

Towards a Personalised Theranostics Platform through an Improved Understanding of the Biological Effects of Radionuclide Therapy

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of

Doctor of Philosophy



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Statement of Originality

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Takanori Hioki

For my grandmother, 日置照子

Acknowledgements

Throughout this long journey, I have been extremely privileged to be mentored by countless brilliant minds in multiple disciplines. I am overwhelmed with gratitude towards every single person I met along the way. If you interacted with me at all, I most certainly asked for your help! Thank you for being a part of my life.

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何時も心配かけてごめんね。皆んなの支え無しでは研究の困難を乗り越える事は出来なかったよ。本当にありがとう。

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Left: Fluffy Truffy, Right: Savvy Cavvy

Abstract

The clinical incorporation of radiobiology into theranostic nuclear medicine for cancer treatment is still an emerging field. The complexities involved in personalising therapy using radionuclides must be investigated from a cellular level, to improve treatment outcomes and predict dose response for patients. This thesis studied an unexplored therapeutic use of an easily accessible diagnostic radionuclide, ^{18}F , which demonstrated a maximum *in vitro* cell kill effect of up to 90% at 30 Gy in a radioresistant prostate cancer cell line, LNCaP C4-2B. The relative biological effectiveness of 0.42 was observed at 50% cell survival, when compared to an external beam x-ray irradiation. The dose rate effects that heavily impact the repair probability of cells were also manipulated for the ^{18}F irradiation, showing a maximum of 12% difference in the cell survival at 15 Gy between a G-factor difference of 0.7 and 0.9, triggering cell cycle arrest in the G2/M phase. Finally, using ^{177}Lu patient images, two dosimetry software platforms, OLINDA/EXM and MIM Encore, were compared in a proof-of-concept biodistribution and activity prescription of ^{64}Cu , ^{68}Ga and ^{18}F , giving MIM/OLINDA activity ratios between 0.5 and 3.5 for six organs of interest. These studies collectively attempted to build a foundation for the personalisation of theranostics in nuclear medicine, while exploring novel modes of therapy using radionuclides with multiple decay pathways.

Works

Publications

Takanori Hioki, Yaser H. Gholami, Kelly J. McKelvey, Alireza Aslani, Harry Marquis, Enid M. Eslick, Kathy P. Willowson, Viive M. Howell, and Dale L. Bailey. Overlooked potential of positrons in cancer therapy. *Scientific Reports*, 11(1):2475 (2021) DOI: 10.1038/s41598-021-81910-4.

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Presentations

Oral Presentation

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Abbreviations

ADT	Androgen Deprivation Therapy
BCR	Biochemical Recurrence
BER	Base-Excision Repair
CRPC . . .	Castrate Resistant Prostate Cancer
CT	Computed Tomography
CTLA4 . .	Cytotoxic T-lymphocyte associated Antigen 4
DNA	Deoxyribonucleic Acid
DOTA . . .	Tetraxetan
DP	Delayed Plating
DSB	Double Strand Break
EBRT	External Beam Radiotherapy
EC	Electron Conversion
EDTA	Ethylenediaminetetraacetic Acid
EQLM . . .	Extended Linear Quadratic Model
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
Geant4 . . .	Geometry and Tracking 4
GS	Gleason Score
HDR	High Dose Rate
HR	Homologous Recombination
IP	Immediate Plating
IS	Intermediate Solution

LET Linear Energy Transfer
LINAC ... Linear Accelerator
LPL Lethal-Potentially Lethal
LQM Linear Quadratic Model
MC Monte Carlo
mCRPC .. Metastatic Castrate Resistant Prostate Cancer
MHC Major Histocompatibility Complex
MRI Magnetic Resonance Imaging
NET Neuroendocrine Tumour
NHEJ Non-homologous End Joining
NK Natural Killer Lymphocytes
NTCP Normal Tissue Complication Probability
PARP Poly Adenosine Diphosphate-ribose Polymerase
PBS Phosphate Buffer Saline
PCa Prostate Cancer
PD1 Programmed Cell Death Protein 1
PERT Positron Emission Radionuclide Therapy
PET Positron Emission Tomography
PPE Personal Protective Equipment
PRRT Peptide Receptor Radionuclide Therapy
PSA Prostate Specific Antigen
PSMA ... Prostate Specific Membrane Antigen
PVC Partial Volume Correction
PVE Partial Volume Effects
RBE Relative Biological Effectiveness
RMR Repair-Misrepair
RNT Radionuclide Therapy

ROS Reactive Oxygen Species
RPMI Roswell Park Memorial Institute
SARRP .. Small Animal Radiation Research Platform
SF Survival Fraction
SIRT Selective Internal Radionuclide Therapy
SPECT .. Single Photon Emission Computed Tomography
SSA Somatostatin Analogue
SSB Single Strand Break
SSR Somatostatin Receptor
TAC Time Activity Curve
TCP Tumour Control Probability
TOPAS .. Tool for Particle Simulation
ULD Ultra Low Dose
US Ultrasound
WS Working Solution

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CHAPTER 1

Introduction

We have entered a burgeoning era of nuclear medicine with the number of potential therapeutic and diagnostic radionuclides dramatically increasing along with the targeting agents, as the race to find the “perfect” *theranostic*, both a THERApeutic and diagNOSTIC, radioisotope or radioisotope pair bound to a highly specific targeting agent is underway. Alpha (α), beta-minus (β^-) and Auger electron emitters are being reconsidered anew in radionuclide therapy, with a myriad of new radioisotopes with varying energies and half-lives present in research and clinical trials. There has been a major shift in the focus of cancer radiotherapy from an external beam radiotherapy unit that targets large tumours or primary sites, towards a more targeted and personalised approach through systemic radionuclide therapy. This can be attributed to the high mortality rate of metastatic cancer, accounting for 90% of cancer related deaths.¹ Targeting specific up-regulated receptors on cancers, as well as trying to understand the immune-triggering doses of radiation treatment has been a topic of interest for the past two decades. More recently, the importance of personalisation of cancer treatment has been highlighted, as a “one-size-fits-all” approach has been used throughout the history of radiotherapy and must be improved upon to increase treatment efficacy.

Radiobiological studies have regained interest in hopes of developing a treatment planning system in radionuclide therapy that allows for personalisation, with parametrisation of patient specific tumour characteristics potentially leading to an improved efficacy.² For external beam radiotherapy, the radiobiology has been relatively well documented due to the constant rate of dose delivery, the method of delivery through a controlled beam, and most importantly, the large amount of patient data available from clinical follow-ups and surveillance.³ Radionuclide therapy differs in such a way that the decay of the radionuclide and thus the constantly decreasing dose rate, the biological uptake, retention and clearance,

inhomogeneous uptake within a tumour volume, longer treatment times leading to a need to take into account tumour dynamics, and finally the off-target effects resulting from the agents' limited specificity must all be taken into consideration when encapsulating radiobiology of radionuclide therapy as a whole.²

The importance of translational research is accentuated when elucidating the radiobiological parameters in radionuclide therapy. Basic scientific research including *in silico*, *in vitro* and *in vivo* experiments effectively simplifies the myriad of variables by working in a controlled environment; a crucial step in isolating the impact of certain effects that can later translate to further work and eventually clinical trials. Thus, ongoing discussions between researchers and clinicians on the concerns of both the lab and the clinic is imperative for a breakthrough in developing a personalised *theranostics* platform through an improved understanding of the radiobiology in radionuclide therapy.

This thesis investigate the methods to improve the efficacy of radionuclide therapy from an *in vitro* standpoint, by formulating a protocol then setting up a series of novel experiments to observe the radiobiological effects and potential treatment ideas for a new radionuclide modality. **Chapter 2** introduces the project's motivation through a literature review on the overarching theme of cancer and its treatment, also focusing on the advancements and the recent improvements in nuclear medicine. **Chapter 3** investigates the use of a potential radiosensitiser for use in radionuclide therapy, and attempts to characterise the suitable cell lines to use in the subsequent *in vitro* experiments. **Chapter 4** formulates a novel protocol on the theoretical and experimental methods in using radionuclides in an *in vitro* setting. **Chapter 5** presents on the potential use of positron emitters as a new radionuclide therapy modality in the treatment of cancer. The experiments were performed as a proof-of-concept. **Chapter 6** follows up on the use of a positron emitter fluorine-18 (¹⁸F), used in the previous chapter, and expands further to investigate the dose-rate effects, the Lea-Catcheside G-Factor, various *in vitro* assays to explore how the cell lines respond in high dose rate irradiation. **Chapter 7** explores the challenges of clinical dosimetry in therapeutic

nuclear medicine, and utilises two dosimetric software platforms to perform a proof-of-concept prescriptive dosimetry from a therapeutic surrogate radionuclide to positron emitting radionuclides. The conclusion and future work from the work in this thesis is summarised in *Chapter 8*.

Literature Review

2.1. Cancer Treatment

Cancer is a disease describing the uncontrolled proliferation of mutated cells that can hinder the function of healthy cells. This is due to the deregulation in cell division as a result of sufficient damage induced in the DNA through endogenous or exogenous factors. Over 14.1 million new cancer diagnoses were made in just 2012 alone, with an estimated 32.6 million people living with cancer, diagnosed in the past 5 years, and a total of 8.2 million deaths that year.⁴ The rising incidence and prevalence has made cancer a global epidemic, reflecting the urgency in the search for a cure.

Described in the highly influential reviews by Hanahan and Weinberg in 2000 with “*The Hallmarks of Cancer*”, and later in 2011 with “*Hallmarks of Cancer: The Next Generation*”, there are six distinct hallmarks, two emerging hallmarks and two enabling characteristics present across a myriad of cancer types.^{1,5} The hallmarks, shown in Figure 2.1, include the cancer cells’ abilities to invade and metastasise, avoid apoptosis, self-sustain growth factors, avoid growth suppression, self-vascularise and lastly, overcome telomere shortening. The emerging hallmarks are believed to be the abilities to avoid immune destruction and change the metabolism of the cells, and the enabling characteristics appear to be the inflammation that promotes tumours and the genomic instability that causes further mutations. To briefly summarise these hallmarks and characteristics, cancer cells have the ability to evade most of the body’s regulatory signals; whether it be the signals that trigger cell death or reduce the over-proliferation, the cells evolve in such a way that they become self-sustaining and self-regulating. The key argument in these reviews is the importance behind deconstructing the underlying principals observed in the enigma that is cancer. Through this process, coupled

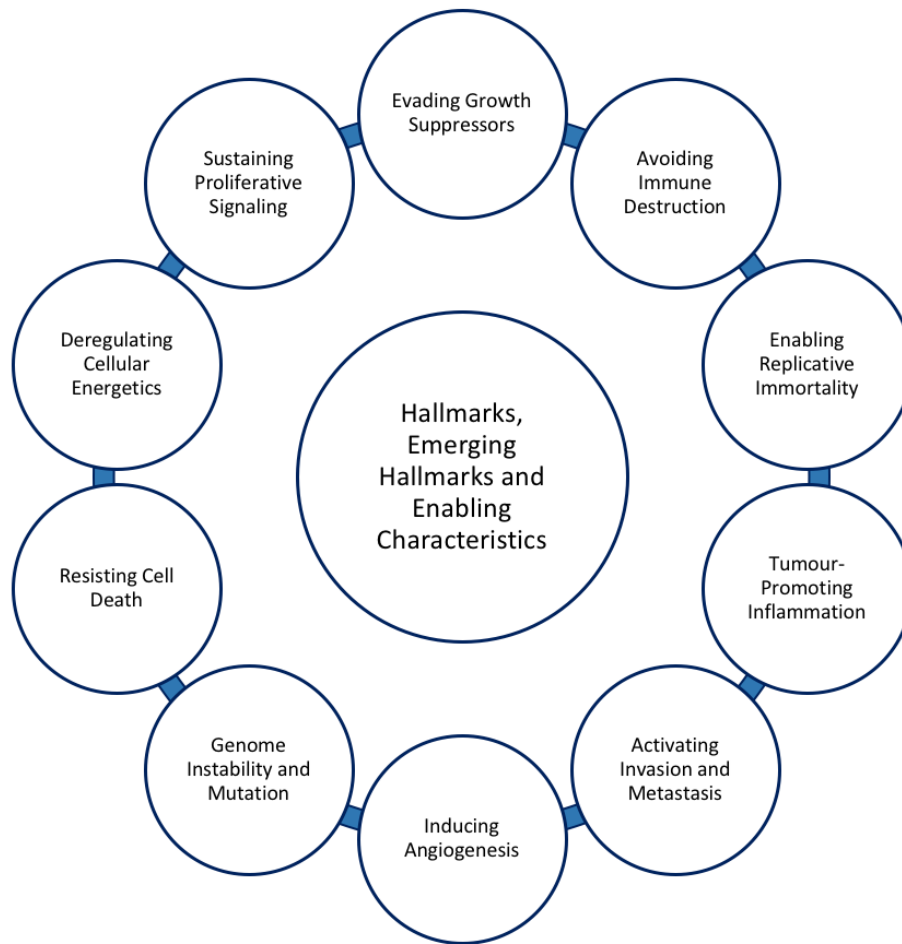


FIGURE 2.1. Reproduced diagram of the hallmarks of cancer¹

with the singular goal of the researchers and clinicians to find a cure, Hanahan and Weinberg strongly believe that the origins of carcinogenesis will be exposed.

Currently, there is no absolute treatment formulated to permanently eradicate cancer cells. Despite the curative intent of the treatments, many result in unpredictable side effects or recurrence. Regardless, major developments have been made in the treatments that are available, with dramatic improvements across multiple modalities. Broadly, these include surgery, chemotherapy and immunotherapy, radiotherapy, radionuclide therapy and hormonal therapy. Coupled with the improvements in the treatment methods, the ability to screen for cancer has also been improved. Early detection has the potential of dramatically

changing the prognosis, as the approach in treating the cancer is not as cost, time or physically demanding for the patients.

For the treatment of cancer, there are two categories referred to as local and systemic. Local treatments deal with the primary tumour site and the closely surrounding areas, whereas the systemic treatments tend to deal with the metastases, although primary sites are treated consequently.

2.1.1. *Local Treatment*

For larger primary tumours, the initial step would be to perform a local treatment, usually in the form of external beam radiotherapy (EBRT), surgery, or a combination of both. EBRT uses a high energy x-ray beam, generated from a linear accelerator (LINAC), to irradiate a volume of tumour whilst avoiding the vital organs and surrounding structures.⁶ Surgical procedures are not performed on large tumours due to a potential risk of harm to the surrounding tissue as well as a risk of promoting metastases, hence a combination of EBRT or chemotherapy and surgery is commonly performed to shrink the cancer before resection, referred to as neoadjuvant therapy.⁷ Resected tumours can then be followed up with adjuvant therapy of the tumour bed, or salvage therapy in the case of recurrence.⁸

Fractionated EBRT is a commonly practiced treatment regimen and its efficacy has improved dramatically in the last 30 years from the improved understanding of the radiobiological effects on the cells.^{9,10} The underlying concept behind the fractionation is to reduce the normal tissue complication probability (NTCP) whilst maintaining a certain level of tumour control probability (TCP) in a given dose delivery.^{11,12} The key components to consider are described in the “five R’s of fractionation”: Repair, Redistribution, Radiosensitivity, Reoxygenation and Repopulation.^{11,12}

Repair is the primary reason for fractionation, as the NTCP is reduced by irradiation using a lower dose. Multiple damage pathways can lead to tumour control, but can be broadly be grouped into lethal, sub-lethal and potentially lethal damage. Lethality is favoured for the

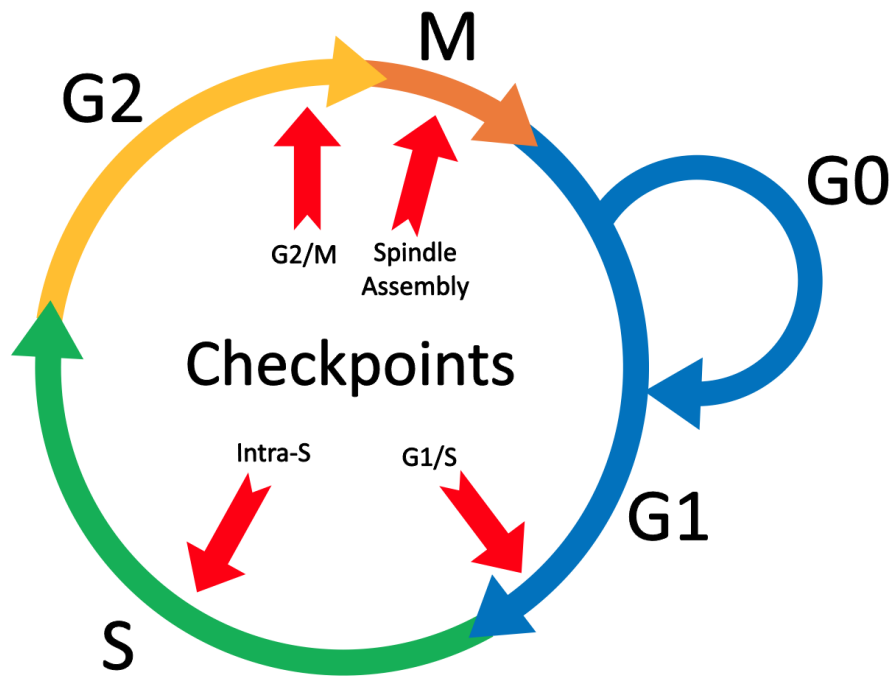


FIGURE 2.2. Cell cycle with four major checkpoints

tumour cells, as opposed to the repairable, sub-lethal damage preferred for the normal tissue. The tissue or DNA repair half-life is a crucial parameter to consider, as the fractionation schedule is heavily dependent on the normal tissues' ability to repair itself to prevent further development of cancer.

Redistribution refers to the cell cycle and the individual cells' distribution within this cycle, as DNA damage is the main target for tumour control in radiotherapy. Figure 2.2 is a simplified cell cycle, where the radiosensitivity varies from the most sensitive in the M and G2 phase, and least sensitive in the S phase. If DNA damage is induced, it activates a cell cycle checkpoint that triggers cell repair or cell cycle arrest that can potentially lead to programmed (apoptosis) or unprogrammed (necrosis) cell death. By fractionating the dose delivery in EBRT, the likelihood of targeting the cells at a more radiosensitive stage may be achieved.

Radiosensitivity is the variation in the intrinsic sensitivity to radiation damage for the both the healthy and cancerous cells and tissue. For the healthy cells, a limitation is placed on

how much dose can be delivered if a target of interest is close to a radiosensitive tissue such as the spinal cord. For cancer cells on the other hand, if a certain cell type is known to be more radioresistant than others in the same heterogeneous tumour volume, if the prescribed dose of radiation does not take this into account the treatment would be considered ineffective.

