activity of bcr-abi tyrosine kinase

encoding for abnormal proteins, leading to human cancer; Gefitinib or erlotinib, a selective oral epidermal growth factor receptor tyrosine kinase inhibitor which is involved in the disruption of EGFR kinase activity by binding the ATP pocket within the catalytic domain, decreasing mitogen-activated protein kinase activity, increasing apoptosis and increasing the level of cyclin dependent kinase p27 responsible for G1 cell cycle arrest. Figure 1.1 Overview of small molecules that are used for targeting specific cellular processes that are predominant in human cancers.

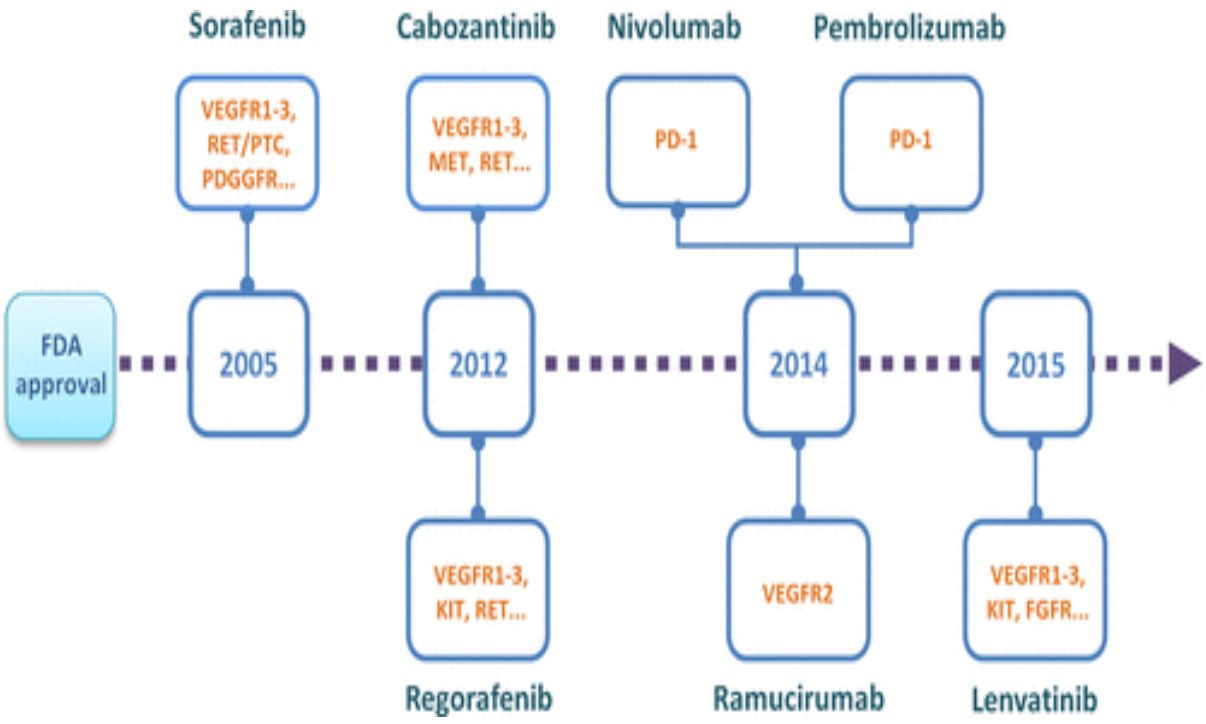

Figure 1.1: Overview of various targeted inhibitors in the market. Small molecules inhibitors target key genes and signaling pathways by reversing the biological behavior of the tumour cells, so as inhibiting cancer proliferation and metastasis (Ma et al., 2020)

1.6.4.1.3. Ligand-Based Targeted Therapy

Most cancer cells share common features with their counterpart normal host cells from which they originated such as a lack of unique molecular targets that would differentiate them from normal cells. Therefore, increasing the therapeutic dose of the tumour cells may lead to the damage of the normal tissue. Ligand targeted therapy offers selective toxicity by increasing the amount of the drug reaching the tumour cells. This technique of tumour targeting, not only limits normal tissue toxicity, but also overcomes obstacles such as elevated interstitial fluid pressure in tumours, drug resistance, and nonspecific drug delivery that are often encountered in chemotherapy (Wu et al., 2006; Li et al., 2016). An example of ligand-based targeted therapy is the nanoparticle-mediated targeted drug delivery system which involves a chemotherapeutic agent, a delivery vehicle targeting the tumour, and a surface ligand that mediates the delivery vehicle's interaction with the tumour at high specificity and high concentration.

Tumour drug resistance and normal cell toxicities are hallmarks of chemotherapy. The development and discovery of targeted therapy was to allow greater tumour specificity and less toxicity of the normal cells (Li et al., 2016). Recently, some attempts have been made to address these therapeutic challenges such as monoclonal antibodies targeted therapy, ligand based targeted therapy and small molecule inhibitors targeted therapy by locating tumour specific receptors, blocking tumour proliferation, and decreasing the level of damage to the normal tissue. However, no matter the promising clinical results from the agents highlighted there is still significant limitations of the concept of "pathway-specific" targeted therapies. It is evident

that most solid tumors are caused by various genetic mutations and the inhibition of a single molecular pathway may not result in a significant therapeutic outcome (Wu et al., 2006; Li et al., 2016). In addition to this, the acquired resistance to protein inhibitors by tumours that initially respond positively is due to mutations in the binding sites of the inhibitors, which lead to the failure of agents to generate any detectable response to subsequent treatment. A classic example is the oncogenic mutation in the case of EGFR kinase where methionine is substituted with threonine at amino acid position 790 (T790 M mutations) of the cancer cells, providing a tumour growth advantage alongside a drug-resistant variant of the targeted protein kinase (Pao et al., 2005; Kobayashi et al., 2005). Therefore, the ongoing obligation to develop therapy that will target many tumour pathways and reduce normal tissue toxicity, is urgent. The combination of targeted therapy with another therapeutic mode, such as radiotherapy, was considered as a novel strategy to respond to this challenge.

1.6.4.2. Combination Therapy

Combined drug therapy is a mode of treatment that consists of increasing the action of one molecule by either increasing its penetration or disrupting its binding activity (Reece et al., 2007, Chanan-Khan et al., 2010). The rationale of this mode of treatment is to use drugs that work by different mechanisms, thereby decreasing the onset of tumour cell resistance. Combination cancer therapies aim to improve the probability and magnitude of therapeutic responses and reduce the likelihood of acquired resistance to single therapy. Multiple drugs may act on different targets to delay cancer cell proliferation or may function together to

improve the therapeutic efficacy by targeting the same molecular pathway (Lee and Nan, 2012; Burris, 2013).

Depending on the type of cancer, the effective approach may be a combination of surgery, radiotherapy, chemotherapy, or other therapy, such as targeted therapy. Radiotherapy may be used prior to another therapy to shrink a tumour to optimise the existing treatment (called neoadjuvant therapy) or may be given after to kill any remaining cancer cells (called adjuvant therapy).

1.6.5. Cell Signalling Pathways of Interest

An evaluation of the effect of inhibiting cell signalling pathways in cellular radiosensitivity might have a beneficial impact on the treatment of lung cancer patients. For this, the cell death and DNA repair pathways, specifically those of Bcl-2 and PARP-1, are of particular interest.