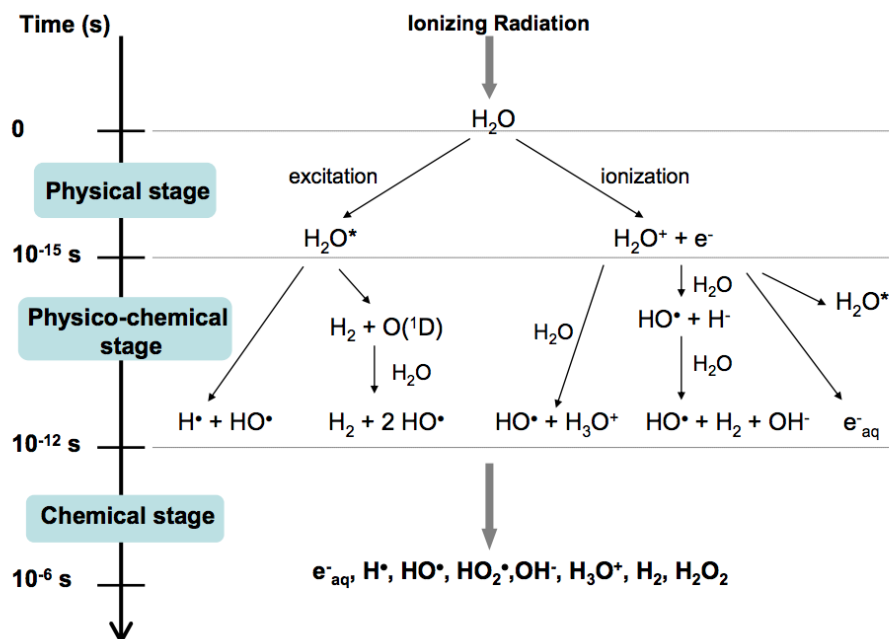


FIGURE 2.3. Diagram of the ROS formation from the radiolysis of water (Reproduced from Le Caer *et al.*, 2011)¹³

Reoxygenation takes into account the vascularisation of the tumour volume; the main damage pathway for EBRT is through the formation of reactive oxygen species (ROS), initiated by the ionisation or excitation of the water molecules in the irradiated target. A schematic of the processes involved is shown in Figure 2.3.¹³ The damage to the DNA or cells is therefore done indirectly, and it heavily relies on the presence of water and oxygen in the vasculature to cause damage to the cancer cells. Low levels of oxygenation in tumour volumes is referred to as hypoxia, and can be a limiting factor when irradiating with indirectly-ionising radiation. Attempts to overcome this is done by fractionating doses of EBRT where the outer, more oxygenated regions of a hypoxic tumour can be targeted with each fraction. The tumour generally becomes inflamed after the first fraction, followed by a

shrinkage if successful. The reduction in the size causes the previously hypoxic volume to be within closer proximity of vasculature, allowing for a gradual complete targeting of the tumour in theory.

Repopulation considers the ability for both the tumours and healthy tissues begin to proliferate or regenerate after the dose is delivered. Healthy cells and tissue can be categorised into early and late responding, where the former impacts the fractionation schedule more so than the latter. Large fractions in a shorter amount of time, or hypofractionation, must be avoided for early responding tissue, as the regeneration of healthy tissue cannot compete with the damage. For late-responding tissue, the time is less of an issue, but the amount of dose per fraction can impact its repopulation. Tumour repopulation is an important factor in the timing of the fractionation schedule. Although there are variabilities between tumour type, they generally have a repopulation rate similar to that of an early responding healthy tissue. One crucial difference is that an accelerated repopulation can be triggered if the time between the fractions is too long, leading to an aggravation of the disease. Taking both factors into account, a suitable dose and interval between the fractions are established.

Advancements in EBRT has led to the use and development of proton and heavy ion therapy, a technique that takes advantage of the Bragg peak to improve localisation of the target lesion while minimising irradiation of the nearby critical structures.^{6,14,15} Although originally proposed in 1946 by Wilson, proton therapy only received United States Food and Drugs Administration (US FDA) approval in 1988 and heavy ion therapy has still yet to be approved, despite multiple centres existing globally.^{14,15} The low entrance dose deposition, the high target dose from the Bragg peak and finally the low post-Bragg peak dose are the three major advantages of using particle EBRT, allowing for the normal tissue to be spared relative to conventional EBRT.¹⁴⁻¹⁶ Recent development of FLASH radiotherapy, a technique that utilises ultra-high dose rate EBRT to produce a phenomenon referred to as the '*FLASH effect*', has roused the scientific community's interests in the radiobiology

of EBRT as limitations such as NTCP and hypoxia can potentially be overcome.¹⁶ The FLASH effect is the reduction in NTCP whilst still maintaining TCP, triggered by using dose rates exceeding 40 Gy/s, several orders of magnitude higher than the conventional dose rate of roughly 5 Gy/min.¹⁶ The exact biological mechanisms of FLASH are still under investigation, but oxygen depletion, ROS generation and immune responses have all been suggested as contributing factors to this phenomenon.^{15,16} The first human clinical trial for proton FLASH radiotherapy, a technique that utilises the benefits of both proton therapy and the FLASH effect, is currently underway at the Cincinnati's Children's/UC Health Proton Therapy Center in the USA, and is gaining increasing interest for further research and clinical translation.¹⁷

2.1.2. *Systemic Treatment*

With the evidence for suspected metastases, whether before or after a local treatment is performed, the patient will have to undergo a systemic treatment. This involves any treatment that targets metastasised cancer cells through the circulatory system and is usually performed concurrently with a local treatment, although it is also used independently. Chemotherapy is the most accessible form of systemic treatment. A commonly used class of cytotoxic chemotherapeutics is the taxane group; the drugs target the cell division function by damaging the tubulins or the microtubules in the cells to promote cell death. Examples of the taxane group currently used include docetaxel (Taxotere®) and paclitaxel (Taxol®).¹⁸ Dependent on the aggressiveness of the tumour, chemotherapy can be performed before any local treatment to stabilise the tumour first. Due to the large amount of physiological stress the drugs place on the patients, chemotherapy may also not be an option for those with rapidly declining conditions and other contraindications.¹⁸

Cancer immunotherapy has been the next advancement in the systemic treatment of cancer, and is used as an alternative to chemotherapeutics despite its relatively slower effects. The basic mechanism of action for immunotherapy in cancer treatment has been

through the blockade of immune checkpoints. A deregulation of the immune-checkpoint proteins in tumours allows for the evasion of the body's innate immune response.^{7,19-21} As briefly summarised in Figure 2.4, there are three possible targets of immunotherapy: increasing the antigen expression of dendritic cells, promoting the production of the T-cells and overcoming of the immunosuppression by the tumours themselves. Regardless of the limitations in efficacy across all cancer types, prime target receptors, including the cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) that causes an immune resistance by reducing the early T-cell activation, and the programmed cell death protein 1 (PD1) and its associated ligand (PD-L1) that limits the effector T-cells' functions therefore allowing tumour proliferation, are the key to treating immunogenic cancers such as skin cutaneous melanoma, colon adenocarcinoma and diffuse large B-cell lymphoma. Examples of CTLA4 and PD-1 checkpoint inhibitors include ipilimumab (Yervoy®) and nivolumab (Opdivo®), respectively.¹⁹

Finally, nuclear medicine, the main field addressed in this thesis, specifically uses radioactive isotopes or radionuclides to either diagnose or treat tumours, although more specifically its metastases. These radionuclides can be bound to a ligand or micro/nanoparticle to target receptors specific to certain cancers or take advantage of the increased metabolic activity of most cancers in general, known as the Warburg effect.²² Some radionuclides such as iodine-131 (¹³¹I) and radium-223 (²²³Ra) are used in its unbound state, utilising the body's uptake mechanism specific to that ion.²³ Details of the main branches of nuclear medicine are outlined in the section below.

2.2. Emergence of Nuclear Medicine

The rapid development in the therapeutic methodologies aforementioned was only possible due to the localisation of the tumours and other diseases through imaging techniques using radioactive isotopes, used in the field of diagnostic nuclear medicine.³ The underlying concept of nuclear medicine is the injection of a bound gamma (γ) or

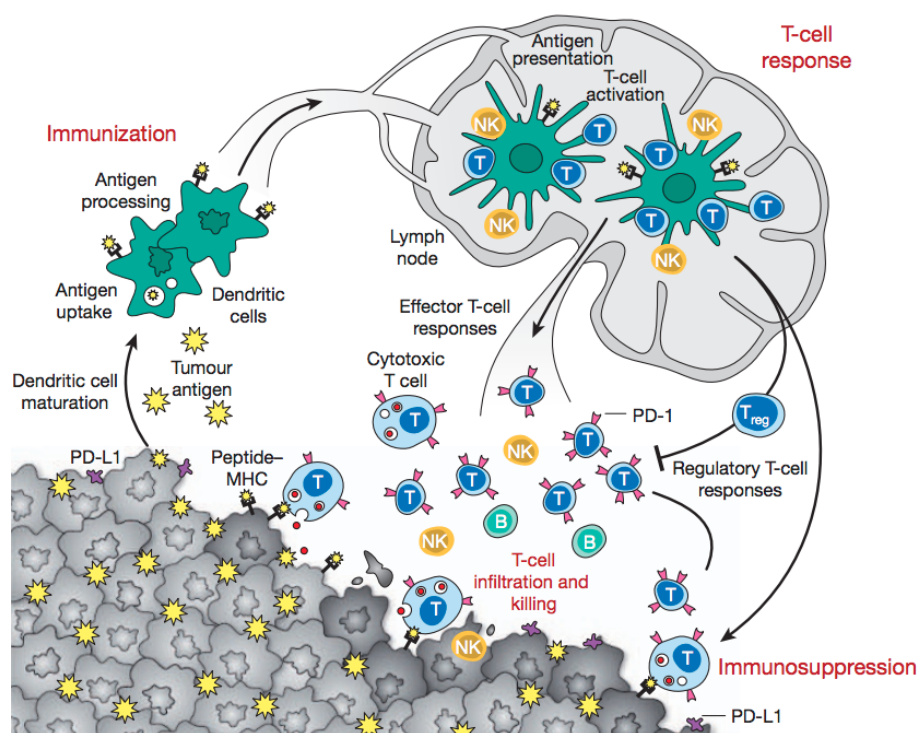


FIGURE 2.4. Diagram of the three immunotherapy targets, where NK is the natural killer lymphocytes, B is B lymphocytes and MHC is the major histocompatibility complex. (Reproduced from Mellman *et al.*, 2011)¹⁹

positron (β^+) emitting radionuclide with a suitable detection modality, either a single photon emission computed tomography (SPECT) or a positron emission tomography (PET), respectively. Dependent on the scan of interest, images are obtained at certain time points to measure uptake or biodistribution of the radionuclide-bound targeting agent to diagnose diseases.³ More recently, with the development of nuclear reactors, the production of a wider range of radionuclides have become possible, extending nuclear medicine into therapeutic applications through the use of beta minus (β^-), Auger electron or alpha (α) particle emitting isotopes.^{24,25}

2.2.1. Diagnostic Nuclear Medicine

The diagnostic radionuclides allow for a functional snapshot of the governing biological mechanisms behind the radiolabelled compounds; co-registration with the anatomical

images of x-ray computed tomography (CT) or nuclear magnetic resonance imaging (MRI) completes the comprehensive diagnostic image. The first notion of medical imaging was initiated through the discovery and study of x-rays by Röntgen in 1895.³ The ability to observe the internal contents of the body non-invasively, although mostly structural features, quickly opened up a new field of diagnostic medicine. The first emergence of nuclear medicine was reported to be between the period of 1910 and 1945, with the initial studies performed by de Hevesy in 1913 with lead nitrate tracers in plants, and by Blumgart and Weiss in 1927 with intravenous radon measurements.^{3,26-28}

Following the increased interest in radioactive isotopes during the Second World War and the improvements in technology that accompanied it, the field of nuclear medicine was established in the late 1950s. With the development of the scintillation γ detector by Anger in 1958 and the first use of the radioactive isotope technetium-99m (^{99m}Tc) by Harper *et al.* in 1964, the foundation of the diagnostic techniques still used in SPECT today, developed by Kuhl *et al.* in the 1970's, were standardised.^{3,26} The basic principles behind SPECT involves the detection of a distinct γ ray emission peak from a radioactive isotope within the body, followed up by a scatter and attenuation correction and a filtered back-projection algorithm to reconstruct an image of the acquired volume. The other major component involved in SPECT, apart from the physical camera itself, such as the collimator, detector type and arrangement and the various algorithms assigned, is the choice of radionuclide. The use of ^{99m}Tc was the initiator for medical imaging physics, mainly due to the extremely favourable characteristics it possesses clinically, and currently continues to be the most commonly used isotope in nuclear medicine.^{3,26}

Stemming from the idea of using positron emitting radionuclides for imaging by Wrenn *et al.* in 1951, along with the computational algorithms of tomography introduced by Kuhl *et al.*, PET was established soon after by Phelps *et al.* during the late 1970s.^{3,29} The concept of acquiring coincidence annihilation γ rays using scintillation detectors coupled with a computational reconstruction algorithm to generate an image in PET is very similar

to SPECT with a few exceptions. The major differences to be noted are the use of a positron emitting radionuclide, the circular arrangement of the detectors and the specific detection of 0.511 MeV annihilation photons.

The underlying mechanism of PET detecting the 0.511 MeV photons is the electron-positron annihilation that occurs after the thermalisation of a positron. When a positron is emitted from the radionuclide source in the body, it will travel a short distance before it collides with its anti-particle, an electron, and converts all of their rest-mass energies into photons. The two photons produced in this event are emitted approximately 180° from each other in a “back-to-back” emission, and the coincidence detection allows for the detectors on either side of the annihilation to locate where the signal originated from. A more complicated algorithm incorporated into the PET scan that is now of common use is the *time-of-flight* PET. This algorithm uses equation 2.1, where Δd is the distance away from the midpoint of the two detectors, Δt is the difference in arrival time of the γ rays, and c is the speed of light, to accurately reconstruct the data obtained from the annihilation events that are not occurring at the centre of the detectors.³

$$\Delta d = \frac{\Delta t \times c}{2} \quad (2.1)$$

When the *time-of-flight* calculates the location, it determines the point at which the annihilation occurred. The exact location of the positron emission is not known, thus the exact location of the tumour volume cannot be defined. Furthermore, when the annihilation takes place, the two photons are not emitted at exactly 180°, referred to as photon noncollinearity, another source of limitation to PET. There are simulation studies currently being performed to develop an algorithm that incorporates positron range correction, although its clinical implementation will take some time.³⁰⁻³² Using the example of a commonly used positron emitter fluorine-18 (¹⁸F), the energy at which the positrons are

emitted are not monoenergetic, as shown in Figure 2.5. The consequence of the emission spectrum is the variability in the distance the positron travels before the annihilation occurs. Not only is there a variability between the positrons of different energies, but the particles with the same energy can have different ranges depending on the path it takes during its energy loss; calculating the probabilities of every single emitted positron is not computationally feasible or required with the limitations in resolution. There will always be a fundamental limit to PET and SPECT's spatial resolutions despite the advancements in algorithms and hardware, but the invaluable tool as non-invasive functional diagnostic modalities have been revolutionary in cancer medicine.

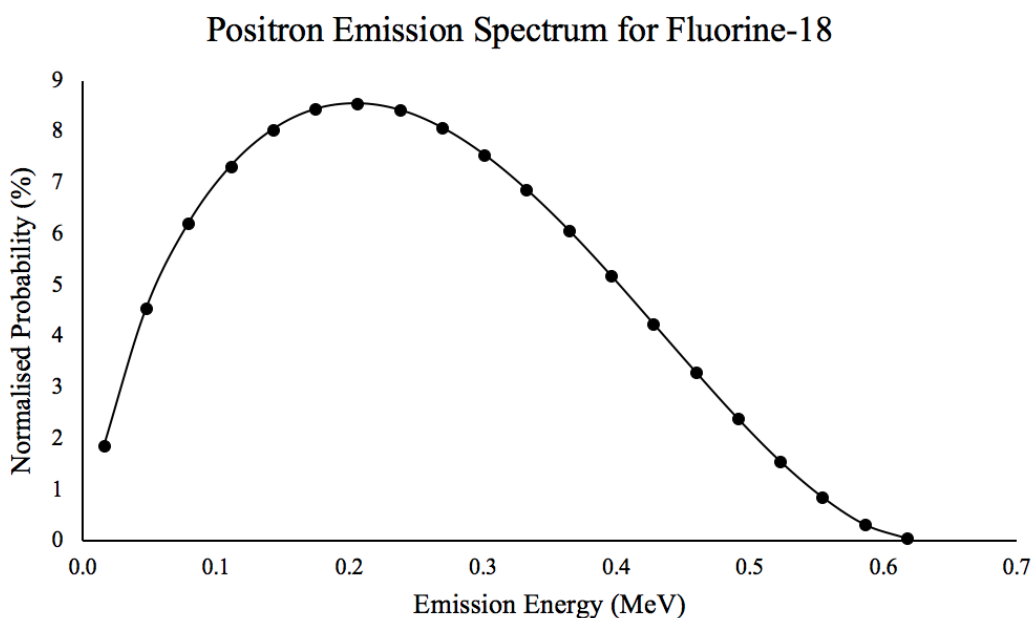


FIGURE 2.5. Positron emission spectrum for fluorine-18³³

2.2.2. *Therapeutic Nuclear Medicine*

Beyond the diagnostic use of radionuclides, the regained clinical interest in the therapeutic branch of nuclear medicine from the improvements and refinements in molecular biology has allowed for an increase in the implementation of radionuclides in the treatment of cancer.³⁴ Although the first clinical use of radionuclides as a form of cancer

treatment was performed using ^{131}I in 1946 on thyroid cancer patients, the availability of nuclear reactors and cyclotrons have limited the production and use of the radionuclides in therapy.³ The available radionuclides in medicine have flourished since the 1940s, especially for cancer therapy, and have allowed for the fields of brachytherapy and radionuclide therapy (RNT), specifically, peptide receptor radionuclide therapy (PRRT) and selective internal radionuclide therapy (SIRT), to be established.^{3,34,35} A non-exhaustive list of radionuclides used the clinic is provided in Table 2.1, where EC is electron capture. The resultant emission of internal conversion electrons, x-ray and γ photons from the Auger effect and the Auger electrons are not listed under “Mode of Decay” for simplicity, although their importance is discussed later.