1.6.5.1. The Programmed Cell Death (Apoptosis) Pathway

The term apoptosis (a-po-toe-sis) was first used in a paper by Kerr et al. (1972) to explain a morphologically different type of cell death and a non-inflammatory biochemical mechanism. This phenomenon is considered a vital component of various processes such as normal cell turnover, normal development and functioning of the immune system, hormone-dependent atrophy, embryonic development, and chemically induced cell death (Kerr, 2002). According

to Kerr, dysfunctional apoptosis (either too little or too much) is a problem in many human conditions, including neurodegenerative diseases, ischaemic damage, autoimmune disorders, and most types of cancers. Much research has been focused on the elucidation and analysis of the cell cycle and signalling pathways that modulate cell cycle arrest and apoptosis because of their tremendous therapeutic potential in the modulation of life or death of a cell (Pucci et al., 2000; Elmore, 2007; Brunelle and Letai, 2009; Dorn, 2012; Carneiro and El-Deiry, 2020; Shahar and Larisch, 2020). Unregulated cell proliferation may lead to pathologic insult, if it is not countered by an appropriate apoptotic response (Pucci et al., 2000). During development and ageing, apoptosis plays a role in homeostasis by maintaining cell populations and in defense mechanisms, such as in immune reactions or when cells are damaged. The main morphological changes following apoptosis, as identified by light and electron microscopy, are: plasma membrane blebbing, cell shrinkage, nuclear fragmentation, pyknosis as a result of chromatin condensation, and cell surface molecule changes to ensure that apoptotic bodies are recognised and engulfed by neighbouring cells or phagocytes in *in vivo* systems (Kerr et al., 1972). The tightly regulated process of phagocytosis ensures either little or no inflammation. The morphological changes that occur during the apoptotic process are depicted in Figure 1.2.

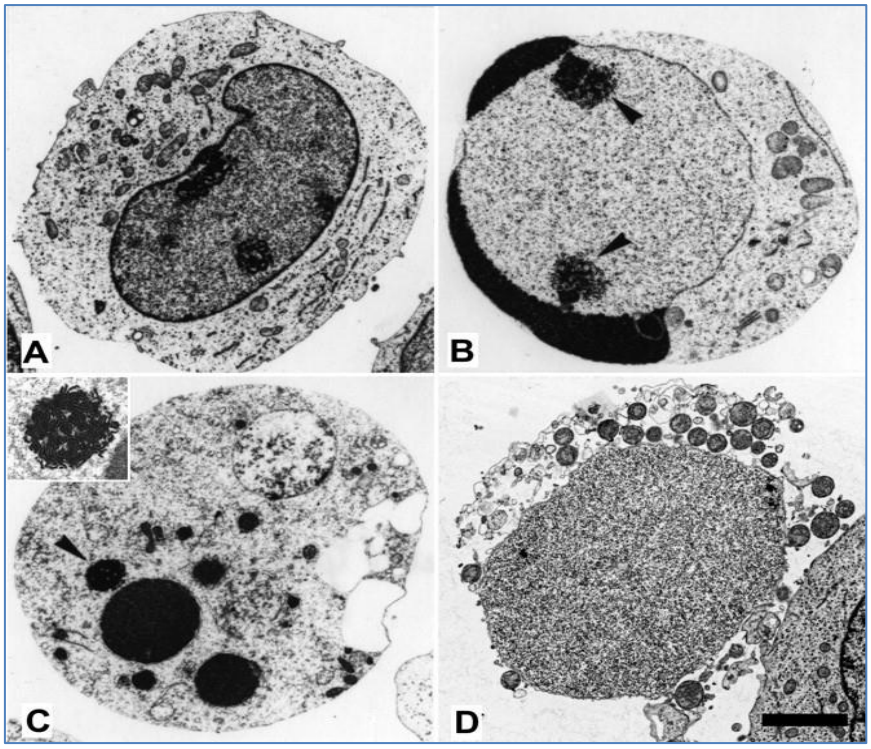

Figure 1.2: Morphological features of cell death (Ziegler and Groscurth, 2004): (A) - Normal cell morphology; (B) and (C) - Blebbing of the cell membrane; and (D) Chromatin condensation, fragmentation of the nucleus, and formation of apoptotic bodies.

There are two main apoptotic pathways, namely, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic signalling pathway involves transmembrane receptor-mediated interactions by activating death receptors which are members of the tumour necrosis factor (TNF) receptor genes superfamily (Locksley et al., 2001; Giussani et al., 2014). These members have a cytoplasmic domain of about 80 amino acids, named the "death domain", and share similar cysteine-rich extracellular regions (Ashkenazi and Dixit, 1998). The death domain is responsible for carrying death signals from the cell surface to the intracellular signalling pathways. The extrinsic phase of apoptosis is characterised with FasL/FasR and TNF-α/TNFRI models, where clustering of receptors and binding of receptors to the homologous trimeric ligand further leads to the activation of a death

inducing complex (DISC), formation of procaspase-8 and caspase 8 to trigger apoptosis (Ashkenazi and Dixit, 1998; Giussani et al., 2014).

The intrinsic pathway involves an array of non-receptor mediated stimuli that produce intracellular signals acting directly on targets within the cell and are initiated by mitochondria. The control of these mitochondrial events happens through members of the B-cell lymphoma 2 (Bcl-2) family of proteins (Cory and Adams, 2002; Shahar and Larisch, 2020), where the tumour suppressor protein p53 plays a critical function in the regulation of the Bcl-2 family of proteins. The extrinsic and intrinsic apoptotic pathways are depictured in Figure 1.3.

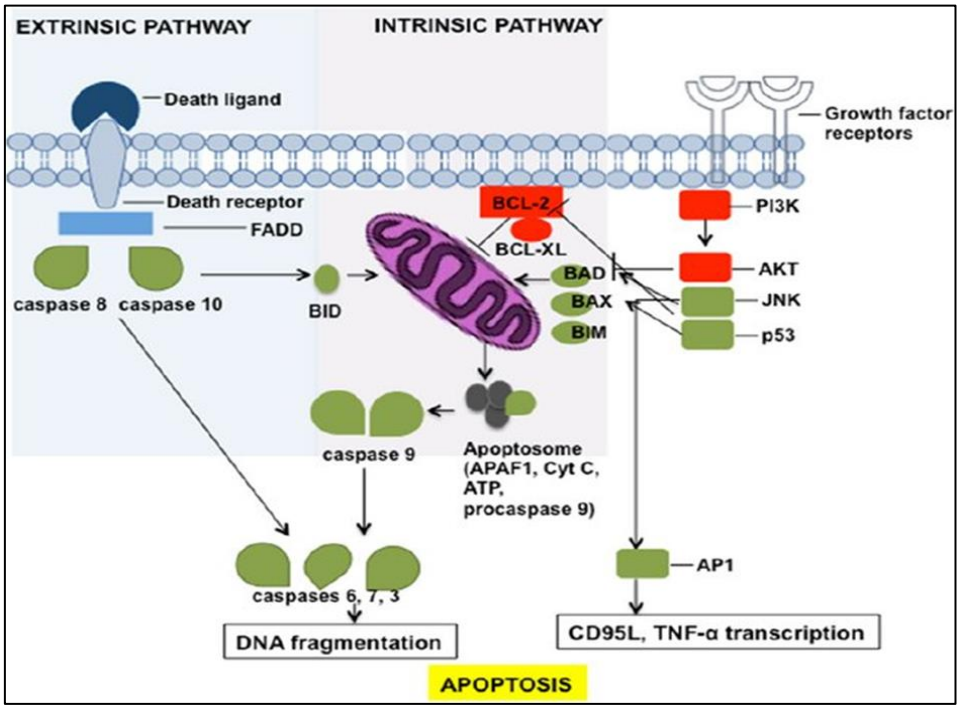

Figure 1.3: Schematic representation of the extrinsic and intrinsic apoptosis pathways with pro-apoptotic regulators in green and anti-apoptotic regulators in red (Giussani et al., 2014).

The mechanisms of apoptosis are highly complex and sophisticated, displaying an energy-dependent cascade of molecular reactions, as shown in Figure 1.4, which may take a mere 15 minutes and is, therefore, difficult to detect in tissue sections (Martin, 1993). A family of mammalian proteins B-cell lymphoma 2 (Bcl-2) may promote or inhibit cell death by regulating the permeabilisation of the mitochondria outer membrane. Bcl-2 anti-apoptotic proteins are represented by Bcl-2 itself, as well as B-cell lymphoma-extra-large (Bcl-xL), and B-cell leukaemia/lymphoma-w (Bcl-W) and myeloid cell leukaemia-1 (Mcl-1), and pro-apoptotic proteins are composed by Bcl-2-like protein 4 (Bax) and Bcl-2 antagonist killer 1 (Bak) (Brunelle and Letai, 2009), as shown in Figure 1.3. It appears that the main mechanism of action of the Bcl-2 family protein is primarily the regulation of cytochrome C release via alteration of the mitochondrial membrane permeability (Zha et al., 1996). This leads to the formation of the ternary complex of cytochrome C, the adapter protein Apaf-1, and the initiator caspase 9, followed by the sequential activation of effector caspases causing endonuclease action resulting in cellular disintegration. Researchers believe that the lack of apoptosis was suggested to be implicated in the pathogenesis of a variety of human diseases including cancer and many other chronic diseases (Thompson, 1995; Reed, 2000; Liu et al., 2004; Wong, 2011). The dysregulation of programmed cell death may be represented by overexpression of Bcl-2 in many cancers and contributes to tumour progression and resistance to therapy. Preferentially, silencing Bcl-2 in tumour cells with specific small molecule inhibitors should render them more vulnerable to the cytotoxic effects of ionising radiation.

1.6.5.2. The DNA Repair Pathway

An alternative strategy to effectively improve ionising radiation at lower doses, may be the use of radiosensitisers to target recognition and repair of deoxyribonucleic acid (DNA) damage. Poly (ADP-ribose) polymerases (PARPs) are a family of enzymes that use nicotinamide adenine dinucleotide (NAD^+) as a substrate to polymerize onto their cellular targets. PARP-1 and PARP-2 isozymes are activated by DNA damage and seem to participate in single-strand DNA break repair by activating the repair protein, X-ray repair cross-complementing protein 1 (XRCC1), base-excision repair, and double-strand DNA break repair, through the influence of both homologous recombination and non-homologous recombination (Veuger et al., 2003), as shown in Figure 1.4. Unlike PARP-2, PARP-1 is the most active protein, responsible for about 90% of cellular PARP formation, and is the highly conserved protein during evolution (Schreiber et al., 2002), and targeting the latter could prove beneficial in cancer therapy. In fact, small molecule inhibitors of PARP-1 have shown significant promise, and third generation PARP inhibitors have been investigated in clinical trials (Lord and Ashworth, 2008; Weaver and Yang, 2013).

The potential use of PARP inhibitors requires an in-depth understanding of their mechanisms of actions and the effects of modifying factors on PARP inhibitor susceptibility. PARP protein is involved in many cellular processes which includes single-strand break repair (SSBR) and double-strand break repair (DSBR). Compared to single-strand breaks (SSBs), DNA double-strand breaks (DSBs) are highly deleterious, with a single unrepaired double strand being

sufficient to trigger cell death (Langelier et al., 2018). Therefore, they represent the most lethal of DNA lesions, eliciting the majority of the cytotoxic effects caused by ionising radiation and certain chemotherapeutic agents. It is stated that healthy or normal cells have minor amounts of DNA damages and possess full DNA repair capacity than cancer cells which have elevated DSBs due to oncogene-induced replication stress and defects in DNA damage response mechanisms. Hence, hyperproliferating cancer cells depend on DSBR for their survival, and this remains a major cause of radioresistance or cancer recurrence. Knowing the fact that DNA damage response (DDR) defects may predispose individuals to developing cancer, this same vulnerability can be exploited to kill tumour cells by targeting the DSB repair mechanism of cancer cells (Bouwman and Jonkers, 2012; O'Connor, 2015). Lung cancer is the leading cause of cancer death worldwide and a potential target of PARP inhibitor therapy. The use of PARP inhibitors to target more than one of the genes promoting cancer cell survival has proved their importance in clinical settings. Recent studies have been reported targeting specific cancer cells deficient in genes such as PARP-1 genes, ATM genes, and mostly BRCA1 or BRCA2 genes, using PARP inhibitors in order to improve the outcome of conventional treatment in clinics (McCabe et al., 2006; Murai et al., 2012; Kantidze et al., 2018; Motegi et al., 2019). This is a concept called "Synthetic lethality", which asserts that not only unrepaired SSBs may lead to potentially lethal damages and become a threat to normal tissue, but that combining PARP inhibitors with others agents would target multiple nodes in DNA damage responses and immune activation, minimize normal tissue toxicity, and also potentiate the result of radiotherapy.

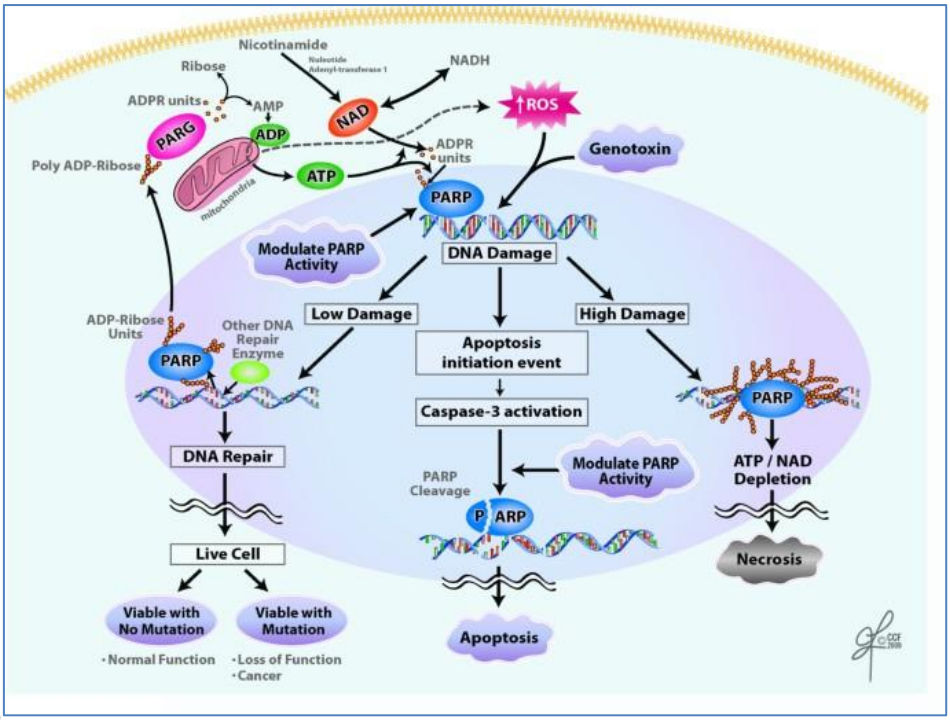

Figure 1.4: The multiple roles of PARP-1 (Agarwal et al., 2009). Following DNA damage, PARP-1 may play roles in DNA repair, apoptosis, and necrosis, depending on the extent of damage.