2.2.3. *Theranostics*

The various innovative ideas developed in delivering radionuclides directly to the tumours through receptor or functional targeting have allowed critical patients to extend their overall survival and potentially improve their quality of life. In 1998, John Funkhouser, the CEO and president of PharmaNetics at the time, coined the term ‘*theranostics*’ describing a material that possess both diagnostic and therapeutic properties.³⁷ Although ^{131}I had been used in such a manner, the prevalence of theranostics had been limited, stemming from the difficulties in discovering molecular targeting agents for cancer.³⁷ PRRT, established in the late 1980s, have regained interest especially through the scope of theranostics in the past two decades, due to the implementations of the new radionuclides available to the clinic, as well as the discovery and improvements in the specificity of targeting agents to the cancer cells.^{34,37,38}

For example, in neuroendocrine tumour (NET) patients, the presence of somatostatin receptors (SSRs) have allowed for a group of compounds called somatostatin analogues (SSAs), such as lanreotide and octreotide, to control carcinoid syndrome; a condition caused by the release of vasoactive peptides and hormones by the NETs leading to diarrhoea,

Radionuclide	Half-life	Mode of Decay	Use
^{11}C	20.4 minutes	β^+ : 99.7669%	PET tracer
^{13}N	9.9 minutes	β^+ : 100%	PET tracer
^{15}O	122.2 seconds	β^+ : 100%	PET tracer
^{18}F	109.8 minutes	β^+ : 100%	PET tracer
^{64}Cu	12.7 hours	EC: 61.5%, β^- : 38.5%	PET tracer, β^- -therapy
^{67}Cu	61.8 hours	β^- : 100%	β^- -therapy
^{67}Ga	78.3 hours	EC: 100%	SPECT
^{68}Ga	67.7 minutes	β^+ : 100%	PET tracer
^{90}Y	64.1 hours	β^- : 100%	Brachytherapy, β^- -therapy
$^{99\text{m}}\text{Tc}$	6.0 hours	IT: 99.9963%, β^- : 0.0037%	SPECT
^{103}Pd	17.0 days	EC: 100%	Brachytherapy
^{123}I	13.2 hours	EC: 100%	Thyroid diagnostic
^{124}I	4.2 days	β^+ : 22.7%, EC: 77.3%	PET tracer
^{131}I	8.0 days	β^- : 100%	Thyroid treatment & imaging
^{131}Cs	9.7 days	EC: 100%	Brachytherapy
^{177}Lu	6.6 days	β^- : 100%	β^- -therapy, SPECT
^{213}Bi	45.6 minutes	β^- : 97.8%, α : 2.2%	α -therapy
^{223}Ra	11.4 days	α : 100%	α -therapy
^{225}Ac	10.0 days	α : 100%	α -therapy

TABLE 2.1. Radionuclides used in medicine^{35,36}

flushing or bronchospasm, seen in roughly 20% of patients.³⁹ Through the modification of an SSR called tyrosine³-octreotate (a compound similar to octreotide) by conjugating a tetraxetan (DOTA) complexing agent, the chelation of radionuclides such as ^{68}Ga and ^{177}Lu has allowed for NET to be treated in the theranostic RNT setting, using an initial PET scan

to plan the treatment and post-injection SPECT scans to monitor the dose delivery as shown in Figure 2.6.^{40,41}

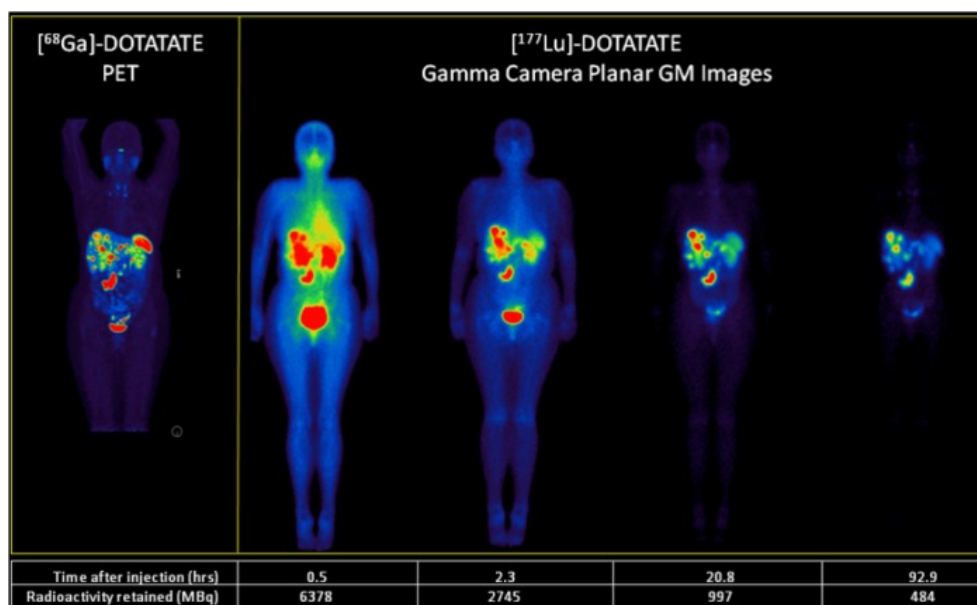


FIGURE 2.6. Image showing examples of a ^{68}Ga -DOTATATE PET/CT pre-treatment scan and ^{177}Lu -DOTATATE SPECT/CT scans at four time points post-injection. (Reproduced from Aslani *et al.*, 2015)⁴⁰

SIRT, also referred to as radioembolisation, is another example of a recently developed technique that has had success in the improvement of quality of life. An example of this modality, marketed as SIR-Spheres[®], utilises ^{90}Y resin microspheres that are embolised into the target of interest to deliver therapeutic β^- irradiation, shown in Figure 2.7.⁴² Unresectable hepatocellular carcinoma and intrahepatic cholangiocarcinoma can be treated using SIRT, generally followed up with chemotherapy or surgical resection if a significant reduction in tumour volume is observed. ^{90}Y not only emits β^- , but also has a small probability for a β^- decay to the first excited state of ^{90}Zr , leading to an internal pair production. Although the exact branching ratio for the pair production process is still under dispute, the process allows ^{90}Y to be imaged by PET, leading to better estimates in the dose quantification to the lesion. Centres not interested in the dose quantification choose to perform bremsstrahlung imaging to obtain images to confirm the embolised volumes using

the SPECT scanners.

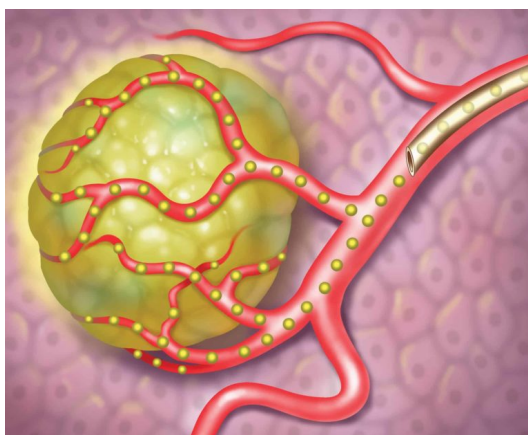


FIGURE 2.7. Image of a catheter delivering the ^{90}Y resin microspheres into the target liver lesion (Reproduced from Sirtex Website)⁴²

With all theranostic pairs of radionuclides, the general process is to use a targeting agent that allows for a chelation of both the imaging and therapeutic radionuclide. Assuming that the saturation of the receptors that are targeted is not present, either managed by the administration timing or disregarded due to the high up-regulation of the receptors in certain cancers, the purpose of the targeting agent is to deliver the radiation to the imaged lesions and potentially the micro-metastases and other small lesions that the images cannot reveal.

Along with the improvements in image reconstruction algorithms and detector hardware, although not up to the standard of the treatment planning software used in EBRT, there has been an improvement in the internal dose calculations or dosimetry, for both the tumours and the healthy tissues.^{43,44} Traditional methods have used a ‘one-size-fits-all’ approach that focuses on avoiding certain critical organs, such as kidney or marrow, but these methods require a major overhaul to allow for the personalisation of radionuclide therapy and theranostics.⁴⁴ To address this, protocols have been developed where clinics rely on dosimetric software, such as the FDA approved dosimetry software Organ Level Internal Dose Assessment/EXponential Modelling (OLINDA/EXM) developed by RAdiation Dose Assessment Resource (RADAR) Task Group of the Society of Nuclear Medicine, in their

analysis.⁴⁵ OLINDA/EXM contains the decay data for over 1000 radionuclides and can be used to calculate the absorbed dose of a target organ at a voxel level for realistic phantoms, but limitations such as the constant tumour volume still needs to be assessed.⁴⁵ These limitations can lead to inaccurate dose-response estimates and toxicity reports and thus crucial to improving the efficacy of treatment planning in RNT.

Furthermore in image-based dosimetry, the partial volume effect (PVE), resulting from the limitations in the spatial resolution of the hardware that causes a blurring in the images obtained, can lead to an over or under estimation in the administered dose or treatment planning.^{3,46} Several partial volume correction (PVC) algorithms, in-reconstruction or post-reconstruction, have been developed to overcome these PVE present in both PET and SPECT imaging, yet varying limitations presented in each method has led to no single algorithm being accepted as a gold standard.⁴⁷

2.3. Prostate Cancer

Prostate cancer (PCa) is the second most incident cancer in men worldwide after lung cancer.⁴⁸ It was estimated that 1.1 million men were diagnosed worldwide in 2012, with the highest rate of incidence occurring in Australia and New Zealand (combined), and contributed to 25.2% of the 5-year prevalence of all cancers.⁴⁸ Specifically within Australia, PCa was estimated to be the most commonly diagnosed cancer in 2017 amongst males, accounting for 23.1% of all new cancers.⁴⁹ In regards to mortality, it was estimated in 2017 that the risk of a man dying from PCa before turning 85 had become 1 in 30, and accounted for 12.7% of all cancer deaths.⁴⁹ In Australia, the rising prevalence of PCa from 1982 (3,607 cases) to 2013 (19,233 cases) was most likely due to the improvements in or implementations of detection methods, along with the increased education and awareness of the disease.^{49,50} This is evident from the lowering rates of mortality despite the increasing incidence.^{48,50}

2.3.1. *Diagnosis and Treatment*

The method in detecting PCa commonly involves a combination of a digital rectal examination and a prostate specific antigen (PSA) serum testing. PSA is a 33 to 34 kDa serine protease (a cleaver of peptides) believed to be involved in liquefying the seminal coagulum.^{51,52} Although present in the prostate of healthy individuals, in the presence of PCa it is increased dramatically by up to 10^5 fold in the blood, thus is used as a primary indicator when screening for the cancer.^{51,53} Certain membrane barrier mechanisms of the prostate prevent PSA from being excreted into the blood circulation in healthy individuals, but with trauma or progression of a prostate disease, they become prone to leakage that result in a rise in serum PSA count.⁵²

When the screening procedure predicts a potential risk of PCa, a histological analysis is recommended on a biopsy of the prostate to determine the type of PCa and its Gleason score (GS).^{7,54,55} The GS is a numerical indicator of the aggressiveness of the PCa and is a summation of two numbers between 1 and 5; 1 indicates normal prostate tissue and 5 indicates poorly differentiated cancer cells.^{55,56} The first number in the summation is used to describe the cells that make up the majority of the prostate biopsy, and the second number describes the minority.⁵⁵ As an example, the same GS of 7 can potentially be a 3 + 4 or a 4 + 3, where the latter has a worse prognosis. Other alternative methods of diagnosis include magnetic resonance imaging (MRI) or ultrasound (US), both dependent on the availability of the different modalities in the clinic as well as the recommendations from the clinician.⁷ Under certain circumstances where the PCa is benign or low-risk, surgery is avoided and an active surveillance is instructed; most PCa patients are over the age of 65 and unnecessary treatment may affect their quality of life.⁷

Over the past three decades, rapid improvements have been made in the treatment of PCa, with over 80% of the patients diagnosed at an early stage of the disease having a good prognosis.⁵⁷ Some of the curative therapies for the primary tumour site include radical prostatectomy, EBRT, brachytherapy and hormone therapy. Brachytherapy, a form of

internal radiation therapy separate to RNT, uses a sealed radionuclide source that is used to irradiate the tumour. The technique, although in its primitive form, was originally suggested as early as 1901 by Pierre Curie, and its development over the past century has resulted in an effective form of cancer treatment, especially PCa, for primary tumours and recurrences.⁵⁸ Fewer side effects, along with comparable 10-year survival rates to a radical prostatectomy and a reduced cost of the patient treatment, has allowed for the procedure to be a standard in assisting the tumour shrinkage and management of PCa for all risk groups.⁵⁸ Various dose-rate brachytherapy implants, ranging from ultra low dose-rate (ULD) (0.01 to 0.3 Gy/h) as permanent, to high dose-rate (HDR) (> 12 Gy/h) for temporary, are used to treat the prostate gland directly.⁵⁸ ULD and other lower dose-rate brachytherapy use sources such as iodine-125 or caesium-131, whereas the HDR brachytherapy use removable catheters with a stepping source with iridium-192 or cobalt-60 to remotely control the source movement within the prostate gland.⁵⁸

Combinatory or adjuvant therapy mentioned above, such as performing a salvage radiotherapy to the surrounding tissue after a radical prostatectomy or using chemotherapy as a follow up to EBRT, is commonly practiced and crucial to intermediate-risk groups due to their potential risk of metastasis.⁵³ Diagnostic imaging and serum PSA follow up checks are recommended every three to six months, to confirm a partial or complete remission.⁵³ Regardless of the curative intent of the therapy, recurrence can occur, detectable either in the form of biochemical recurrence (BCR), diagnostic or symptomatic examination.⁵⁹ BCR is diagnosed through three consecutive PSA serum examinations that display a rise in PSA levels each time, and is currently a main indicator of PCa recurrence.⁵⁹

If the diagnosis of PCa was given at a later stage in the disease progression, there would be a higher chance of metastasis and the prognosis is typically worse due to the aggressive nature of the tumour, and thus will be classified in the high-risk group.⁵⁹ For a PCa metastasis, it is common to use chemotherapy and androgen-deprivation therapy (ADT) to prevent further metastasis and attempt to slow down the proliferation that rely

on testosterone.⁷ As with all cancers, metastasised PCa is capable of developing resistance to chemotherapy and ADT, such that using the two modalities become futile and further implementations must be made in planning a new treatment.⁷

2.3.2. *Castrate-Resistant Prostate Cancer*

Castrate-resistant PCa (CRPC) has the ability to proliferate at castrate levels, or low levels of testosterone (< 1.7 ng/mL), and patients have a mean overall survival of two years.⁶⁰ Approximately 85% of the patients that are diagnosed with CRPC will have mCRPC, and palliative therapy using EBRT, chemotherapy, ADT and PRRT are trialled to extend the overall survival.⁶⁰ The bone is a common site of metastasis in metastatic PCa patients, evident in up to 90% of mCRPC patients.⁶¹ This is primarily due to the PCa cells favouring hypoxic conditions in the bone for its growth.⁶² A common symptom of metastatic PCa is bone pain associated with the erosion of the bone, although more prominent in mCRPC patients.⁶² Currently, the FDA approved Xofigo®, a radium-223 dichloride ($^{223}\text{RaCl}_2$) α -particle emitting therapeutic, has been an effective radio-pharmaceutical to suppress the disease.²³ Due to the short range of the α -particles (< 100 μm) in tissue, those emitted from the $^{223}\text{RaCl}_2$ will not penetrate though to the bone marrow, resulting in low myelotoxicity whilst successfully targeting the cancer cells.⁷ Regardless of the effectiveness in targeting the metastasised PCa, it is still not a curative treatment but aids in removing the symptomatic bone pain that is often reported in mCRPC.⁵⁹

Quite recently in the literature, the discovery of the prostate specific membrane antigen (PSMA) has taken off as a primary target for the treatment of metastasised PCa.^{63–65}

2.3.3. *Prostate Specific Membrane Antigen PRRT*

PSMA is an 84 kDa, 750 amino acid chain glycosylated type II transmembrane protein, also known as glutamate carboxypeptidase II, found in various tissues throughout the body.^{63,65} The term “prostate specific” in this transmembrane protein was coined despite

its expression in other tissues such as the central and peripheral nervous systems, salivary glands and kidneys, from its use as a biomarker for PCa and its metastases.^{63,65,66} The up-regulation of PSMA expression in PCa makes it an ideal target for both diagnostic and therapeutic agents, and many radiolabelled ligands are currently being developed and trialled.⁶⁷⁻⁶⁹ Despite increasing interest of PSMA, its exact function has yet to be elucidated.

The biggest precursor to the recent boom in theranostics is the clinical translation of ¹⁷⁷Lu-labelled PSMA ligands, most commonly ¹⁷⁷Lu-PSMA-617, for the treatment of mCRPC.⁷⁰⁻⁷² The variety of available PSMA ligands allow for the molecule to be labelled with different radionuclides whilst retaining very comparable uptake into the tumours expressing PSMA.⁶⁹ Thus, at least two different PSMA ligands are used throughout the course of mCRPC patients' treatment, starting with the ⁶⁸Ga-labelled PSMA ligand to assess the suitability of RNT via PET imaging, and following up with ¹⁷⁷Lu-PSMA therapy and the subsequent imaging using SPECT/CT.^{69,72}

2.4. Radiobiology in RNT

Radiobiology is the study on the biological effects and responses from radiation when exposed to a specific dose from a particular type of radiation. In radiobiological studies, it is crucial to compare the effects of the radiation of interest to that of a standard or a previously studied radiation type, referred to as the relative biological effectiveness (RBE), given by equation 2.2.

$$\text{RBE} = \frac{\text{Dose of the Reference Radiation Type for a given survival}}{\text{Dose of the Test Radiation for the same survival as the Reference}} \quad (2.2)$$

Traditionally, it is compared to a standard of 225 or 250 kVp x-rays, but in radionuclide studies, it is more appropriate to compute the RBE between the radionuclides, for example between two β^- emitters with different mean energies like ⁹⁰Y and ¹⁷⁷Lu, by comparing

the survival fraction (SF) at a given dose from a survival curve.^{10,25} Radiobiology of radiation oncology is relatively well-established due to the prevalent use of LINACs, further justified by the limited parameters of the radiation used, leading to the development of the five R's of fractionation and radiobiology described in *Section 2.1.1*.^{11,12} Radiobiology in radionuclide therapy on the other hand, the core theme of this thesis, has to incorporate the differences in linear energy transfer (LET), the half-life and dose rate of the radionuclide, and the energy profile of the emitted particles from the decay. In the clinic, this is further expanded to biological uptake (inhomogeneous), retention and clearance, binding or targeting mechanisms, cross-dose and more recently, radio-immune response, bystander effect and Abscopal effect.^{20,73}

2.4.1. *Linear Energy Transfer and DNA Damage*

The most obvious difference between the dose delivery in RNT to EBRT is the use of directly ionising radiation sources. Whilst EBRT relies only on the reactive oxygen species (ROS) to damage the cells' DNA and organelles, RNT's use of radionuclides that decay through α , β^- and Auger electron emission, with an estimate that approximately 30% of the DNA damage is from direct damage and remaining 70% is from the indirect damage from the ROS generation.^{35,74} The differences in the range and the average energy deposited per unit path length, called LET (keV/ μ m), dictates the choice of radionuclide to use for a particular type of cancer. Table 2.2 compares the approximate LET of the major radiation types used in nuclear medicine, and the approximate range for each.⁷⁵

A simple schematic shown in Figure 2.8 illustrates the major differences between the therapeutic particles and their LET.³⁵ The RBE increases with LET until approximately 100 keV/ μ m, where the recombination process of the free radicals with each other takes over and begins to reduce the RBE. For lower LET particles, whilst the damage may be sparse,

Decay Radiation	Approximate LET (keV/ μm)	Approximate Range in Soft Tissue/Water
β Particle	≈ 0.2	Roughly half of the energy expressed in MeV, in cm
Auger Electron	$\approx 4 \sim 25$	Up to 100 μm
α Particle	≈ 100	50 to 100 μm

TABLE 2.2. LET and range of radiation types used in therapeutic nuclear medicine.⁷⁵

its longer range allows for cross-fire dose delivery and direct irradiation of hypoxic regions.