CHAPTER 2

2. Materials and Methods

2.1. Study Location and Ethics Consideration

This research as well as all the laboratory experiments were carried out in the Division of Radiobiology, in the Faculty of Medicine and Health Sciences (FMHS), at the University of Stellenbosch. No ethics approval was required since only established cell lines were used.

2.2. Cell Lines and Culture Maintenance

To conduct this study, two human lung cell lines (normal: L132; cancer: A549) were used. Cells routinely stored in liquid nitrogen (-196°C) were retrieved and cultured in their respective growth media.

2.2.1. L132

The human normal lung epithelial cell line, L132 (ATCC® Number: CCL-5™), is a p53 wild-type cell line. It was a gift from Dr T. Robson (University of Ulster, UK). L132 was used to represent normal tissue. The cells were routinely grown as monolayers in 25- or 75-cm^2 tissue culture flasks in Roswell Park Memorial Institute (RPMI-1640) medium (Sigma-Aldrich, USA, cat # R8758) supplemented with 10% foetal bovine serum (HyClone, UK, cat #SV30160.03) and 1% penicillin-streptomycin solution (Lonza, Belgium, cat # DE17-602E), and incubated at

37°C in a humidified atmosphere (95% air, 5% CO_2). Cells were used for experiments upon reaching 70-80% confluence. L132 was used between passage numbers 30-45 for the study.

2.2.2. A549

A549 is an adenocarcinoma human alveolar basal cell line collected from a 58-year old Caucasian male. It has an epithelial-like structure and is often utilised for the study of lung cancer. It is hypotriploid and grows as a monolayer in RPMI-1640 medium (Sigma-Aldrich, USA, cat # R8758), mixed with 10% heat-inactivated foetal bovine serum (HyClone, UK, cat #SV30160.03), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Lonza, Belgium cat # DE17-602E). The cells were routinely cultured at 37°C in a humidified atmosphere (95% air, 5% CO_2). The A549 cell line has been used extensively in cancer proliferation studies and has served as a testing ground for novel drug development (Giard et al., 1973). The cell line was purchased from Cellonex (Johannesburg, South Africa, cat # CA54-C) and passages ranging from 25-65, at 70-80% confluence, were used in this study.

2.3. Target Inhibitors

Two inhibitors, ABT-737 and ABT-888, were used to target vital cell survival proteins, namely Bcl-2 and PARP-1, respectively.

2.3.1. ABT-737

ABT-737 ($C_{42}H_{45}ClN_6O_5S_2$; MW = 813.4 g; cat # 11501, Biocom Africa, South Africa) is a small molecule drug that inhibits two members of the Bcl-2 family, namely Bcl-2 and Bcl-xL. The chemical structure of ABT-737 is shown in Figure 2.1. ABT-737 is not orally bioavailable which can limit its administration, especially in the case of multiple fractionated doses or as an adjuvant. In this study, 3.1 mM stock solution of ABT737, dissolved in DMSO, was stored at -20ºC until used.

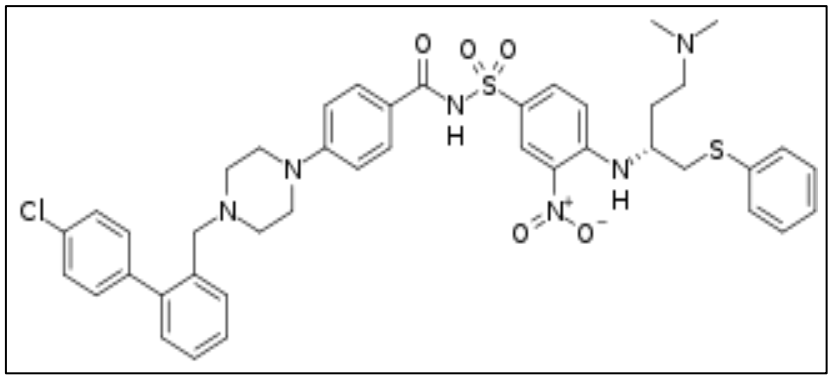

Figure 2.1: Chemical structure of ABT-737.

2.3.2. ABT-888

ABT-888 ($C_{13}H_{16}N_4O$; MW = 244.29 g; gift from the Drug Synthesis and Chemistry Division (National Cancer Institute, USA)) inhibits the PARP activity in cells. ABT-888 inhibits the DNA repair mechanism by blocking the action of the polymerase enzyme PARP-1 and reduces clonogenic survival (Bouchard et al., 2003). This makes ABT-888 an attractive drug in the treatment of recurrent resistant tumours (Bouchard et al., 2003; Yuan et al., 2017; Bhute et al.,

2016). Figure 2.2 shows the chemical structure of ABT-888. The stock solution consisted of 2.1 nM of ABT888, dissolved in DMSO, and was stored at -20ºC until used for experiments.

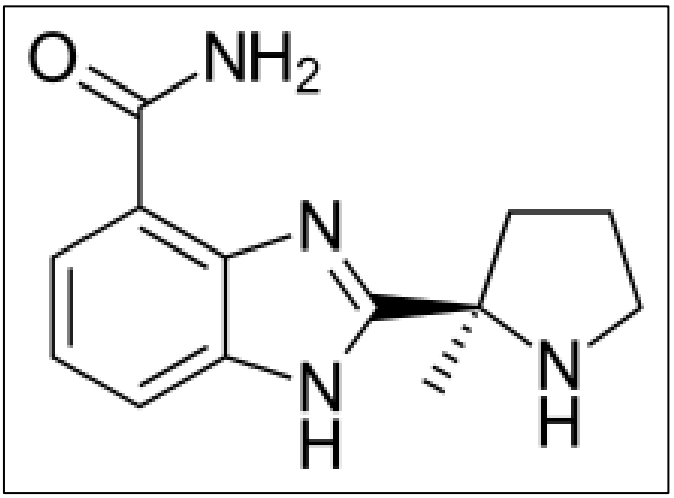

Figure 2.2: Chemical structure of ABT-888.

2.4. Irradiation of Cell Cultures

Cells were irradiated to doses ranging from 0 to 10 Gy at a dose rate of 1.0 Gy/min, using a Precision MultiRad 160 X-ray irradiator (Precision X-Ray Inc., Branford, CT, USA) at the Division of Radiobiology (Faculty of Medicine and Health Sciences, Stellenbosch University). The irradiation of cells was performed at a source-to-sample distance of 65 cm at room temperature (22°C), with 10 ml of culture medium in the 25-cm^2 flasks serving as build-up material.

2.5. Clonogenic Cell Survival Assay

The colony formation assay was used to assess cell survival following irradiation and specific inhibitor treatment. Briefly, 70-80% confluent stock cultures were washed with sterile phosphate buffered saline (PBS) (Lonza, Belgium, cat # 17-512F), trypsinised, and the cells counted using a haemocytometer. Cells were then seeded in triplicate per experiment in 25-cm^2 tissue culture flasks at 100 - 10 000 cells per flask (for X-ray exposure, depending on the radiation absorbed dose: 0 - 10 Gy) and from 200 to 6000 cells per flask (for inhibitor treatment, depending on inhibitor concentration: 0 - 10000 nM). After 4-5 hours of incubation at 37°C for the cells to attach, the cultures were subjected to the respective radiation doses or inhibitor concentrations. The cell cultures were exposed to inhibitors for the entire duration of experiments. The cultures were then incubated for 7 or 14 days (depending on cell line) for colony formation. The normal cell line were incubated for 14 days, while its counterpart cancer cell line was incubated for 7 days. The growth media were decanted and the colonies washed with PBS, fixed with a mixture of glacial acetic acid, methanol, and deionized water (v:v:v:) for 10 minutes, stained with 0.01% Amido Black in fixative for 30 minutes. The stained colonies were then washed in tap water and left to dry. The colonies were counted using a stereoscopic microscope (Nikon, Japan; Model #: SMZ-1B). The average of the surviving fractions (*SF*) for three independent experiments were determined, following each respective radiation dose and inhibitor concentration, as follows:

$$SF=n_{col}(D)/\{[n_{col}(u)/n_{cell}(u)]\times n_{cell}(D)\} \text{ or } SF=n_{col}(C)/\{[n_{col}(u)/n_{cell}(u)]\times n_{cell}(C)\} \quad (2.1),$$

where $n_{col}(u)$ and $n_{col}(D)$ (or $n_{col}(C)$) are the number of colonies scored in non-irradiated (or untreated with inhibitor) (control) samples and those irradiated to dose D (or treated with inhibitor at concentration C), respectively. $n_{cell}(D)$ and $n_{cell}(u)$ are the number of cells seeded in irradiated (or treated with inhibitor at concentration C) and control cultures, respectively.

To obtain cell survival curves for radiation experiments, the surviving fractions for doses D (in Gy) were fitted to the linear-quadratic model:

$$SF = \exp[-\alpha D - \beta D^2] \qquad \text{(Eq. 2.2)},$$

where α and β are the coefficients of the linear and quadratic cell components of cell kill. From these curves, the radiosensitivity of the cell lines was determined and expressed in terms of the surviving fractions at 2 Gy (SF_2: for low fraction dose response) and 6 Gy (SF_6: for large fraction dose response), and mean inactivation dose which is the area under the cell survival curve ($\bar{D}$: for integrative low-high dose response).

Inhibitor cytotoxicity was depicted by plotting the surviving fractions (SF) against the logarithms of inhibitor concentrations (L_c) and fitting the data to a a 4-parameter logistic equation of the form:

$$SF = B + \frac{T-B}{\{1-10^{[(logEC_{50}-L_c)HS}\}} \qquad \text{(Eq. 2.3)},$$

where *B* and *T* are the minimum and maximum of the sigmoidal curve, respectively, EC_{50} is the equivalent concentration for 50% cell kill, and *HS* is the steepest slope of the curve.

2.6. Effect of Bcl-2 and PARP-1 Inhibitors on Radiation Response

To evaluate the effect of inhibiting Bcl-2 and PARP-1 on radiosensitivity, the colony assay was performed in irradiated cell cultures that were pre-treated with ABT-737 and ABT-888, respectively. For this, pre-prepared cultures (seeded at 300 to 10000 cells per 25-cm^2 flask) were treated with inhibitors, singly or combined, at the EC_{50} estimated from the toxicity experiments (Eq. 2.3) 30 min prior to irradiation (at 2 and 6 Gy). The cells were then incubated for colony formation (7 days for A549; 14 days for L132), and surviving fractions were determined as described under Section 2.5. Inhibitors were left in cell culture media for the entire duration of experiments. The radiomodulatory effect of the inhibitors was expressed in terms of a survival modifying factor (MF) for small and large fractional doses, defined as:

$$MF_2 = SF(2\ Gy)/\{SF(EC_{50}+ 2\ Gy)\}\ or\ MF_6 = SF(6\ Gy)/\{SF(EC_{50}+ 6\ Gy)\} \qquad (2.4).$$

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

2.7. Data Analysis

Data were analysed using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Data were presented as the mean (± SEM) from three independent experiments. For each experiment and data point, 3 replicates were assessed. Where necessary, errors were determined using appropriate error propagation formulae. For the comparison of two data sets, the unpaired two-sided *t*-test was used. A $P < 0.05$ indicated a statistically significant difference between the data sets.

CHAPTER 3

3. Results

3.1. Intrinsic Radiosensitivity

The response of the human lung cell lines (normal: L132; cancer: A549) used in this study to exposure to ionising radiation was determined using the colony formation assay (14 days for the normal cell line; 7 days for the cancer cell line). The respective cell survival curves, as obtained by fitting survival data to the linear-quadratic model (Eq. 2.2) are shown in Figure 3.1. On average, the normal cell line is more radiosensitive and exhibits a steeper survival curve than its cancer counterpart. This is also apparent from the radiobiological parameters presented in Table 3.1.

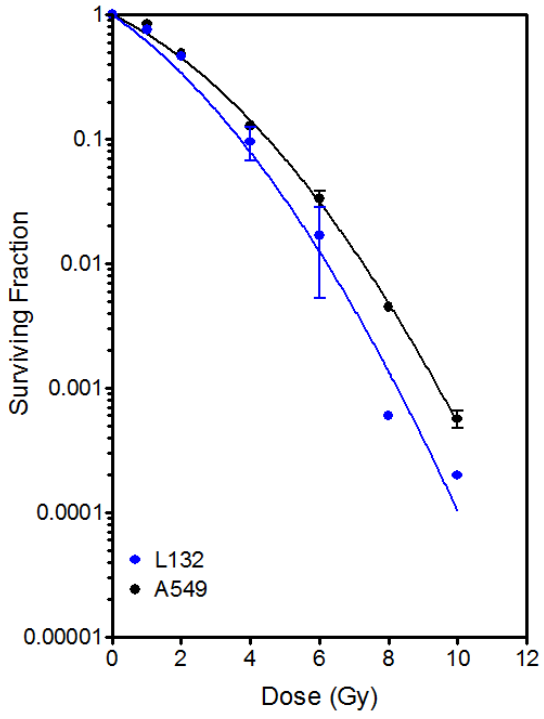

Figure 3.1. Clonogenic cell survival curves for 2 human lung cell lines (normal: L132; cancer: A549) after X-ray irradiation. Symbols represent the mean surviving fraction ± SEM from three independent experiments. Survival curves were obtained by fitting experimental data to the linear-quadratic model.

However, the value of SF_2 and $\bar{D}$ as predictors of differential radiosensitivity between L132 and A549 is minimal as the differences in these parameters did not reach statistical significance. A comparison of the SF_2 and $\bar{D}$ values of the cell lines yielded *P*-values of 0.7158 and 0.1756, respectively. The normal cell line is significantly more radiosensitive at large fractional radiation doses of the order of 6 Gy than its malignant counterpart ($P = 0.0011$). The α/β ratio which is routinely used in the clinic as a tool for gauging the radiation response of tissue also emerged as a sensitive predictor of radiosensitivity, with L132 being approximately 40% more radiosensitive than A549 (Table 3.1).