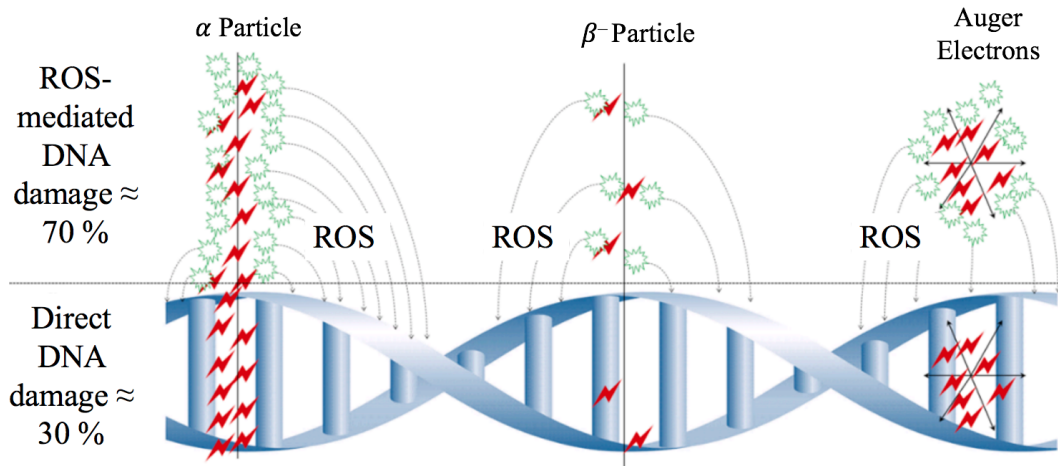


FIGURE 2.8. Diagram of the different particles' damage to the DNA (Reproduced from Gudkov *et al.*, 2015)³⁵

The therapeutic aim of RNT is the same as EBRT; to damage the DNA of the tumours to cause cell death, whilst limiting off-target effects. The basic description of DNA damage is the same for both RNT and EBRT, categorised into single-strand breaks (SSBs) and double-strand breaks (DSBs). This can be broken down further into direct DSBs and indirect DSBs through multiple SSBs. There is a higher chance to induce both direct DSBs and indirect DSBs compared to EBRT if the mean separation between two ionisation events are comparable to the diameter of the DNA double helix (≈ 1.8 nm), but can be compensated

by the use of a higher dose-rate.^{76,77} The dose-rate effect is therefore a crucial parameter that affects the multiple SSBs that result in a DSB, both temporally and spatially. Due to the intrinsically decreasing dose rate of radionuclides after administration, if the SSBs occur at a rate slower than the repair time or if two breakages are too far away, the damage is more likely to be repaired. This is explored further in *Section 2.4.2.* below.

The details of the repair pathways are beyond the scope of this thesis, but Figure 2.9 below briefly summarises the steps taken after DNA damage.⁷⁸ As SSBs are induced, the DNA undergoes base-excision repair (BER), a process used to correct the DNA damage caused by oxidation, deamination and alkylation. DNA glycosylases recognise the damage and remove the bases at the point of strand breakage, which is followed by a short or long patch repair mechanism. With DSBs, a homologous recombination (HR) or a non-homologous end joining (NHEJ) occurs at the site of damage, where HR is specific to the S and G2 phases of the cell cycle and NHEJ is present across all phases. HR is more likely to lead to accurate repair as it uses a homologous template to replicate the damaged portion of the strands, whereas the NHEJ is intrinsically mutagenic from the processing and ligation steps involved in repair. Although less likely than a HR or NHEJ, BER can still lead to cell death, especially if the process is overloaded. An unsuccessful repair of the DNA and thus cell death resulting from apoptosis or necrosis, is the prime target to be considered in the DNA-based approach in cancer treatment.

2.4.2. *In vitro Experiments*

Cell or tissue culture (*in vitro*) research is frequently used in a series of proof-of-concept experiments for novel studies, to observe the cellular or functional impact of the particular intervention that is being implemented. Obtained data can be used to model cellular response for further experimentation and is commonly used as evidence and a foundation for initiating animal (*in vivo*) research and clinical trials.

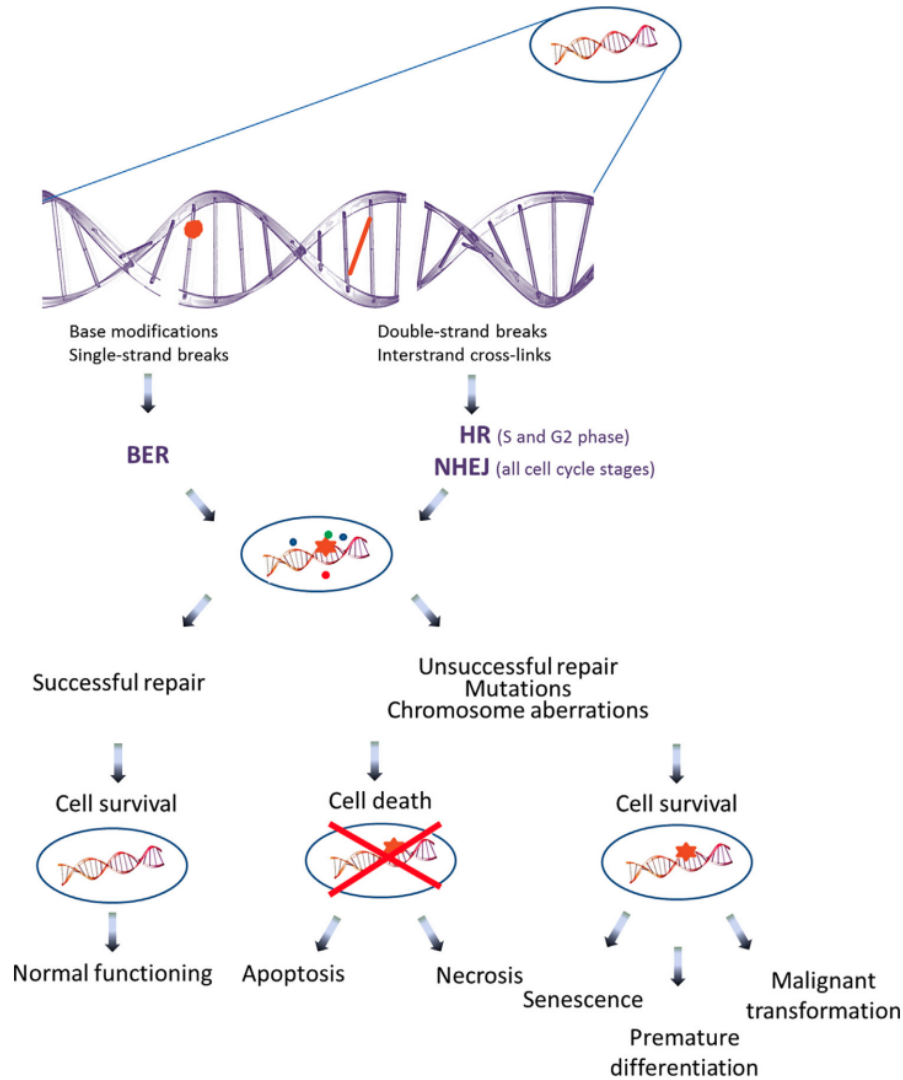


FIGURE 2.9. Diagram of the successful and unsuccessful repair pathways from the radiation induced SSBs and DSBs (Reproduced from Arena *et al.*, 2014)⁷⁸

In *in vitro* radiation studies, although the key target is the DNA double helix as mentioned in the section above, the isolated system of a tumour cell culture is significantly different to that in a patient due to the lack of the immune system that the tumours are able to successfully evade. Regardless, the primary function of *in vitro* studies in radiation research is the parametrisation of cell behaviour, as fundamental data is obtained from the work is the

survival fraction (SF), along with the radiobiological parameters α and β derived from the linear quadratic model (LQM) (Eq. 2.3) fitted onto the survival data.

$$SF = e^{-(\alpha D + \beta D^2)} \quad (2.3)$$

For a range of irradiated doses D (Gy), the α (Gy^{-1}) represents the linear component depicting the lethal, irreparable damage to the cells (DSBs, spatially and temporally close SSBs), whereas the β (Gy^{-2}) represents the quadratic component depicting the sub-lethal, repairable damage (SSBs).

The SF curve has universally been used to quantify the impact that the radiation has *in vitro*, and is obtained from the gold standard radiobiological assay, the clonogenic (colony formation) assay. The clonogenic assay is a basic assay that allows for the investigator to isolate the survival of the cells after irradiation.

A colony is defined as a collection of at least 50 cells originating from a single seeded cell, and thus requires the cell to cycle through 6 mitotic phases to be defined as clonogenic. The SF (Eq. 2.4) is then plotted against the range of doses used to irradiate the cells before the LQM is fitted.

$$SF = \frac{\text{Number of colonies formed in the irradiated sample}}{\text{Number of colonies formed in the control sample}} \quad (2.4)$$

The macroscopic value of the colonies formed allows for the simplification of the impact on the cell survival without having to consider the microscopic and functional impact that is also involved, such as a change in cell metabolism or cell cycle arrest. By obtaining the α and β parameters along with the α/β ratio, the cell lines' radiosensitivity can be observed and compared. Finally, from the SF curve, the dose in which the impact of the radiation on the cell becomes relevant can be revealed, and further *in vitro* assays can be performed on the particular doses of transition.

Other major assays commonly used to supplement the clonogenic assay include cell cycle and oxidative stress assays, and are used in **Chapter 6** of this thesis to elucidate the impact of the β^+ emitting radionuclide's irradiation on the cells. The cell cycle assay quantifies the distribution of the cells in the particular phase of the cycle through the use of propidium iodide and its ability to fluoresce at different intensities dependent on the phase. It can be utilised to indicate the dose in which cell cycle arrest is pronounced, as well as the phase in which the arrest occurs.⁷⁹ The oxidative stress assays measure the percentage of cells that are undergoing oxidative stress through the use of a dihydroethidium reagent. This cell permeable reagent measures the intracellular superoxide radicals present by reacting to form a fluorescing marker detected in the flow cytometer.⁸⁰

A simplified schematic is given below in Figure 2.10, showing the pathways that can be studied through the use of a compact flow-cytometric machine, the Guava[®] MUSE[®] Cell Analyzer, as used throughout this project for performing the assays along with the essential data such as cell viability and count.

2.4.3. Dose-Rate Effects and the Lea-Catcheside G Factor

Various binary misrepair formalism such as repair-misrepair (RMR) and lethal-potentially lethal (LPL) models exist in the literature based off of the LQM.^{82,83} These stem primarily from the differences in dose rate and range that influence the DNA repair kinetics. As shown by Brenner *et al.*, the time related factors that are present in the RMR and LPL models all simplify down to the form known to most as the Lea-Catcheside time factor, also known as the “G-factor”, shown in Equation 2.5.^{76,84}

$$\begin{aligned}
 G &= 2 \left(\frac{\dot{D}_0}{D} \right)^2 \int_0^T e^{-\lambda t} dt \int_{-\infty}^t e^{-\lambda t'} e^{-\mu(t-t')} dt' \\
 &= \frac{2}{\lambda - \mu} \left(\frac{\lambda}{1 - e^{-\lambda T}} \right)^2 \left(\frac{1 - e^{-(\lambda+\mu)T}}{\lambda + \mu} - \frac{1 - e^{-2\lambda T}}{2\lambda} \right),
 \end{aligned} \tag{2.5}$$

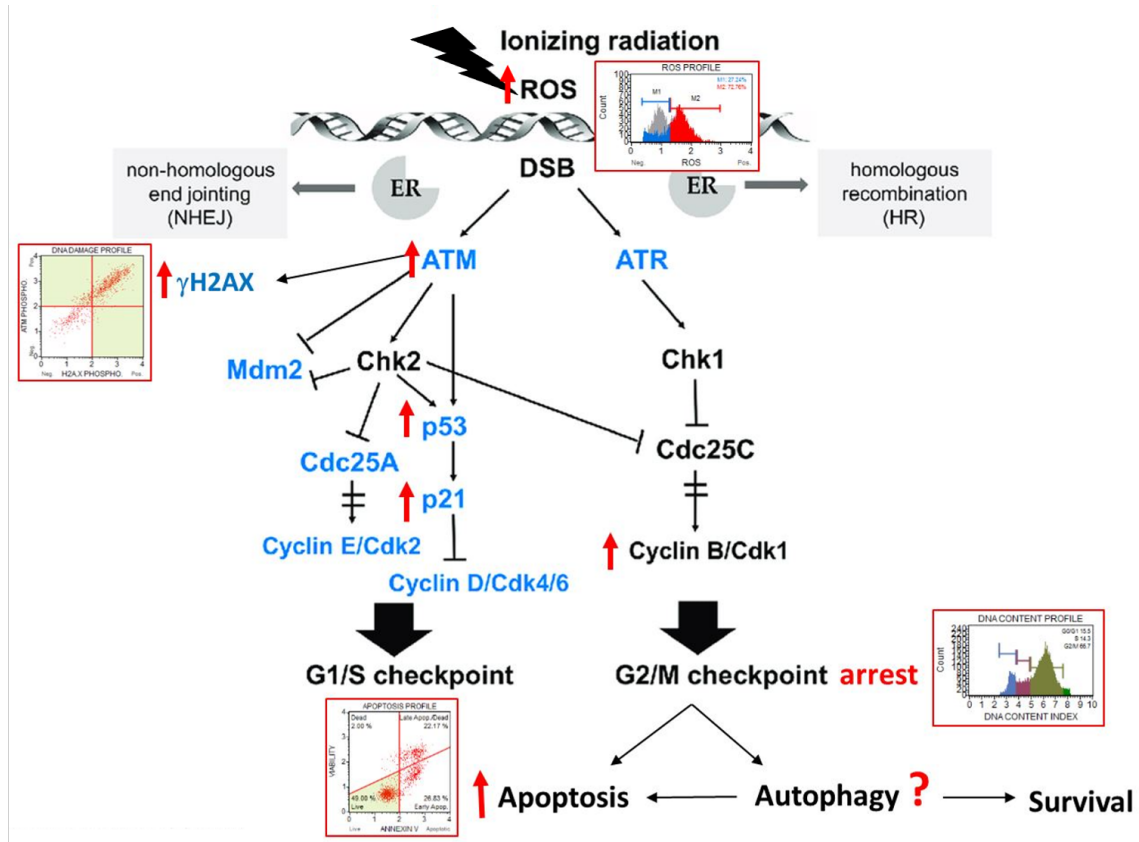


FIGURE 2.10. Diagram of the pathways of damage or repair in the context of biological assays (Adapted by McKelvey, 2021, from Pawlowska *et al.*, 2018)⁸¹

where SF is the survival fraction, D is the total dose, α and β are the linear and quadratic radiobiological parameters (derived from the EBRT cell survival data), respectively, T is the total irradiation time, λ is the decay constant, μ is the DNA repair half-life, t and t' are the times of the first and second SSBs that results in a DSB, respectively.

As with most radiobiological modelling, these applications were developed in the concept of radiotherapy, and the incorporation of the G-factor is primarily reported for fractionation schedules, in particular for 2 to 10 Gy per fraction, and dose protraction in brachytherapy.^{84,85} Gholami *et al.* postulated that radionuclide dose-response modelling is analogous to dose protraction, providing G-factors for radionuclides commonly used in the clinic.⁷⁶ Similarly, in order to simplify the repair kinetics and in the attempt to correlate

dose response of novel theranostic radioisotopes, this thesis focuses on the manipulation of the G-factor in attempt to approach the limit of a radionuclide's therapeutic efficacy.

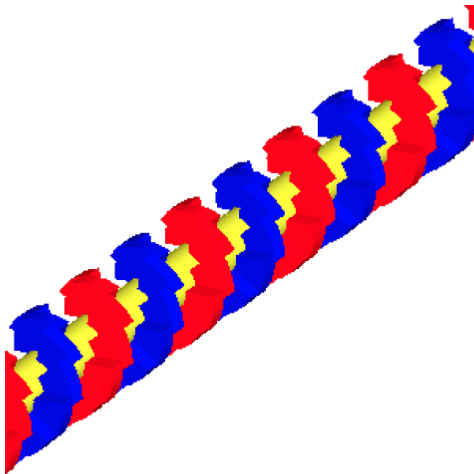
2.5. Computational Modelling

The development of Monte Carlo (MC) simulations in the context of tracking particle transport in physics has been a revolutionary step in fields ranging from high energy physics and astrophysics to medical physics. In particular, the GEometry ANd Tracking (Geant4) is a MC toolkit developed by CERN that simulates the interaction events of particles through matter.^{86–88} Whilst originally intended for use in high energy physics, its integration into medical physics quickly followed, becoming a crucial tool for dosimetry in RNT. TOOl for PArticle Simulation (TOPAS) is the primary Geant4-based simulation toolkit used in this project, specifically, the radiobiology extension: TOPAS-nBio.^{89,90}

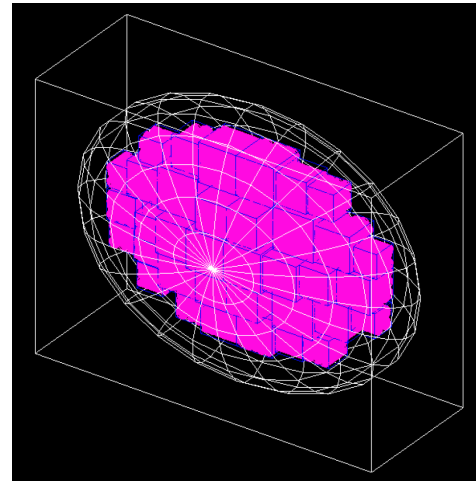
TOPAS-nBio extends the Geant4-DNA toolkit, which includes low-energy particle interactions to a sub-cellular level. Instead of simulating a condensed history of particles, TOPAS-nBio simulates every particle interaction in a given geometry. The models that are available in the extension, used to simulate and correlate the radiobiological effects, include the cell, DNA, nucleus and organelle models. In the interest of a simplified radiobiological outcome, a linear DNA model (Figure 2.11a) is sufficient, although a highly comprehensive Geant4-DNA full nuclear model (Figure 2.11b) is also available for systems incorporating the use of computer clusters.⁹⁰

2.6. Project Aims

The projects reported in this thesis were proposed with the intention of elucidating the radiobiological effects of radionuclides from the cellular level in the hopes of developing a personalised platform for the treatment of mCRPC as well as other metastatic diseases using RNT. Furthermore, the novel method of using β^+ emitters as a potential therapeutic agent is explored, not only to implement a new theranostic modality, but to standardise an approach



(A) Linear DNA model, where the red and blue are bases and the yellow is the sugar-phosphate backbone.⁹⁰



(B) Geant4-DNA full nuclear model encompassing the whole genome, where the magenta boxes represent 23 chromosome territories of varying size.⁹⁰

FIGURE 2.11. Examples of the available DNA models in TOPAS-nBio⁹⁰

in the way radiobiology is studied at an *in vitro* level for radionuclides. The final project aims to tie the *in vitro* concepts in a patient dosimetry framework for future clinical application.