Table 3.1. Summary of radiobiological parameters for the L132 and A549 cell lines. SF_2 and SF_6 denote the surviving fraction at 2 and 6 Gy, respectively. α and β are the linear and quadratic coefficients of cell inactivation, respectively. $\bar{D}$ denotes the mean inactivation dose (area under the cell survival curve). Data are presented as the mean ± SEM from 3 independent experiments.

Cell Line	SF_2	SF_6	$\bar{D}$ (Gy)	α (Gy^{-1})	β (Gy^{-2})	α/β (Gy)[#]
L132	0.44±0.03	0.0143±0.0012	2.18±0.12	0.45±0.10	0.047±0.013	9.57±3.40
A549	0.45±0.02	0.0320±0.0031	2.41±0.08	0.31±0.03	0.045±0.003	6.89±0.81

[#]Errors calculated from a standard error propagation formula.

3.2. Cytotoxicity of ABT-737 in L132 and A549 Cells

Inhibition of Bcl-2 with ABT-737 yielded a concentration-dependent cell kill in the L132 and A549 cell lines (Figure 3.2). At toxic concentrations of the inhibitor, the normal cell line (L132) shows a much steeper cytotoxic response than the cancer cell line (A549), indicating that small increments in ABT-737 result in more cell kill in the former.

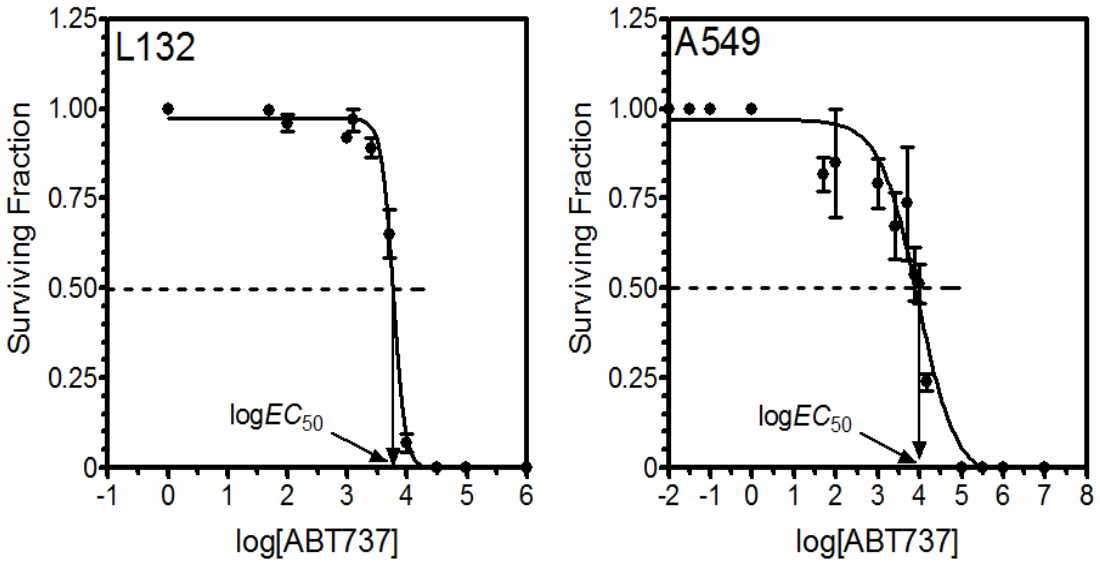

Figure 3.2. Cytotoxicity curves of the L132 and A549 cell lines when treated with a Bcl-2 inhibitor (ABT-737). Curves were obtained by plotting the cell survival as a function of log(inhibitor concentration). Cell survival was determined by the colony assay, and data were fitted to a 4-parameter logistic equation (Eq. 2.3). Data points are means ± SEM of 3 independent experiments. The log of the concentration at which 50% of cells survive (logEC_{50}) is that at which each survival curve intersects the horizontal dashed line (as indicated by the arrow heads).

The observation that ABT-737 is more toxic in the normal than the cancer cells is also apparent from the inhibitor cytotoxicity parameters that are summarised in Table 3.2, with the L132 requiring about 65% of the inhibitor concentration needed by the A549 for 50% cell kill. This is confirmed by the larger slope (*HS*) of the curve for L132.

Table 3.2: Summary of cytotoxicity data for the L132 and A549 cell lines treated with Bcl-2 inhibitor (ABT-737). 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 3.2). *HS* is the steepest slope of the curve (negative sign indicates a left to right decline in response with increasing concentration).

Treatment	Cell line	EC_{50} (nM)	*T*	*B*	*HS*
ABT-737	L132	5819±163	0.97±0.01	-0.002±0.001	-4.46±0.49
	A549	8996±1323	0.97±0.02	-0.030±0.029	-0.97±0.15

3.3. Cytotoxicity of ABT-888 in L132 and A549 Cells

When PARP-1 was inhibited by ABT-888, a concentration-dependent cytotoxic effect was also observed in both cell lines (Figure 3.3). As in the case of Bcl-2 inhibition, the normal cell line (L132) was more sensitive to PARP-1 inhibition than its cancer counterpart (A549).

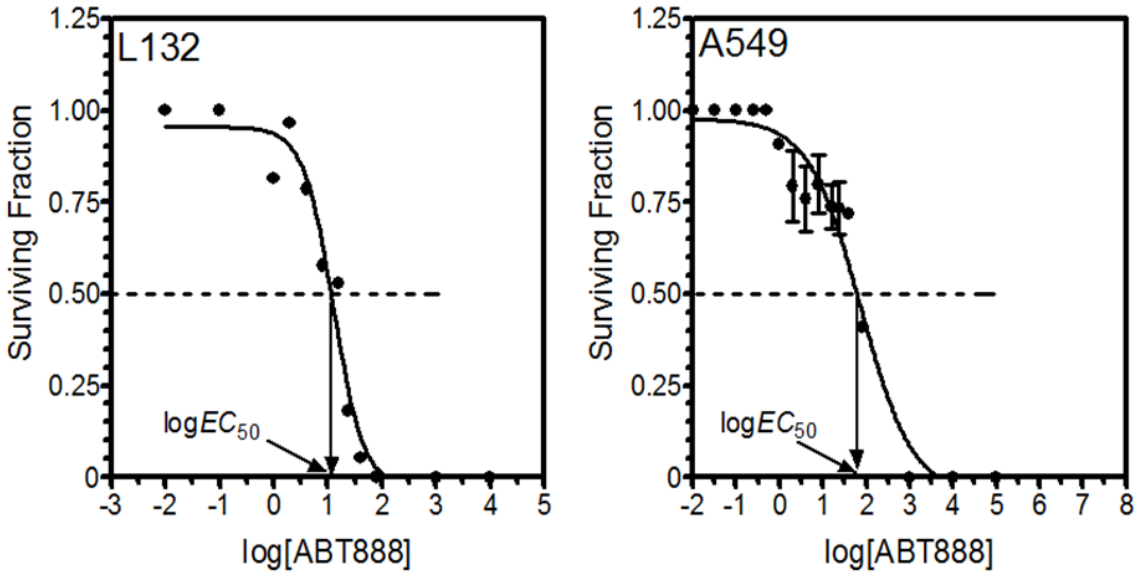

Figure 3.3. Cytotoxicity curves of the L132 and A549 cell lines when treated with a PARP-1 inhibitor (ABT-888). Curves were obtained by plotting the cell survival as a function of log(inhibitor concentration). Cell survival

was determined by the colony assay, and data were fitted to a 4-parameter logistic equation (Eq. 2.3). Data points are means ± SEM of 3 independent experiments. The log of the concentration at which 50% of cells survive (logEC_{50}) is that at which each survival curve intersects the horizontal dashed line (as indicated by the arrow heads).