The specific aims of the project were:

- a) To determine the radiobiological parameters for the cell lines in the in-house laboratory conditions,
- b) To develop a standardised protocol to perform radiobiological *in vitro* experiments using short half-life radionuclides,
- c) To perform a pilot experiment examining the potential therapeutic effect of the positron-emitting radionuclide using ^{18}F as an example *in vitro*,
- d) To investigate the cellular damage that ^{18}F has *in vitro* through the manipulation of the Lea-Catcheside G-factor, and to determine the biological impact from the varying dose rates,
- e) To explore the feasibility of prescriptive dosimetry for β^+ emitters as a therapeutic, using multiple time-point images of a patient injected with ^{177}Lu as a surrogate.

As cancer therapy is a race against the evolutionary pressure the treatment places upon it, the more treatment variation that is given to a patient in a shorter amount of time becomes crucial. Despite the difficulties in completely eradicating cancer, with enough proof to support the treatment combination, the patient can be offered a potentially better quality of life until a curative treatment pipeline is developed.^{67,68,91}

CHAPTER 3

***In vitro* study of Radiosensitising Effects of Idronoxil® on Prostate Cancer Cells**

This chapter presents the *in vitro* experiments performed using a radiosensitising agent, 3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol (Idronoxil®), a synthetic flavonoid derivative, on three prostate cancer cell lines: LNCaP, LNCaP C4-2 and LNCaP C4-2B. It expands on the internal report that was prepared for Noxopharm, a Sydney-based start-up biotech company, that approached our group as an industry collaboration to demonstrate their product's radiosensitising effects *in vitro* on prostate cancer cells.

This project acted as an introduction to the prostate cancer cell lines that could be used in future investigations, and allowed for the determination of the radiosensitivity associated with each variant. The main assay performed in this chapter used to determine the efficacy of the radiosensitiser, incorporated a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) metabolic assay for a survival fraction.⁹² For the subsequent chapters, this was optimised and adapted to a colony formation (clonogenic) assay in a 6-well plate, in preparation for the use of radionuclides that would generate large amounts of contaminated waste if performed in 96-well plates.

3.1. Introduction

Noxopharm is a Sydney-based start-up biotech company with a putative radiosensitising agent, 3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol (Idronoxil®), a synthetic flavonoid derivative, for prostate cancer (PCa) radiation therapy. They engaged our group to examine whether radiosensitising effects could be demonstrated *in vitro* on prostate cancer cells with varying levels of radiosensitivity. The mechanism of action has not yet been fully elucidated, but the radiosensitising effect is hypothesised as the promotion of apoptosis from the inhibition of DNA repair, resulting from the reduction of the Sphingosine-1-phosphate receptors.⁹³ Off-target effects have not been reported for this compound.

Cell and tissue culture, known as *in vitro* studies, require a multitude of optimisation steps to achieve consistent and reliable results. This is heavily due to the culture environment that the cell lines must adapt to before it begins behaving characteristically as reported in literature. Furthermore, if any minor changes are made, such as medium composition or temperature regulation is altered, the impact on the growth of the cells can be detrimental.⁹⁴ Variability of the robustness between cell line exist, even those with minor phenotype differences, and only becomes evident after comprehensive optimisation.

3.2. Methods

Three major optimisation steps needed to be performed before the main aim of the project could be addressed: cell density, dimethylsulfoxide (DMSO) tolerance and IC₅₀ of the Idronoxil®.

3.2.1. Cell Lines

The PCa cell lines used in this section are presented below in Table 3.1. The LNCaP and their derivative cell lines were used to due their expression of the prostate specific membrane antigen (PSMA) receptor, varying dependency on androgen and natural progression of the disease.^{62,95-98} Although these factors are not relevant when determining the radiobiological

parameters, with the intention of a clinical translation from the work described in this thesis, a target such as the PSMA receptor is crucial in PRRT, further justifying their use *in vitro*.

Cell Line	Tissue	Details	Source
LNCaP	Human	Androgen Dependent PSMA Receptor Present 48 Hour Doubling Time	Garvan Institute of Medical Research
LNCaP C4-2	Human	Androgen Independent PSMA Receptor Present 24 Hour Doubling Time	American Type Culture Collection
LNCaP C4-2B	Human	Androgen Independent PSMA Receptor Present 24 Hour Doubling Time	Garvan Institute of Medical Research

TABLE 3.1. Cell lines used throughout this project

3.2.2. Cell Culture

The three PCa cell lines were cultured in either T25 or T75 flasks in Roswell Park Memorial Institute (RPMI, Life Technologies) medium with 10% v/v foetal bovine serum (FBS, Sigma Aldrich). Cells were maintained in an incubator at 37°C with 5% CO₂. Cells were sub-cultured approximately twice a week, ranging from a ratio of 1:4 to 1:20, by first washing with phosphate buffered saline (PBS, Astral Scientific), then adding trypsin (Life Technologies) and placing in the incubator for 5 minutes or until the cells detached. The standard medium (RPMI with 10% v/v FBS) was added to inhibit the trypsin and then detached cells were pelleted by centrifuging at 300 g for 3 minutes. Cells were then resuspended in the medium and reseeded into new flasks at the desired ratio.

3.2.3. Cell Count and Viability Assay

25 to 50 μ L of cells resuspended in medium was added to the Count and Viability reagent to make up a total volume of 200 μ L, and was incubated for 5 minutes at room temperature.

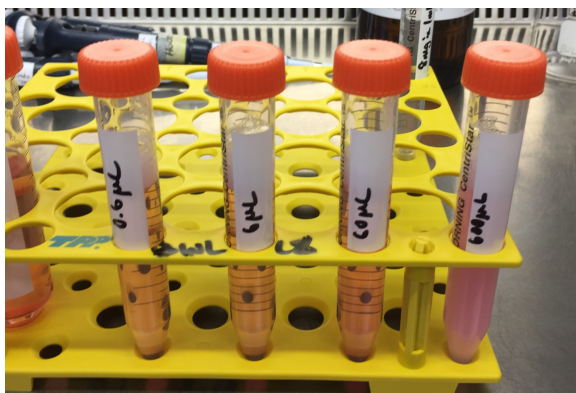
Cells were resuspended by pipetting before counting on the Guava[®] Muse[®] Cell Analyser (Luminex) following the general protocol.

3.2.4. Cell Density Optimisation

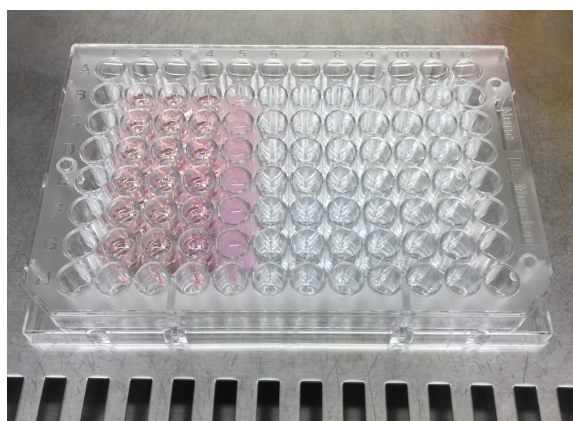
The cell density calculations were performed for an 8 day incubation to ensure the cells' growth were at the exponential phase when plating. In cell culture biology, the confluence refers to the percentage of the surface of a culture dish that is covered by adherent cells. If the cells reached 100% confluence, the amount of FBS would not have been sufficient to sustain a regular pattern of growth and the cells would lose viability from the lack of sustenance.

3.2.5. DMSO Tolerance

DMSO was necessary for the solubilisation of Idronoxil[®] before adding to the culture media. Despite the low concentrations of the radiosensitiser that were to be administered to the cells, DMSO had the possibility of affecting the cell growth and could damage the membranes and alter the results.^{99,100} The clarity of the solution was also visibly affected with DMSO and solubilised Idronoxil[®], as shown in figures 3.1a and 3.1b. Thus, a tolerance experiment was essential to ensure that the values obtained were not influenced by the presence of DMSO.



(A) Varying levels of Idronoxil[®] concentration in tubes



(B) Varying levels of Idronoxil[®] concentration in a 96-well plate

FIGURE 3.1. DMSO and Idronoxil[®] effects on solution clarity

3.2.6. *IC₅₀ Determination*

The optimisation step for the IC₅₀ were performed to observe the range of values required for the actual administration of Idronoxil® as a radiosensitising agent. For the IC₅₀ measurements, the control media had DMSO at a percentage that matched that of the highest concentration of Idronoxil®. The IC₅₀ values were computed through a non-linear regression (log(inhibitor) v.s. response - Variable slope (four parameters)) on GraphPad Prism Version 7. This used the logarithm of the concentration and the survival fraction that was obtained from the MTS assay absorbance values at 490 nm using a spectrophotometer.

3.2.7. *SARRP Irradiation and Cell Survival*

The SARRP (Xstrahl Inc, GA, USA) irradiation was performed with cells in a T25 flask containing 5 mL of standard medium.¹⁰¹ The basic setup is pictured in Figure 3.2, which has the flasks placed on top of a 3.6 cm Perspex base. An open-field setup (20 × 20 cm²) was incorporated, with a source-to-axis distance (SAD) of 33.4 cm, giving a dose rate of 4.45 ± 0.06 Gy/min using the default x-ray beam energy spectrum of 225 kVp. The doses delivered using the setup were confirmed using the EBT3 GAFchromic™ films with the assistance from radiation oncology medical physicists in the hospital.

The Idronoxil® was administered 3 hours post-plating of the cells at a concentration at the IC₁₀ value calculated in the IC₅₀ determination step. 24 hours after the administration of the radiosensitiser, the cells were irradiated from 0.5 to 3 Gy in 0.5 Gy increments. The cells were then incubated for 5 days post-irradiation, before the addition of MTS. The absorbance readout was taken on the spectrophotometer, and the survival fraction (SF) was calculated for each dose. The SF was determined by the ratio of the absorbance values in the samples to that of the control as shown in equation 3.1, and was fitted to a linear quadratic relation (Eq. 3.2), to obtain the radiobiological parameters α and β .

$$SF = \frac{\text{Absorbance of 490 nm wavelength for sample}}{\text{Absorbance of 490 nm wavelength for control}} \quad (3.1)$$

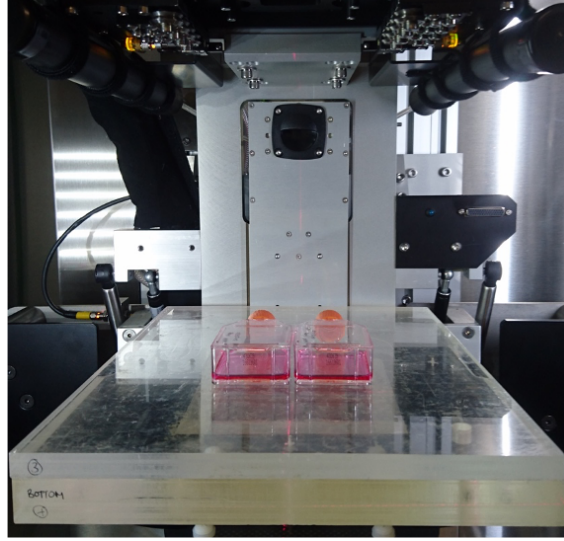


FIGURE 3.2. SARRP irradiation setup

$$SF = e^{-(\alpha D + \beta D^2)} \quad (3.2)$$

The α and β are the linear and quadratic parameters in Gy^{-1} and Gy^{-2} , respectively, and D is the dose in Gy.

3.3. Results

The first column in figure 3.3 shows the final optimised cell densities that were used in all subsequent experiments. LNCaP, LNCaP C4-2 and LNCaP C4-2B had optimal cell densities of 500, 300 and 300 cells per well, respectively. All cell lines had no impact from DMSO below 0.5% concentration, as shown in the second column. The IC_{50} values of Idronoxil[®] for the three cell lines, shown in the third column, were 1.5 ± 0.2 , 0.9 ± 0.2 and 1.1 ± 0.1 μM , respectively.

Figure 3.4 shows the intrinsic radiosensitivity factor (α) for the three cell lines in the control and treated cell cultures. The control and treated α values for LNCaP, LNCaP C4-2 and LNCaP C4-2B were 0.48 ± 0.02 and 0.47 ± 0.03 , 0.36 ± 0.03 and 0.55 ± 0.02 , and 0.18 ± 0.04 and 0.30 ± 0.05 Gy^{-1} , respectively. The corresponding α/β ratios derived in the

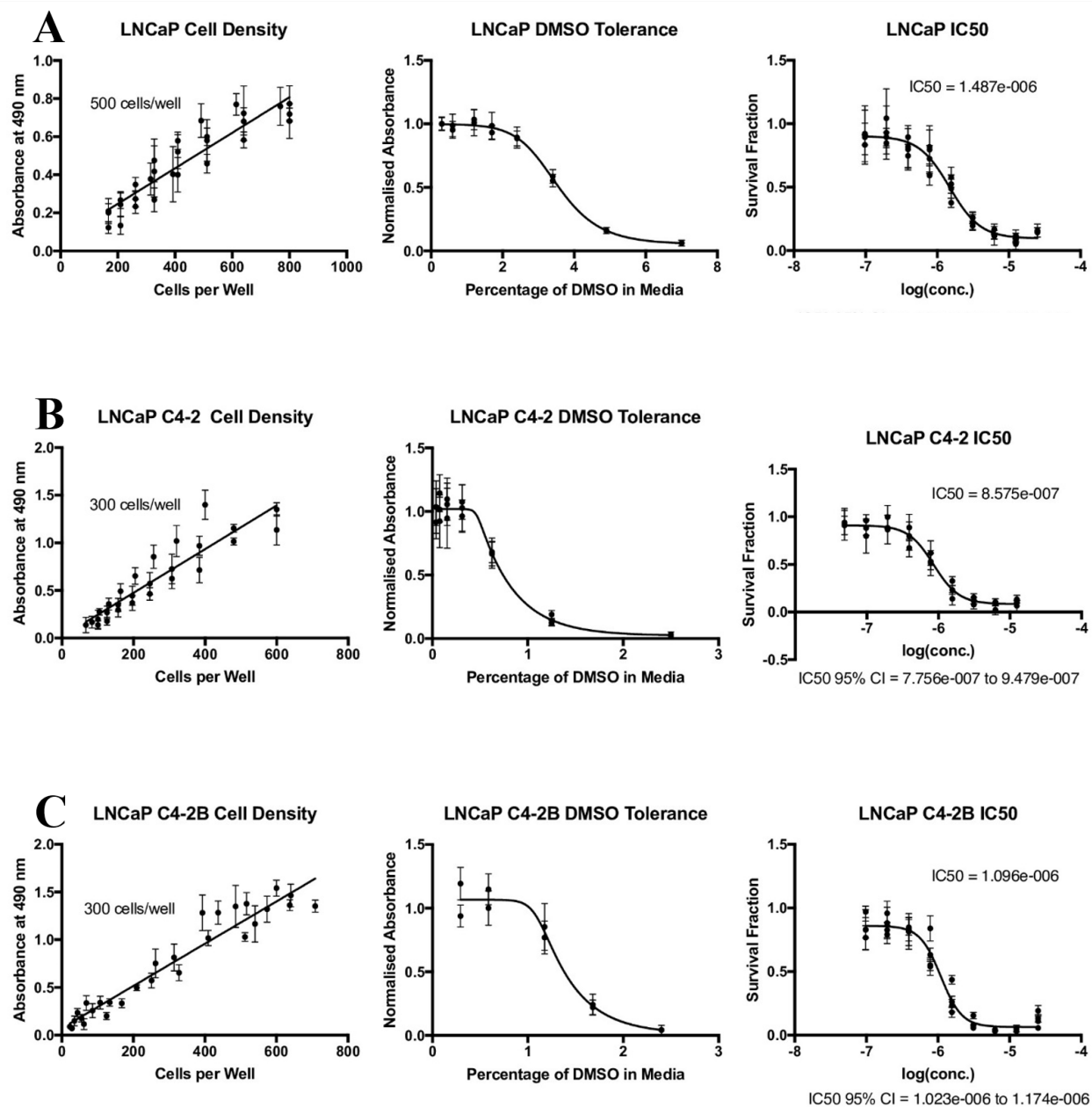
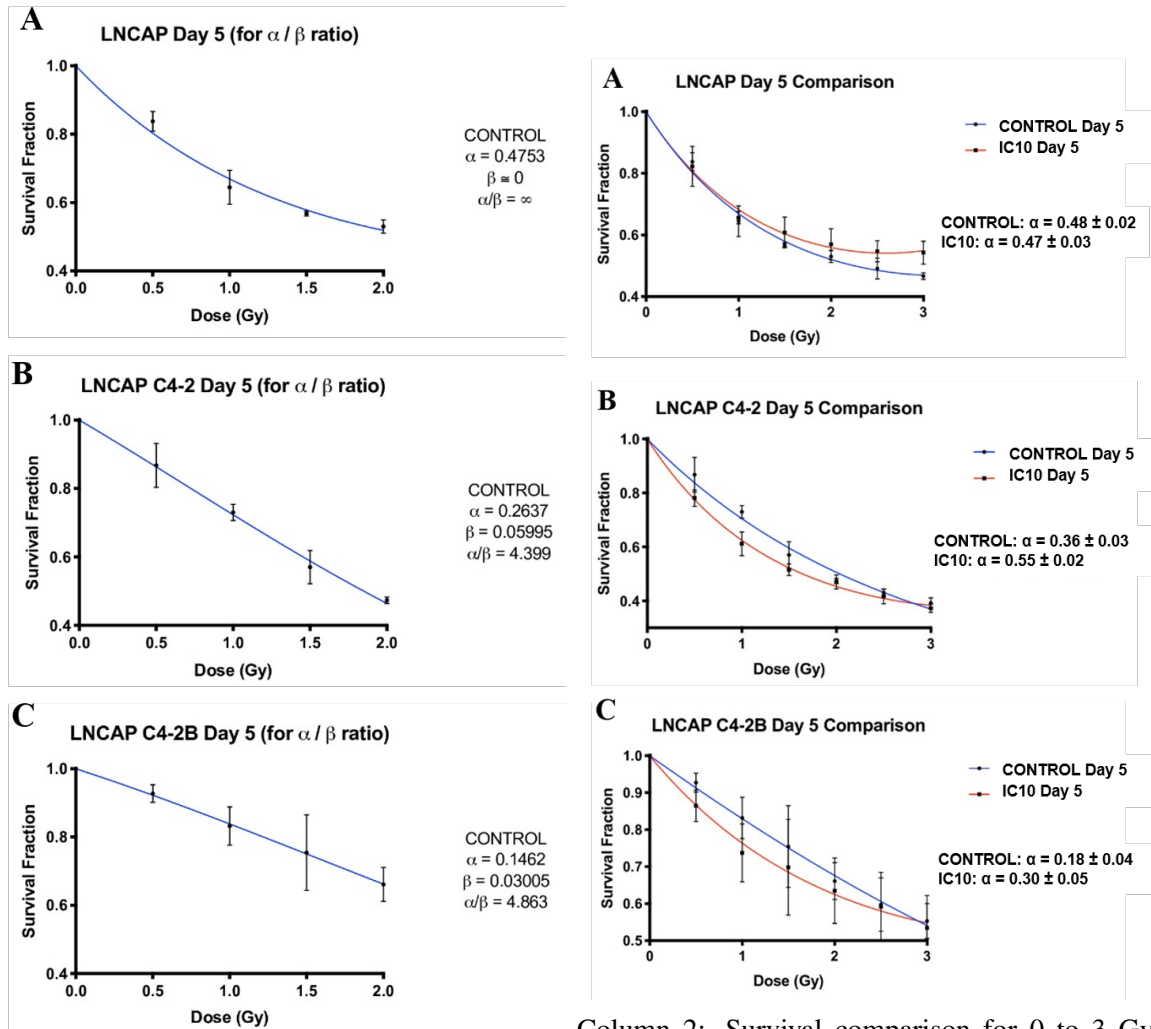


FIGURE 3.3. Cell density, DMSO tolerance and IC₅₀ results for A) LNCaP, B) LNCaP C4-2 and C) LNCaP C4-2B

0 to 2 Gy region for the purpose of extracting a linear-quadratic relation were 4.4 ± 2.7 and 4.9 ± 7.4 for LNCaP C4-2 and LNCaP C4-2B, but could not be determined for LNCaP.



Column 1: α/β Ratios for 0 to 2 Gy

Column 2: Survival comparison for 0 to 3 Gy:
Control v.s. IC₁₀ of Idronoxil®

FIGURE 3.4. Survival fraction results for A) LNCaP, B) LNCaP C4-2 and C) LNCaP C4-2B

3.4. Discussion

The comparison of the effects of the radiosensitiser on the cells has focussed on the α parameter only, shown in column 2 of figure 3.4, as the dominance of the α in the fit of the curves can lead to a zero or negative β value that will result in an unreasonable α/β ratio.

The α represents the lethal and non-reparable damage to the cells at lower doses, and is biologically much more important than the β parameter, which reflects DNA repair. The α value is the linear component in the LQ model, and thus the higher the value, the steeper the slope, indicative of a more radiosensitive cell line. This is reflected in the phenotypic progression of the three cell lines presented, as the α value is highest for LNCaP, and lowest for LNCaP C4-2B, indicating the increasing radioresistance with progression.¹⁰²

For the cell lines LNCaP C4-2 and LNCaP C4-2B, there is an evident effect of Idronoxil® on the α parameter, indicative of increasing radiation response in comparison to the control. Due to the high radiosensitivity of the LNCaP cell line, no significant effect of Idronoxil® on the cell survival was seen, as the radiation independently may have saturated the observable impact. The maximum difference on the survival for LNCaP C4-2 and LNCaP C4-2B was found to be at 1 Gy, with 12 and 9% difference in survival between the control. The convergence of the cell survival data past this point was most likely due to the irreparable damage negating the repairable, effectively masking the radiosensitiser's effects. For LNCaP, the intrinsic radiosensitivity has made the β component irrelevant, and so would more likely be modelled using a linear model over the LQ model.⁸⁴

The values for 2.5 and 3 Gy were omitted in column 1 of figure 3.4 for the generation of the α and β parameters for the control. This was due to the same biological endpoints observed that would affect the β parameter when calculating the radiobiological parameters.

For the α/β ratio derivation where the uncertainty in the values is larger than the value itself, it is indicative of the low radiosensitivity of the cell lines irradiated for the range of doses that they were exposed to. The higher the α/β ratio, the more radiosensitive it is. Thus, the implications of this high uncertainty is that the range of doses used to irradiate the cell lines were insufficient to properly quantify the cell lines' response to irradiation. For the sake of this project, the effect of the radiosensitiser was already present at the lower doses, but for a more robust dataset, a wider range of absorbed doses should be explored.

3.5. Conclusion

Idronoxil[®] has shown promising results in the more radioresistant prostate cancer cell lines used in this study. However, to ensure that the effects can be translated successfully into *in vivo* models, the actual mechanistic pathways of the drug should be further elucidated. This would then lead to the utilisation of an appropriate assay to quantify the effects of Idronoxil[®], as a metabolic or proliferative assay may underestimate the impact that the drug has on the cell radiosensitivity.

To explore the efficacy of the radiosensitising effect of Idronoxil[®] in radionuclide therapy, the assay must be altered to a colony formation assay due to the production of contaminated waste from the procedure. Longer-lived radionuclides will require extended periods of decay-in-storage for the materials used, and exposure risks and shielding measures must be assessed for up-scaling the administered radioactivity to achieve dose targets.

CHAPTER 4

^{18}F *in vitro* Experimental Protocol

This protocol chapter has been prepared with the following headings with the intention of submitting to the Nature Protocols journal: *Abstract, Introduction, Development of the Protocol, Applications, Overview, Limitations, Materials and Equipment, Setup, Procedure, Timing* and finally, *Anticipated Results*.

The chapter discusses the necessary steps needed to perform *in vitro* experiments using short half-life radionuclides for radiobiological studies, and the importance of developing a methodology that can be utilised as a gold standard. The key in experimental success is to be aware of the limitations posed by the radionuclide or cell line of choice, and thus is heavily emphasised at the beginning of the protocol. The results presented as examples and expected results are from experiments performed using a positron emitter ^{18}F as the radionuclide due to its widespread availability and relatively low cost, on the metastatic castrate resistant prostate cancer cell line LNCaP C4-2B. The novelty of this protocol is in the incorporation of the radiobiological parameters at an early stage of planning that can be implemented into the user's desired assays, along with practical solutions to overcome issues of short half-life radionuclides.

The protocol in this chapter was finalised after the completion and review of the experiments performed in **Chapter 6** and therefore developments have been made after publication in **Chapter 5**. The proof-of-concept of fractionation experiment in the context of RNT performed in **Chapter 6** was also based on this protocol.

The tentative author list is as follows: **Takanori Hioki**, Yaser H. Gholami, Kelly J. McKelvey, Kathy P. Willowson and Dale L. Bailey.

PROTOCOL: *in vitro* Irradiation using Short Half-life Therapeutic Radionuclides for Radiobiological Studies

4.1. Abstract

In the isolated system of *in vitro* studies, irreparable damage to the cell can be optimised through the choice of radionuclide used; the energy or range along with the yield of the emitted particles, the number of decays before stability and the half-life are some key elements that are considered. The focus in this protocol is on the shorter half-life radionuclides, as the higher intrinsic dose rate has the potential of overcoming the DNA repair pathways and mechanisms triggered both temporally and spatially. Despite this, the difficulties faced with handling these radionuclides with strict time constraints limit the prevalence of such *in vitro* studies performed in the literature. Here, we introduce an efficient methodology to manage the processes required for the full radiobiological characterisation of a cancer cell line, using a short half-life radionuclide of choice. Although certain limitations of equipment and radionuclide availability exist, the manipulation of specific radiobiological parameters such as the Lea-Catcheside G-factor allows for the comparison against the theoretically achievable cell survival in a standardised protocol for an insight into the radiobiological dose-response effects.

4.2. Introduction

In vitro work has been a foundation for many pilot experiments and proof-of-concept studies, used to justify further cell studies to pave the way for *in vivo* studies or clinical trials. The isolated system of a cell culture environment allows for researchers to investigate the raw impact of the chosen intervention, thus allowing for a simpler parametrisation crucial in biological modelling.

The fundamental form of radiobiological studies using radionuclides has been present since the initial discovery of radioactive isotopes in medicine.^{38,103} With the improvement in technological advances, there was a shift from radionuclides to external beam radiotherapy (EBRT) in the form of x-ray machines and linear accelerators (LINACs), leading to the radiobiology of such controlled sources of radiation to be substantially developed and implemented into treatment planning systems, whilst the radiobiological studies of radionuclides fell behind.^{16,104}

In the past two decades, the discovery and implementation of highly specific radionuclide tracers have highlighted the importance of the radiobiological effects in therapeutic nuclear medicine.^{2,105-107} Some of these include dose-rate effects, Lea-Catcheside G-factor and other spatial and temporal effects relating to the radionuclide, to antigen expression or immune related responses such as the bystander and the Abscopal effects.^{73,108-110} Although a myriad of *in vitro* radiobiological studies for radionuclides have been performed and reported throughout the literature, there has not been an effective development towards a protocol that is used as a gold standard.¹¹¹⁻¹¹⁴ Thus, this protocol aims to begin standardising, for the cell lines and radionuclides that fit a therapeutically desirable criteria, a protocol that may be used for closer cross-comparisons for further insight into the radiobiology of radionuclides, in particular, those with shorter half-lives.

4.2.1. *Development of Protocol*

The development of the protocol was conducted throughout the *in vitro* investigation performed in the article *Overlooked potential of positrons in cancer therapy* in the novel concept of utilising positrons in a therapeutic setting.¹¹⁴ The protocol was fully realised through further optimisation and experimentation performed in a currently unpublished work. The clonogenic assay protocol is based off of the *Clonogenic assay of cells in vitro*, and the initial work of the radiobiological studies extended in this protocol was performed in *Comparison of radiobiological parameters for ⁹⁰Y radionuclide therapy and external*

beam radiotherapy in vitro, by our team.^{76,115} The foundations behind the procedure is built on the linear quadratic model (LQM, Eq. 4.1) of the EBRT irradiation, and the extended linear quadratic model (ELQM, Eq. 4.2) that incorporates the Lea-Catcheside G time-factor (Eq.4.3) for the use of radionuclides.^{76,84,108}

$$SF = e^{-(\alpha D + \beta D^2)}, \quad (4.1)$$

$$SF = e^{-(\alpha D + \beta G D^2)}, \quad (4.2)$$

$$\begin{aligned} G &= 2 \left(\frac{\dot{D}_0}{D} \right)^2 \int_0^T e^{-\lambda t} dt \int_{-\infty}^t e^{-\lambda t'} e^{-\mu(t-t')} dt' \\ &= \frac{2}{\lambda - \mu} \left(\frac{\lambda}{1 - e^{-\lambda T}} \right)^2 \left(\frac{1 - e^{-(\lambda+\mu)T}}{\lambda + \mu} - \frac{1 - e^{-2\lambda T}}{2\lambda} \right), \end{aligned} \quad (4.3)$$

where SF is the survival fraction, D is the total dose, α and β are the linear and quadratic radiobiological parameters (derived from the EBRT cell survival data), respectively, T is the total irradiation time, λ is the decay constant, μ is the DNA repair half-life, t and t' are the times of the first and second single strand breaks (SSBs) that results in a double strand break (DSB), respectively. The DNA repair half-life was not calculated throughout the examples provided in this protocol but was assumed to be 1 hour in the derivation of the G-factor. This arbitrary number was chosen for mathematical ease, but also due to the high variability of doubling time and repair times that can be observed among cell lines; shorter repair half-life range anywhere from 1 to 30 minutes, and longer times range from an hour up to 20 hours.¹¹⁶⁻¹¹⁸

4.2.2. Applications

This protocol can be used as a base for a myriad of radiobiological studies *in vitro*, including cell survival, relative biological effectiveness (RBE) comparisons and other radiobiological characterisations stemming from survival curves and the α and β

parameters.^{14,119} Dependent on the half-life of the radionuclide chosen for the investigation, the early-effect of irradiation can be observed, although the most accessible and reproducible effect is the delayed-effect of radiation damage beyond 24 hours post-irradiation.¹¹⁵ Repair enzyme targeting pharmaceuticals such as poly adenosine diphosphate-ribose polymerase (PARP) inhibitors may potentially be incorporated after the characterisation of the cell lines.¹²⁰

4.2.3. *Experimental Design*

a) *Understanding the logistics*

Not all cell lines are suitable for performing a clonogenic assay. This is purely dependent on the physical characteristics of the cell line, where the more suitable cells form an adherent monolayer provided there is enough space between adjacent cells. The survival fraction is calculated with Eq. 4.4, and is used to convert the number of colonies formed within the clonogenic assay to a survival curve.

$$SF = \frac{\text{no. of colonies in irradiated sample}}{\text{no. of colonies in control sample}} \quad (4.4)$$

Regardless of the data available, the cells' characteristics on doubling time, cell density for growth, and robust adherence through multiple growth medium changes, should be thoroughly studied before proceeding. This information will dictate which radiobiological experiments can be performed, especially within the time frames of the entire protocol. Cell densities for the chosen sized wells and the flasks must be determined in such a way that the cells are constantly in an optimal state.

b) *Benchmarking experiment with EBRT*

The radiobiological impact of EBRT has been established for decades, as the dose delivery is controlled in comparison to radionuclide therapy. Thus, the LQM has

been formulated from the EBRT effects. To create a bridge between EBRT and RNT, the initial characterisation of the cell lines must be performed using external beam irradiation. The α and β radiobiological parameters will be obtained for the cell lines using the clonogenic assay, which will be used to determine the RBE of the radionuclide that will be used in the investigation.

c) *Screening radionuclide doses and calculation*

From the benchmarking experiment, an estimate of the absorbed dose for a substantial or desired cell kill will be revealed. A wide range of absorbed doses need to be administered in the clonogenic assay setup to ensure that a relevant incremental doses will be administered when observing the effects of the Lea-Catcheside G-factor. Using the α parameter extrapolated from the screening of doses, the G-factor can be calculated, with an estimated DNA repair time constant. With the screening dose experiment's G-factor as a reference, higher or lower values can be chosen. This dictates the irradiation time required for any given dose to give the G-factor of interest, and effectively the activity required to achieve the target absorbed dose.

d) *Performing the optimised irradiation and assays*

Depending on the volume of activity available and the laboratory's capacity to store radioactive flasks, preparation and experimental time will vary significantly. When choosing the radiobiological assays to perform, ensure to minimise the number of flasks being prepared by noting the compatibility of assays that can be performed together in cells acquired from one flask. Certain assays are not compatible with others, such as an apoptotic marker assay, as the growth medium must be retained to count the entire population of cells after a specific time interval. A buffer time in administering the desired activity must be incorporated when streamlining this step,

and will be detailed in the steps below. The assays will yield results at different times, and thus the limitations of the data obtained must be taken into account.

4.2.4. *Required Expertise*

A high proficiency in cell and tissue culture technique is required throughout the entire experiment, as the time constraints of short half-life radionuclides leave little room for delay and any contamination will invalidate results obtained. To perform experimental procedures involving radiation implicates the investigators' competence and understanding on the use and handling of unsealed radioactive sources. The benchmarking involving the use of an EBRT machine can be performed either by a medical physics specialist in a radiation oncology department using a LINAC, or trained personnel if using a small animal irradiator such as the Small Animal Radiation Research Platform (SARRP).¹⁰¹ It is highly recommended the steps involving the handling of the unsealed radionuclides is performed by two people. The recording of the activity, time and any other data by a separate individual to the one performing the physical experiment is crucial for reducing exposure time, and limits the error that may be introduced with time sensitive steps.

4.2.5. *Limitations*

Most limitations can be addressed by choosing a radionuclide and cell line suitable for the clonogenic assay setup. As the title of the protocol suggests, short half-life radionuclides are preferred due to the amount of waste that can be generated throughout the protocol. If your facility is capable of storing large quantities of longer half-life radioactive waste, the protocol can be appropriately adapted. When using radionuclides with relatively longer half-life, the early and immediate effects of the radiation on the cells will not be observable; the DNA damage before the initial repair. Furthermore, the delayed effects will be need to be determined over a proportionally longer time frame compared to the shorter half-life radionuclides.

Utilising shorter half-life radionuclides may present difficulties in the repeatability of the experiments. For comparison between multiple experiments, or research groups, the only way to achieve an acceptable level of consistency with the dose delivery is to report all of the residual activity collected, and by applying decay correction. Although a small amount of variability will not impact the overall results as there is no clear-cut threshold dose between cell kill and survival, proper reporting of all activities, volumes and times of measurement is an absolute necessity.

The mode of decay of the chosen radionuclide will dictate the extent of cross-irradiation from the emitted gamma (γ), and therefore must be taken into consideration for higher energy γ emission. This implies that a separate control experiment must be performed comparing the additional impact the “stray” γ may have on the overall survival, as this becomes highly dependent on the branching ratios of emission and the emitted energies.

Some cell lines are not suitable for a colony formation assay. This may be due to the way the cells proliferate; a monolayer colony formation is preferred over clusters due to the ability to distinguish and identify a single colony, defined as being comprised of a minimum of 50 cells originating from a singular cell. Other cell lines, despite forming monolayers, are not as strongly adherent to the bottom of the well. Unless a treatment of the wells are performed before the seeding or the laboratory has a cell-plate centrifuge, the staining and washing process can unintentionally extract the colonies. Each cell line must be tested thoroughly before proceeding. This particular protocol has not been verified using a 3-dimensional culture irradiation such as spheroids.

Certain assays require additional steps in the methodology of preparation. For example, an apoptotic assay that counts the cells in particular stages of apoptosis, and thus, is essential that the cell debris is collected for a valid result. A separate flask must be prepared exclusively for such assays, consequently increasing the amount of activity required. Ensure to note the compatibility of assays that may be performed with one flask to reduce the amount of resources required.

Finally, the calculations used in this protocol are based off of the ELQM (Eq.4.2) that incorporates the Lea-Catcheside G-factor (Eq.4.3). This is a semi-empirical extension to the standard LQM, and here, assumptions such as the DNA repair time and cell doubling time are not measured.

4.3. Materials

Biological Materials:

- Cell line(s) of interest: *We used a highly radioresistant, metastatic castrate resistant prostate cancer cell line, LNCaP C4-2B*

Reagents: Below does not list the required reagents for the various assays that may be conducted.

- Suitable cell culture medium
- Phosphate-buffered saline (PBS)
- Trypsin/Ethylenediaminetetraacetic acid (EDTA)
- 1% w/v crystal violet in Methanol

Equipment: Below does not list the standard PPE required for radionuclide experiments. All listed equipment is sterile unless otherwise mentioned.

- EBRT machine: *We used the SARRP*
- Dosimetry film or dose verification device: *We used a GAFchromic EBT3 Film*
- 6-well (or any other preferred) plates
- Short half-life radionuclide: *We used an unsterilised saline ^{18}F source, which was sterilised with a micro-filter prior to use*
- Syringes and screw-needles
- Micro-filters
- Calibrated Pipettes
- Pipette tips
- T25, T75, T175 flasks

- Centrifuge tube
- Centrifuge
- Dose calibrator
- Dose meter
- Shielded fume hood: *We used a Pb shielding slab to line the fume hood, and worked behind a lead shielding panel with a viewing window*
- Shielded CO₂ incubator: *We formed a Pb shielding container and ensured that no radiation was leaking from the incubator*
- Flow cytometer: *We used a Guava MUSE Cell Analyser for all assays including cell count and viability*
- Data analysis and graphing software: *We used GraphPad Prism 8*

4.4. Procedure

4.4.1. Understanding the Logistics

Estimated total time: Up to 3 months (each optimisation taking approximately 1 week)

NOTE: Figure 4.1 shows a snapshot of the space required to perform optimisation steps, which should also be considered in the steps below.

1. Choose a suitable cell line, a relatively short half-life radionuclide, and a series of assays as these will determine the overall scheduling of the tissue culture.
 - (i) A pilot clonogenic assay should be performed on the cell line to confirm that individual colonies formed are distinguishable from each other. This can be done by seeding a range of cells (i.e., 200 to 1200 in 200 cell increments) and observing the stability of the cell growth over 6 cell cycles. The methods described in *Franken et al.*, or an in-house alternative such as the method described in *Hioki et al.* should be used.^{114,115} The time between irradiation and harvesting for this assay should be kept consistent throughout the entire

protocol where possible, and any variations should be noted to observe any significant effects.