The ABT-888 cytotoxicity parameters are listed in Table 3.3. The normal cell line requires only about 17% of the EC_{50} of the cancer cell line to reach a 50% cell death, indicating that use of ABT-888 alone might lead to extensive normal tissue toxicity. The high sensitivity of the L132 cell line to ABT-888 treatment is also clear from its cytotoxicity curve showing a ~2-fold slope relative to that of the A549 cell line.

Table 3.3: Summary of cytotoxicity data for the L132 and A549 cell lines treated with PARP-1 inhibitor (ABT-888). 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 (negative sign indicates a left to right decline in response with increasing concentration).

Treatment	Cell line	EC_{50} (nM)	*T*	*B*	*HS*
ABT-888	L132	12.79±2.35	0.96±0.05	-0.027±0.055	-1.56±0.38
	A549	74.83±23.04	0.98±0.03	-0.046±0.048	-0.73±0.14

3.4. Modulation of Radiation Response by Inhibitors

To evaluate the effect of inhibition of Bcl-2 and PARP-1 with ABT-737 and ABT-888, respectively, on the radiation response of L132 and A549 cells, radiomodifying factors were estimated as described under Section 2.6 (Eq. 2.4). Single or concomitant inhibition of Bcl-2 and PARP-1 in the normal cell line (L132) prior to a 2-Gy exposure resulted in an insignificant

radiosensitisation (Table 3.4; $0.22 \leq P \leq 0.56$). Although the inhibition yielded up to a 2-fold radiosensitisation and corresponding modifying factors ranging from 2.05 to 3.40, the reduction in the radioresistance of L132 did not reach statistical significance. This is likely due to the large errors in surviving fraction, especially those for irradiation alone and pre-treatment with ABT-888. The surviving fractions following irradiation alone in the radiomodulation experiments (Table 3.4) were generally higher than those obtained in the preliminary intrinsic radiosensitivity experiments (Table 3.1), but the two data sets were comparable within the limits of experimental error.

For the cancer cell line (A549), inhibition of Bcl-2 and PARP-1 (singly or in combination) consistently resulted in moderate to strong radiosensitisation, with modifying factors ranging from 1.52 to 5.30 (Table 3.4). Specifically, pre-treatment with ABT-737 alone or a combination of ABT-737 and ABT-888 yielded a significant reduction in cell survival of the order of 2.3 to 5.3-fold (Table 3.4; $P \leq 0.0187$).

Table 3.4. Modifying factors (*MF*), relative to X-ray treatment alone, derived from clonogenic survival at 2 Gy, for 2 human lung cell lines (normal: L132; cancer: A549) irradiated in the presence of ABT-737 and ABT-888.

Cell line	Treatment	*SF*	*P-value*	*MF*[#]
L132	2 Gy	0.4935 ± 0.3035	-	-
	ABT-737+2 Gy	0.2143 ± 0.0308	0.3111	2.30 ± 1.45
	ABT-888+2 Gy	0.2405 ± 0.2185	0.5685	2.05 ± 2.25
	ABT-737+ABT-888+2 Gy	0.1453 ± 0.0211	0.2240	3.40 ± 2.15
A549	2 Gy	0.5930 ± 0.0853	-	-
	ABT-737+2 Gy	0.2597 ± 0.0177	0.0187*	2.28 ± 0.36
	ABT-888+2 Gy	0.3913 ± 0.0383	0.0973	1.52 ± 0.26
	ABT-737+ABT-888+2 Gy	0.1120 ± 0.0496	0.0082*	5.30 ± 2.46

*Significant difference in cell survival, relative to survival at 2 Gy; [#]Errors calculated from a standard error propagation formula.

Pre-treatment of the L132 cells with Bcl-2 and PARP-1 inhibitors (singly or concomitantly) before a 6-Gy irradiation resulted in a moderate ($P \leq 0.0556$) to large ($P \leq 0.0315$) reduction in cell survival, yielding modifying factors of 2.67 to 9.73 (Table 3.5). Similarly, pre-treatment of the cancer cell line with ABT-737 or ABT-888 led to significant radiosensitisation at 6 Gy, with modifying factors of 3.0 and 5.25, respectively (Table 3.5; $P = 0.0141$; $P = 0.0242$). A pre-irradiation treatment of A549 cells with a combination of ABT-737 and ABT-888 seemed to be too toxic and it was not possible to determine a surviving fraction, and a corresponding modifying factor. The addition of the inhibitors at a high dose of 6 Gy showed strong evidence against the null hypothesis, as significant radiosensitisation was observed in both cell lines, and could negatively impact on normal tissues.

Table 3.5. Modifying factors (*MF*), relative to X-ray treatment alone, derived from clonogenic survival at 6 Gy, for 2 human lung cell lines (normal: L132; cancer: A549) irradiated in the presence of ABT-737 and ABT-888. n.d. denotes parameters that could not be determined.

Cell line	Treatment	*SF*	*P-value*	*MF*[#]
L132	6 Gy	0.1070 ± 0.0000[&]	-	-
	ABT-737+6 Gy	0.0401 ± 0.0250	0.0556	2.67 ± 1.66
	ABT-888+6 Gy	0.0110 ± 0.0001	<0.0001*	9.73 ± 0.09
	ABT-737+ABT-888+6 Gy	0.0262 ± 0.0249	0.0315*	4.08 ± 3.88
A549	6 Gy	0.0420 ± 0.0050	-	-
	ABT-737+6 Gy	0.0140 ± 0.0031	0.0141*	3.00 ± 0.75
	ABT-888+6 Gy	0.0080 ± 0.0020	0.0242*	5.25 ± 1.45
	ABT-737+ABT-888+6 Gy	n.d.	n.d.	n.d.

*Significant difference in cell survival, relative to survival at 6 Gy; [#]Errors calculated from a standard error propagation formula; [&]error less than 0.0001 (actual error used in error propagation).

CHAPTER 4

4. Discussion

Ionising irradiation as monotherapy has been tremendously explored together with surgery and chemotherapy in clinics as an alternative therapeutic mode for cancers (Hastak et al., 2017). However, no matter the therapeutic potential of radiotherapy, significant limitations have been recorded due to tumour resistance to treatment, cancer cell regrowth and normal tissue damage. To address this challenge, a novel therapeutic approach employing cocktails of biomolecules in combination with ionising irradiation was evaluated in this study, with the ultimate aim of identifying biomolecule mixtures that could radiosensitise cancer cells to radiotherapy. Specifically, the effect of inhibiting anti-apoptotic Bcl-2 protein with ABT-737 and the DNA repair protein, PARP-1, with ABT-888, singly or in combination, on cellular radiosensitivity was evaluated. Inhibitor-induced changes in radiosensitivity of two human cell lines (cancer: A549; apparently normal: L132) were assessed using the colony forming assay. Inhibitors were used at their respective concentrations for 50% cell inactivation.

4.1. Intrinsic Cellular Radiosensitivity

A preliminary assessment of the relative cellular radiosensitivity showed no apparent difference in the radiation response the cell lines at 2 Gy, but the apparently normal cell line (L132) emerged more radiosensitive than its malignant counterpart (A549) at a higher dose of 6 Gy (Table 3.1). A similar trend in the radiosensitivity of these cell lines was previously observed (Hamid, 2019). However, the mean inactivation dose which accounts for the net cellular radiosensitivity over the entire dose range only showed that the cancer cells were marginally more radioresistant than the apparently normal cell line; and did not differ

significantly among the two cell types. The lack of a clear distinction in the radiosensitivity of the cell lines used here has also been demonstrated by other investigators (Verheye-Dua and Böhm, 1998; Hamid, 2019). This is also apparent in the absence of marked difference between the α/β ratios which are indicators of overall cell lethality in radiotherapy (Karagounis et al., 2017). These findings cannot be corroborated by the report that the A549 highly expresses complement factor H (CFH) while the L132 does not (Yoon et al., 2019). Overexpression of CFH would lead to faster replication in the cancer cells compared to their normal counterparts, which should render the former cells more susceptible to radiation insult. In the clinical realm, genetic factors play a crucial role in the variation in tissue response to radiotherapy (Andreassen et al., 2002; Ho et al., 2006; Barnett et al., 2009; Tung et al., 2015). The level of expression of the DNA repair genes and the activity of the encoded proteins are determinants of radioresistance. That the radiosensitivities of these cell lines are comparable may be explained by the fact that they both have wild-type p53 (Takeyama et al., 2004; Berglind et al., 2008), and should be expected to respond similarly to radiation-induced damage. The marginally higher radioresistance of the A549 cell line may be attributable to its downregulation of p53 expression (Reddy et al., 2020), which could potentially suppress p53-mediated cell inactivation following radiation exposure.

4.2. Cytotoxicity of ABT-737 and ABT-888

Based on clonogenic cell survival, the cancer cell line (A549) emerged more resistant to Bcl-2 inhibition with ABT-737 than its normal counterpart (L132), requiring ~55% more inhibitor for 50% cell inactivation than the latter (Figure 3.2; Table 3.2). The resistance to Bcl-2 inhibition seen in the A549 cells is consistent with that reported elsewhere (Han et al., 2015; Hamid, 2019). In the study conducted by Hamid, ABT-263 was used instead of ABT-737

(Hamid, 2019). Although both are Bcl-2 inhibitiors, ABT-263 appeared to be more effective (molar per molar) than ABT-737. This was confirmed by the higher concentrations of ABT-737 needed by both cell lines to reach 50% of the cell killing in the current study compared to those of Hamid (Hamid, 2019). It is conceivable that such relative resistance is due to expression of Bcl-2 in these cells (Han et al., 2015; Reddy et al., 2020). Higher levels of Bcl-2 expression have been reported in normal lung tissue (Pezzella et al., 1993). This could act as a precursor for the sensitivity to Bcl-2 targeting observed in the apparently normal L132 cells, as high expression of Bcl-2 would be an indication of a high level of dependence of the cells on this anti-apoptotic gene for survival. The apparent resistance of the cancer cells (A549) to ABT-737 treatment might also be due the observation that the inhibitor is effective on Bcl-2 and Bcl-xl, but not Mcl-1 (Yang et al., 2009). This may offer the malignant cells another anti-apoptotic pathway of escape.

A similar trend emerged when PARP-1 was inhibited with ABT-888, with the A549 cells requiring about 6-fold higher concentration of inhibitor to reach the 50% level of cell kill when compared with the apparently normal L132 cells (Figure 3.3; Table 3.3). This finding is in contrast with the report that the L132 cell line was more resistant to PARP-1 inhibition with a novel lipopeptide (Hajare et al., 2013). This inconsistency cannot be explained by differences in PARP-1 expression, as PARP-1 is minimally expressed in the cancer cell line, relative to its normal counterpart (Lee et al., 2013). The disparity in findings may be associated with factors such as an alteration of the drug target and heterogeneity in target cell population (Kvinnsland et al., 2001; Akudugu et al., 2011; Akudugu and Howell, 2012; Torres-Martinez et al., 2021). However, the cytotoxicity induced by ABT-888 in cell lines used here may be attributable to PARP-1 inhibition markedly resulting in the inhibition of other key survival pathways like the

protein kinase B, extracellular signal-regulated kinases (ERK1/2), and p38-mediated EGFR pathways (Chowdhury et al., 2019).

4.3. Radiomodulation by ABT-737 and ABT-888

In this report, pre-treatment of the Bcl-2 and PARP-1 inhibitors followed by a 2-Gy irradiation yield modifying factors of statistical significance only in the cancer cell line (A549). This emerged when cells were treated with either ABT-737 alone or an ABT-737/ABT-888 cocktail prior to irradiation, where the A549 cells were radiosensitise by ~2- to 5-fold (Table 3.4). At a larger dose of 6 Gy, prior inhibitor treatment resulted in an even higher radiosensitisation of ~4- to 10-fold in the apparently normal cell line (L132) for single ABT888 and ABT-737/ABT-888 cocktail exposure (Table 3.5). A radiosensitisation of ~3- to 5-fold was observed in the A549 cells following pre-treatment with ABT-737 alone or an ABT-737/ABT-888 cocktail; and was comparable to that seen in the 2-Gy irradiation.

The significant levels of radiosensitisation seen in the normal and malignant cell lines can be attributed to multiple factors that mediate the inherent sensitivity of the cells to the inhibitors. These include, but not limited to, the target expression profiles (Pezzella et al., 1993; Han et al., 2015; Reddy et al., 2020) and inactivation of non-targeted survival pathways (Chowdhury et al., 2019). Silencing of specific and non-specific targets by the inhibitors should be expected to render the cells more susceptible to radiation insult. The current findings, however, seem to indicate that use of these Bcl-2 and PARP-1 inhibitors may not be advisable in hypofractionated radiotherapy where large fractions of absorbed radiation doses are employed. This, as the apparently normal cells are radiosensitised to a much larger extent compared to their malignant counterparts; a likely indication that use of ABT-737 and ABT-888 with large

fractional doses might exacerbate normal tissue effects. The findings appear to suggest that inhibition of Bcl-2 alone or a concomitant inhibition of Bcl-2 and PARP-1, when combined with conventional radiotherapy (2 Gy per fraction) could be beneficial in the management of NSCLC.

CHAPTER 5

5. Conclusion

This study demonstrates that both the apparently normal and malignant cell lines (L132 and A549, respectively) are highly radiosensitised when Bcl-2 and PARP-1 are inhibited at a large fractional dose of radiation. However, inhibition of Bcl-2 alone or combined inhibition of Bcl-2 and PARP-1 at a lower dose of 2 Gy results in significant radiosensitisation in only the lung cancer cell line (A549). It can be suggested that inhibition of Bcl-2 and PARP-1 might be of benefit when combined with conventional radiotherapy, but not with hypofractionated radiotherapy.

Possible Future Avenues

To fully elucidate the potential benefits of combining Bcl-2 and PARP-1 inhibition with radiotherapy, it would be of interest to explore the following avenues:

1. Conduct in-depth studies on the mechanisms underlying the radiosensitisation exhibited by the Bcl-2 and PARP inhibitors, using techniques like western blotting, real time polymerase chain reactions, and flow cytometry.
2. An expansion of the panel of cell lines (multiple normal and malignant cell lines) could shed more light on cell-type specific radiosensitisation by the inhibitors.
3. Consideration of non-specific key parameters that may play a potential role in the explanation of the difference in radiosensitivity and alternative therapeutic modality of lung cancer.

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