- (ii) The chosen radionuclide should be easily accessible to your institution, with any logistical considerations including delivery dates and times must be incorporated into the scheduling.
 - (iii) The radiobiological information that is to be determined will dictate the assays that will be performed. This will vary the incubation times for the cell lines' post-irradiation, dependent on the doubling time. One assay may need the immediate harvesting of the cells and another may require a further 24 hour incubation or even an entire cell cycle before the assay can be performed.
2. The initial seeding of the smallest flasks (i.e., T25) to be irradiated is calculated by working backwards from the longest incubation time between the seeding to the harvesting of cells. To ensure that the control flask of the longest incubation does not go beyond 80% confluence, an appropriate seeding number is determined. The limiting factor is the number of cells that is available to be seeded from the larger flask, and therefore an even larger flask may be required (i.e., T175 instead of a T75). Two examples on the following page gives possible scenarios for different doubling times.
- (i) Using a simplified arithmetic approach in determining the cell density, it is possible to continue working backwards to determine whether or not a T75 or T175 is required for the stock cell flask. This is dependent on how many flasks will be required (i.e., how many different doses will be administered simultaneously, assays performed and the respective control flasks needed for each assay). This approximate number needs to be experimentally verified.
 - (ii) Depending on the cell line, there is a variability of proliferation rate post-seeding due to the changing environment; some cells are capable of adhering and return to normal proliferation rate quickly, whereas more sensitive cell

lines may require a few hours before adhering properly. Again, this will need to be investigated through day-to-day observations when passaging (reseeding into new flasks).

3. By this point, the following should be established to ensure that the remainder of the protocol runs smoothly.
 - (i) Observed doubling time of each cell line
 - (ii) The assays to be performed for the radionuclide irradiation
 - (iii) The number of flasks needed per radionuclide experiment (inclusive of the control)
 - (iv) Cell seeding number required to achieve 80% confluence of the large stock flask (i.e., T75 or T175)
 - (v) Cell seeding number required for the small flask (i.e., T25) to be irradiated to achieve 80% after the longest incubation time
 - (vi) Cell seeding number required for the clonogenic assay (i.e., in a 6-well plate)
 - (vii) The timing between irradiation and harvesting cells for the chosen assays



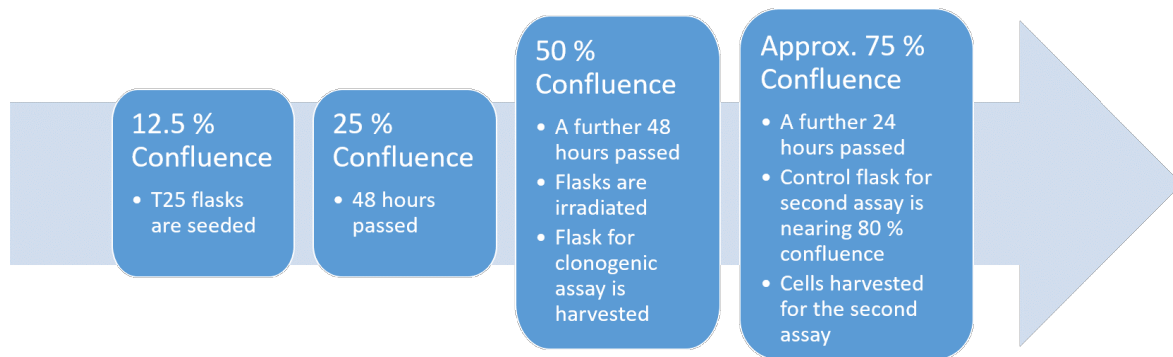
FIGURE 4.1. Incubator with flasks and plates for optimisation

NOTE: The percentage confluence in Examples 1 and 2 are guidelines and do not reflect the true cell count increases over the times described; this can only be verified and scaled via a counting assay.

Example 1.

Cell doubling time : 48 hours

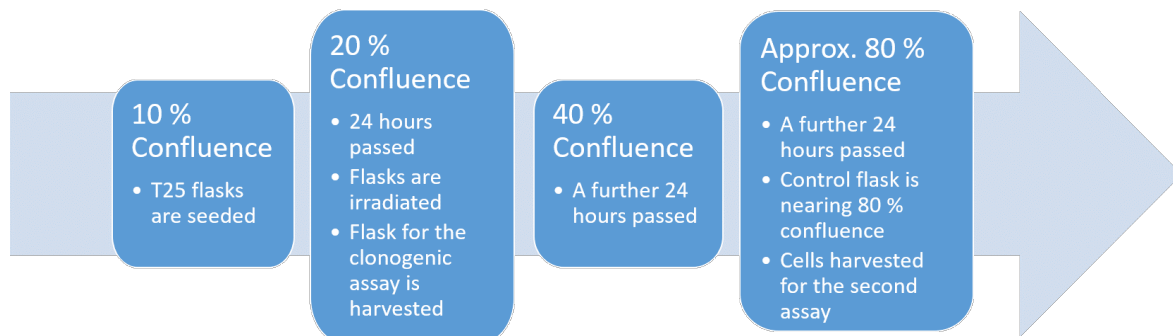
Longest incubation time post-irradiation : 24 hours



Example 2.

Cell doubling time: 24 hours

Longest incubation time post-irradiation: 48 hours



4.4.2. Benchmarking Experiment with EBRT

Estimated total time: Up to 1 month (each optimisation taking approximately 1 week)

1. Establish an irradiation setup for the chosen EBRT machine, as shown in figures 4.2 for the SARRP or 4.3 for as LINAC (i.e., field size, source-to-surface distance, build-up and backscatter material, amount of cell medium being irradiated, etc.), and verify the beam profile and uniformity to ensure that the desired dose can be delivered. Our dose delivery was confirmed with an EBT3 Gafchromic film. The setup must be validated by a radiation oncology medical physicist if the irradiation of a T25 flask has not been established at your centre.

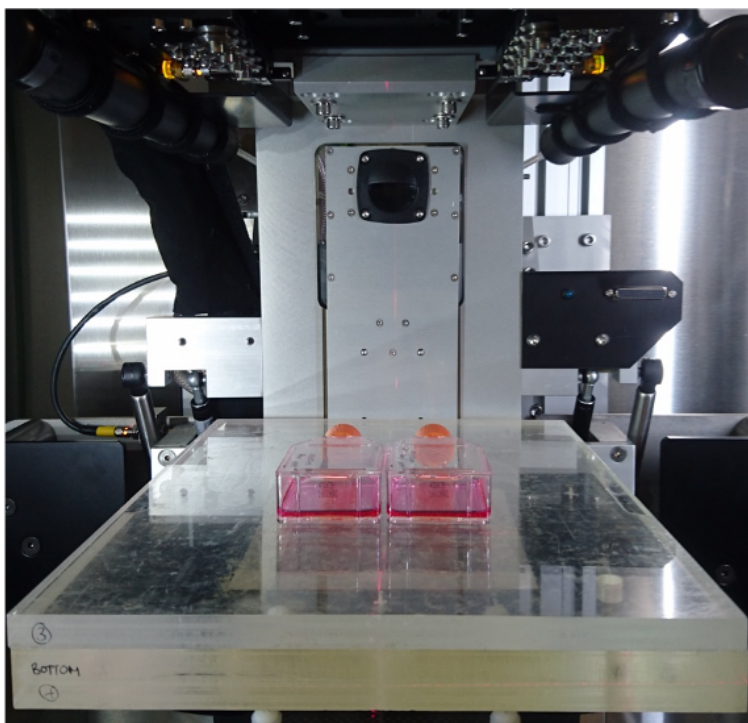
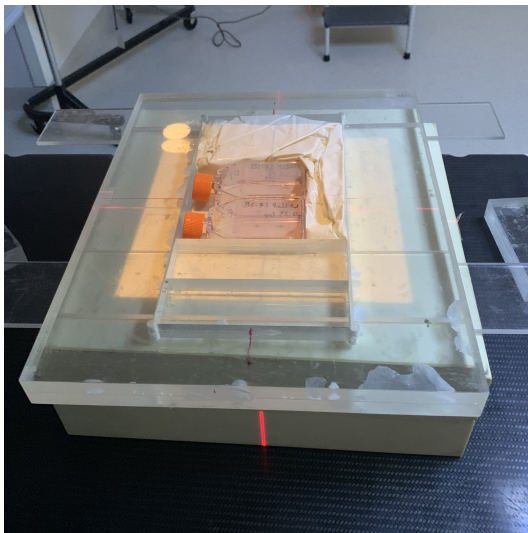
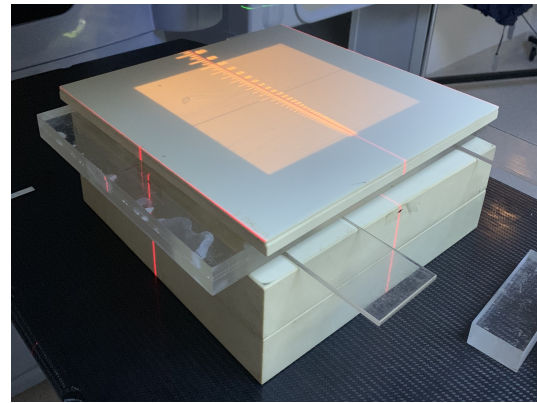


FIGURE 4.2. SARRP irradiation setup with T25 flasks

2. After the verification of dose delivery, irradiate the T25 flasks with one of the three dose range options below. This step is a screening exercise, thus only one set is required as opposed to the standard minimum of a triplicate.
 - (i) For reported radioresistant cell lines, 0 to 10 Gy in 2.5 Gy increments.



(A) Flasks with solid water filling



(B) Solid water build-up setup for the irradiation

FIGURE 4.3. LINAC irradiation setup with T25 flasks

- (ii) For reported radiosensitive cell lines, 0 to 6 Gy in 1.5 Gy increments.
 - (iii) For cell lines with unreported radiosensitivity, 0 to 10 Gy in 2 Gy increments.
3. Perform a clonogenic assay for the flasks using the seeding numbers obtained in Procedure 4.4.1 and calculate the survival fraction for each dose using equation 4.4. Three possible trends will be present from the survival curves as shown below in figure 4.4.
- (i) If the trend corresponds to b), then proceed to perform triplicates of the irradiation, while adding smaller incremental range of absorbed doses at the “shoulder” of the curve as this heavily dictates the β parameter
 - (ii) If the trend corresponds to a) or c), repeat the pilot screening by increasing or decreasing the dose range and increment to encompass the linear and quadratic region of the survival curve until a trend shown in b) is achieved.
 - (iii) If the trend still corresponds to c) after repetition, the cell line may potentially be too radiosensitive to derive the radiobiological parameters for *in vitro* irradiation and should be re-evaluated.

Survival Curve Examples for EBRT

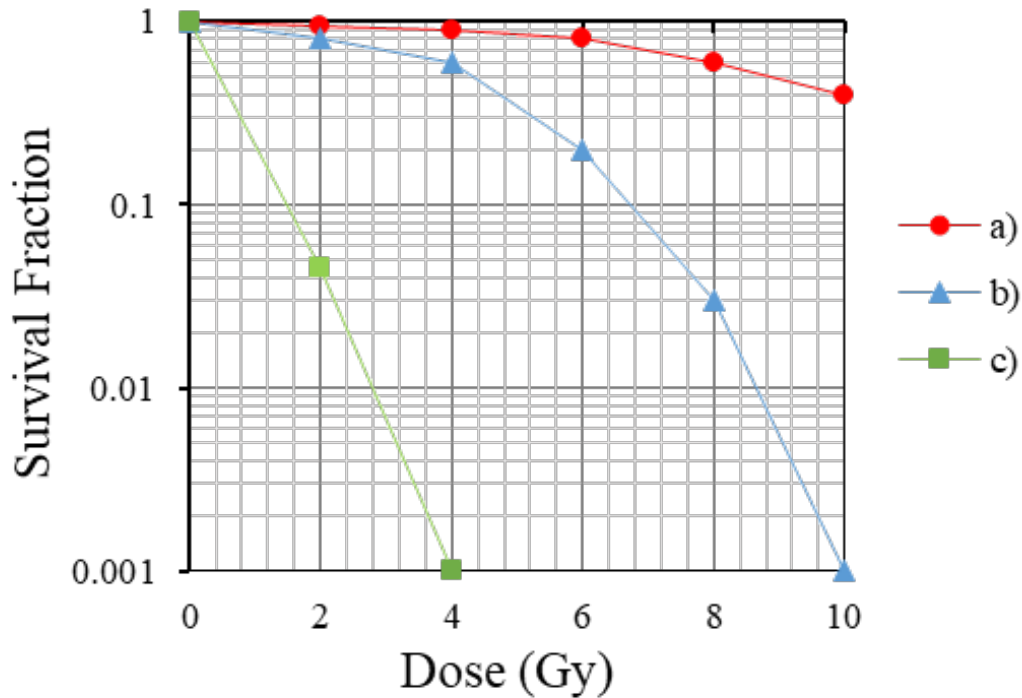


FIGURE 4.4. Example survival curves; for the range of irradiated doses: a) represents a radioresistant cell line requiring higher doses to obtain accurate radiobiological parameters, b) represents an optimal dose range, and c) represents a radiosensitive cell line requiring more intervals between 0 and 2 Gy.

4. Once a minimum of three clonogenic assay results are collected for the determined range of doses, fit the LQM (Eq. 4.1) to obtain the α and β values.

4.4.3. Screening Radionuclide Doses and Calculation

Estimated total time: Up to 2 months (each optimisation taking approximately 1 week)

As the cell kill from the EBRT modality is significantly more pronounced than from the irradiation using radionuclides, a relatively large dose must be delivered to obtain a comparable survival/kill. This must be determined in a similar process as Procedure 4.4.2,

but in larger incremental doses up to, and potentially beyond 50 Gy, where practicable. Ensure that the incubator and work area is prepared and shielded before commencing the practical component of this section, beginning at step 4).

NOTE: In some circumstances, the amount of activity required to deliver a dose that has the same endpoint as the EBRT for an RBE comparison is not feasible; it is known that the efficacy in cell kill is superior for EBRT due to its high dose rate compared to radionuclides. Although RBE comparisons can be calculated at any survival fraction between the two modalities, attempt to aim for a point of comparison achievable with all of your cell lines, for example, 50 or 80% cell kill instead of 99.9% cell kill.

1. Keeping the irradiation time constant (i.e., 24 hours) for all of the doses administered, calculate the initial activities, and the total activity of the entire batch, needed to deliver the desired absorbed doses of your chosen radionuclide using the equations below:

$$D = \frac{\tilde{E}}{m} \quad (4.5)$$

$$\tilde{A} = A_0 \int_0^T e^{-\lambda t} dt \quad (4.6)$$

$$= \frac{A_0}{\lambda} (1 - e^{-\lambda T}) \quad (4.7)$$

$$\tilde{E} = \tilde{A} \sum_i^j \bar{E}_i \kappa_i \quad (4.8)$$

$$A_0 = \frac{D \lambda m}{\sum_i^j \bar{E}_i \kappa_i (1 - e^{-\lambda T})} \quad (4.9)$$

Where:

- D = target absorbed dose (Gy)

- $\tilde{E}$ = total energy delivered (J)
- m = mass of the volume of cell culture medium in the flask (kg)
- $\tilde{A}$ = cumulative activity or total number of disintegration
- A_0 = initial activity required to deliver D (Bq)
- T = total irradiation time (s)
- λ = decay constant (s^{-1})
- $\bar{E}$ = average energy of the decay (J)
- κ = branching ratio or fraction of total decay that produces the particle of interest

NOTE: The irradiation time should be chosen in such a way that the activity required to deliver the highest dose is feasible. The summation in Eq. 4.7 should be simplified for energy deposited by the particles of interest only for simplicity. For radionuclides with multiple decay chains, the summation must be performed for every individual daughter radionuclide that contains the particle of interest, with a κ summing to unity per daughter radionuclide.

Example 3. Using ^{18}F , its decay to ^{18}O goes through two paths in a single step. Each path has a corresponding branching ratio of 0.9673 and 0.0327 for β^+ emission and electron capture, respectively. Although the electron capture leads to the emission of Auger electrons along with characteristic x-rays, for the sake of simplicity, purely the β^+ component of the decay was incorporated into the absorbed dose calculation:

$$\tilde{E} = \tilde{A}\bar{E}\kappa = \tilde{A} \times 249800 \text{ eV} \times 1.602 \times 10^{-19} \text{ J} \times 0.9673$$

Example 4. In a more complicated example of radioactive decay, the ^{225}Ac decay to ^{209}Pb has multiple steps of α and β^- decays, along with the associated characteristic x-rays, Auger and conversion electrons for most intermediate steps. Although it is

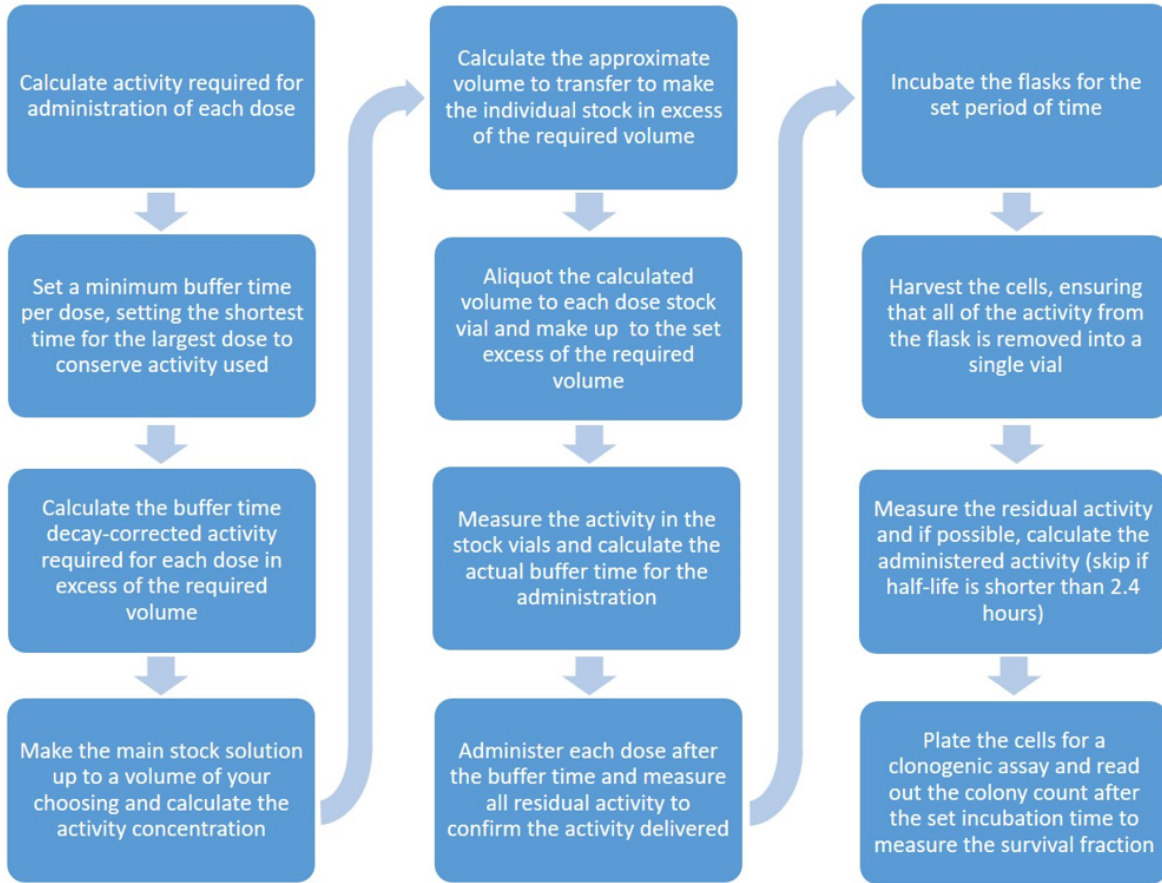


FIGURE 4.5. Flowchart of the key steps in Procedure 4.4.3.

entirely dependent on the aim of the study, a simulation to quantify the contribution of dose from x-ray and electron emissions should be performed as the total absorbed dose is highly dependent on the geometry. If one decides to simplify this to the contribution of dose from only α and β^- particles, then a combination of Eq. 4.8 will be implemented into Eq. 4.10, as shown below:

$$\tilde{E} = E_{\alpha} + E_{\beta^-} = \tilde{A} \left(\sum_i^j \bar{E}_i \kappa_i + \sum_m^n \bar{E}_m \kappa_m \right), \quad (4.10)$$

where the summations correspond to the α and β^- components, respectively.

NOTE: The flowchart given in figure 4.5 summarises the steps elaborated throughout Procedure 4.4.3.

2. Choose a buffer time; the interval of time between the activity administrations of each flask, to use in the determination of the stock vial preparation. This buffer achieves an accurate initial activity that aligns with the calculated values.

NOTE: Ensure that the buffer time is shortest for the largest activity to administer as it requires less activity to prepare, and therefore manages cost.

3. Calculate the decay corrected activity inclusive of the buffer time for each dose, making the stock volume in slight excess of the volume administered into the flask; this volume can be altered if limited activity is available.

Example 5. The below makes a stock volume of 1.5 times the required volume in the flask:

- Required activity: 200 MBq
- Buffer time decay-corrected activity: 230 MBq
- Required radioactive medium volume: 2 mL
- Total volume of stock: 3 mL
- Activity in stock of 3 mL: $230 \times 1.5 = 345$ MBq

4. Make up the main stock solution up to a volume of your choosing (i.e., 10 mL) and calculate the activity concentration of the solution to approximate the volume to transfer into the dose stock vials.
5. Aliquot from the main stock solution to the individual dose stock vials, recording the activity using the dose calibrator.
6. Calculate the actual buffer time from the activities in the dose stock vials, adjusting the volume to match the desired activity if the buffer time is not feasible.
7. Make up each dose stock vial to the total desired volume (i.e., 1.5 times the required volume).

8. Before the buffer time expires for a particular dose stock vial, remove the culture medium from the flask, ensuring that as little liquid remains to allow for an accurate dose delivery.
9. Administer the dose into the corresponding flask and place it into the shielded incubator.
10. Repeat steps 8 and 9 for all prepared flasks.
11. After the set incubation period (i.e., 24 hours), harvest the cells ensuring that all the activity from the flask is removed by gently washing multiple times using PBS, collecting it into a single tube.

NOTE: For low-adhering cell lines, step 11 can remove cells that may have been successfully damaged by the irradiation. When measuring the viability, the cells can either: be combined together after centrifuging the cells collected into the tube after the PBS wash and performing step 12, or plated separately in the clonogenic assay to determine whether the irradiation further impacted the adherence of the cells, or, discarded. This will all depend on the nature of the study.
12. Measure the residual activity in the collected tube in the dose calibrator and decay correct to find an estimate of the administered dose; if the half-life is shorter than 10 times the incubation period, then this step can be skipped.
13. Plate the cells for a clonogenic assay and read out the colony count after the incubation time (i.e., 7 days).
14. Calculate the survival fraction for each flask and plot the survival curve; if the dose response of the cell lines does not achieve the desired endpoint, the dosing interval must be re-evaluated.
15. Repeat steps 4 to 14 at least two more times and plot with the EBRT results from Procedure 4.4.2.

16. Determine the RBE of the radionuclide against the EBRT at a desired end-point (i.e.; 50% survival), and calculate the Lea-Catcheside G-factor of the RNT irradiation using Eq. 4.3.

Example 6. For the irradiation using ^{18}F and a Small Animal Radiation Research Platform (SARRP, Xstrahl), the RBE was calculated at the end point of 50% survival, shown in figure 4.6.

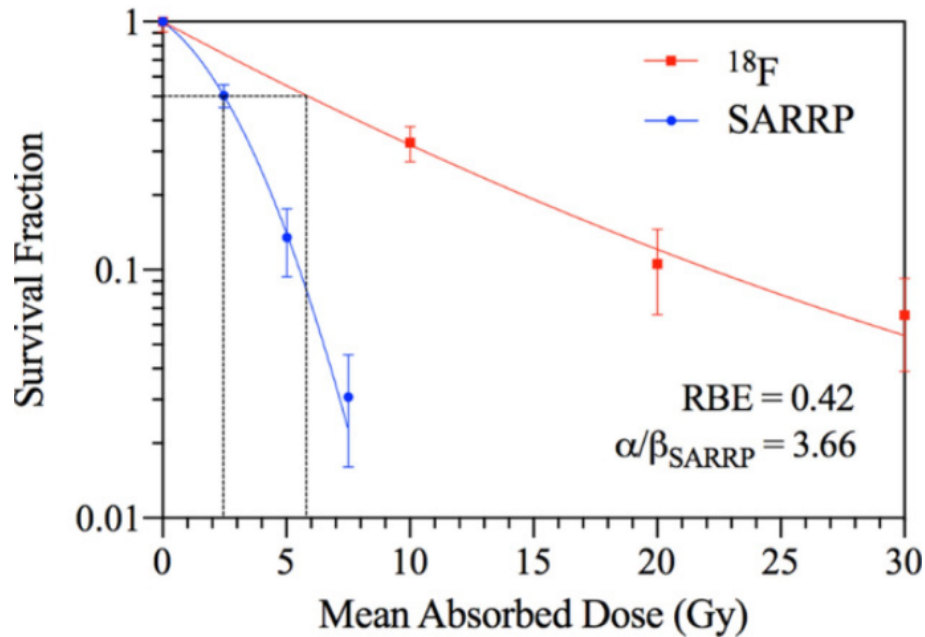


FIGURE 4.6. Example of the RNT and EBRT survival curves for the RBE analysis: endpoint at 0.5 survival.¹¹⁴

4.4.4. *Performing the Optimised Irradiation and Assays*

Estimated total time: Up to 2 months (each optimisation taking approximately 1 week)

The steps in this section of the procedure are identical to those in Procedure 4.4.3, but is scaled up to incorporate the biological assays along with the investigation into the G-factor effects. The incubation time and number of simultaneous flasks set up are the key differences to be noted. As the irradiation can disrupt the cells' proliferation, adherence and sensitivity

to medium changes, if observations such as the cell count upon harvesting was noted, this must be taken into account prior to commencement of this section.

1. Choose the doses to administer dependent on the nature of the investigation; the dose that has already achieved a considerable cell kill can be used as an endpoint.
2. Using the G-factor calculated in Procedure 4.4.3 as a point of reference, determine the range of G-factors to use and calculate the irradiation time required using Eq. 4.3, along with the associated activities required to achieve the target doses in this time.

NOTE: The higher the Lea-Catcheside G-factor, the shorter the irradiation time to deliver the same dose, thus resulting in a higher initial activity. For relatively higher absorbed doses, some G-factor values may not be feasible due to the scaled activities required.

3. Plan the assays to perform, ensuring to note assays that can be performed at the same time to utilise as many cells irradiated as possible; the number of flasks to irradiate double for each assay that cannot be performed simultaneously.

Example 7. For assays such as the apoptotic marker, the harvesting can occur simultaneously with the clonogenic plating. For other assays such as the cell cycle requiring further incubation post-irradiation, cells must be harvested from a separately irradiated flask.

4. Follow steps 4 to 12 in Procedure 4.4.3 and harvest the cells according to the protocols of the chosen biological assay(s).

4.5. Timing

As the timing of this protocol at every experimental step is dependent on the half-life of the radionuclide, doses to deliver, G-factors investigated, doubling time of the cell line, assays chosen, and number of replicates, there is no indication on how long each procedure will take. An estimate is provided in each Procedure section, based on a 24

hour doubling time cell line. The preparation of activity, along with the harvesting of the cells post-irradiation is purely dependent on the proficiency of the individual performing the experiments.

4.6. Anticipated Results

Direct comparison of EBRT and RNT irradiation using the clonogenic assay provides an RBE as a benchmark to evaluate the efficacy of the radionuclide in affecting cell survival. It should be noted that this is done to allow for the comparison between different radionuclides, as the RBE will always be less than unity for therapeutic radionuclides. Figure 4.7. displays an example of the decreasing clonogenicity of cells with increasing absorbed dose, with a potential for saturation of efficacy around the higher total absorbed doses.

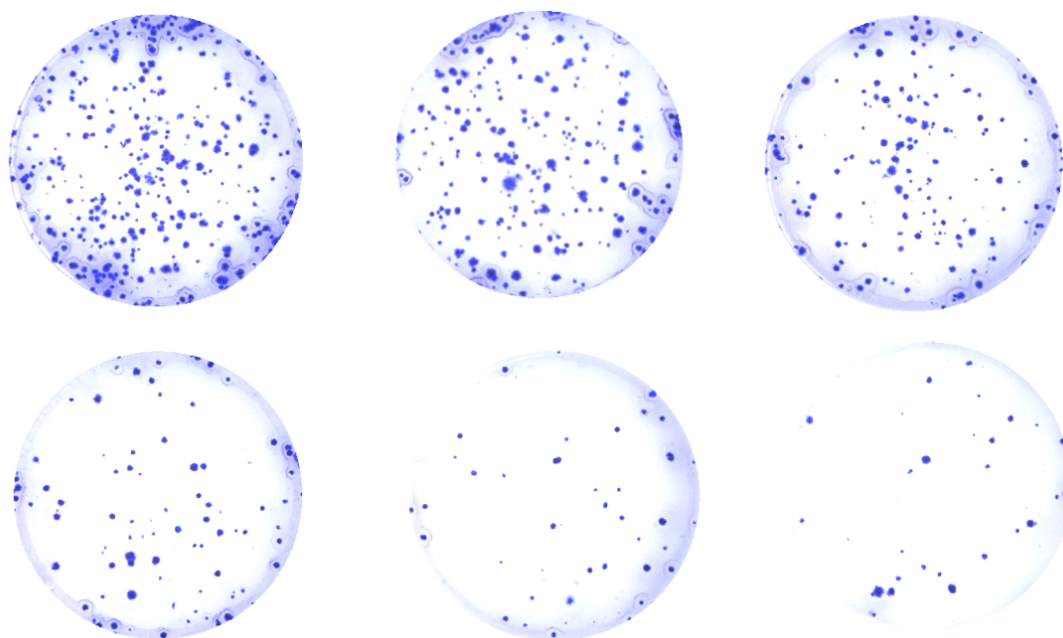


FIGURE 4.7. Decreasing colony formation with increasing dose

4.7. Conclusion

Radiobiological characterisation of a cell line is a crucial step towards maximising the efficacy of using radionuclides in therapy. Although the current method in treating cancer

patients in trials are not personalised, through the information obtained using a standardised *in vitro* protocol, a patient specific prescription of activity for cancer therapy in nuclear medicine can be achieved.

Overlooked Potential of Positrons in Cancer Therapy

5.1. Statement of Joint Authorship

The article *Overlooked Potential of Positrons in Cancer Therapy* has been published in *Scientific Reports (Springer Nature)* with the following as the contribution from the authors:

Author	Specific Contributions	Percentage Contribution (%)
Takanori Hioki	Developed and performed experiments, built and ran simulations, analysed results, wrote manuscript	75
Yaser H. Gholami	Assisted in simulation design and results analysis and edited manuscript	5
Kelly J. McKelvey	Assisted in experimental design and results analysis and edited manuscript	5
Alireza Aslani	Edited manuscript	1
Harry Marquis	Edited manuscript	1
Enid M. Eslick	Edited manuscript	1
Kathy P. Willowson	Edited manuscript	1
Viive M. Howell	Edited manuscript	1
Dale L. Bailey	Assisted in both experimental and simulation designs along with results analysis, edited manuscript	10

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Dale Bailey

28th of February 2024

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Takanori Hioki

28th of February 2024



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Overlooked potential of positrons in cancer therapy

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Positron (β^+) emitting radionuclides have been used for positron emission tomography (PET) imaging in diagnostic medicine since its development in the 1950s. Development of a fluorinated glucose analog, fluorodeoxyglucose, labelled with a β^+ emitter fluorine-18 (^{18}F -FDG), made it possible to image cellular targets with high glycolytic metabolism. These targets include cancer cells based on increased aerobic metabolism due to the Warburg effect, and thus, ^{18}F -FDG is a staple in nuclear medicine clinics globally. However, due to its attention in the diagnostic setting, the therapeutic potential of β^+ emitters have been overlooked in cancer medicine. Here we show the first in vitro evidence of β^+ emitter cytotoxicity on prostate cancer cell line LNCaP C4-2B when treated with 20 Gy of ^{18}F . Monte Carlo simulation revealed thermalized positrons (sub-keV) traversing DNA can be lethal due to highly localized energy deposition during the thermalization and annihilation processes. The computed single and double strand breakages were $\sim 55\%$ and 117% respectively, when compared to electrons at 400 eV. Our in vitro and in silico data imply an unexplored therapeutic potential for β^+ emitters. These results may also have implications for emerging cancer theranostic strategies, where β^+ emitting radionuclides could be utilized as a therapeutic as well as a diagnostic agent once the challenges in radiation safety and protection after patient administration of a radioactive compound are overcome.

Main

Approximately 90% of cancer related deaths are from metastatic disease and thus the focus of cancer radiotherapy (RT) in both the scientific and clinical communities is shifting towards a more targeted, personalized approach¹. While the radiobiology of gamma, MV X-rays and particles (e.g., β^- , α^{2+} , Auger electron) for therapy have been well investigated, there is a paucity of in vitro and in vivo studies on the biological impact of the positron (β^+) which could provide a new modality for cancer RT^{2–5}. The β^+ is the anti-matter counterpart of the electron and its emission from a radionuclide such as fluorine-18 (^{18}F), leads to the conversion of a proton within the atomic nucleus to a neutron accompanied by a release of an electron neutrino. This decay process results in two 0.511 MeV photons due to the annihilation of the β^+ when coming to rest in matter and making contact with an electron. β^+ lose their kinetic energy in discrete quantities (≈ 100 eV) producing ‘rabbits’ of excited and ionized molecules⁶. They create an excess-electron/positive-ion pairs inside a spherical nano-volume along its radiation track (called the β^+ ‘spur’) and during the post-thermalization process (called the terminal β^+ ‘blob’) with further annihilation ionization when they come to rest after positronium (Ps) formation (Fig. 1)^{7–9}.

Each spur and blob on the particle track are a temporal and spatial microcosm of highly reactive species capable of locally depositing energies up to 100 and 5000 eV respectively⁹. Thus, a relatively large radiation dose (i.e., large energy deposition in a small volume) can be delivered when a spur or blob interacts with biomolecules such as DNA⁹. Despite these unique radiation properties, the fundamental interactions of β^+ particles before the annihilation process and its potential therapeutic impact in vivo/vitro, have not been considered due to its prominent role in PET to image annihilation photons. The aim of our study was to derive the radiobiological parameters for ^{18}F β^+ emission and demonstrate proof-of-concept cytotoxicity data for β^+ emission in radionuclide therapy (RNT). These results may have implications for novel RNT treatment planning strategies, where β^+ emitting radionuclides are used as a theranostic.

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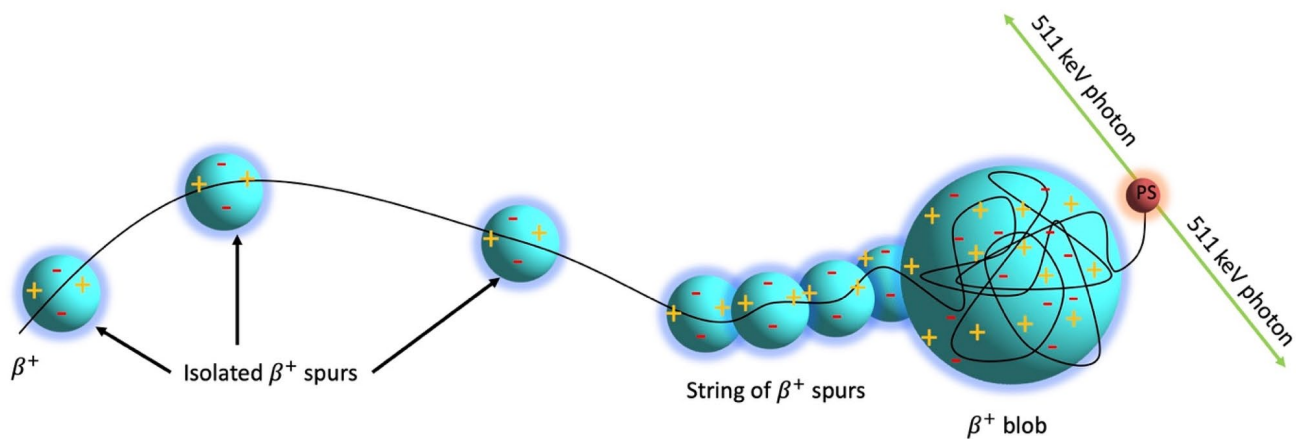


Figure 1. β^+ track deposits energy in water in through the formation of “spurs” and the terminal “blob”, before a positronium (Ps) formation and annihilation to generate the 0.511 MeV photons.

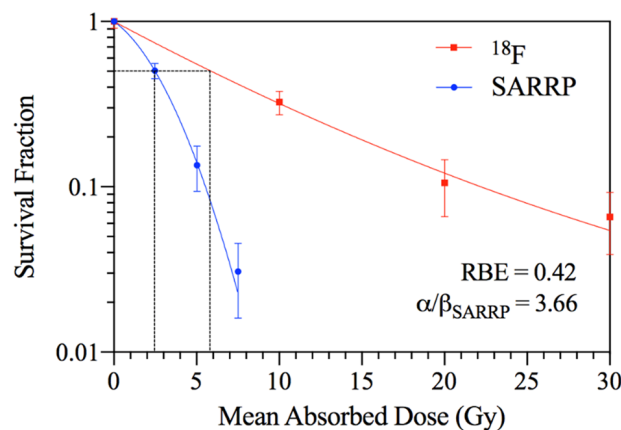


Figure 2. LNCaP C4-2B Clonogenic Cell Survival: ^{18}F β^+ emission versus SARRP EBRT. Black dotted line depicts the mean absorbed dose required to reduce cell survival fraction to 0.5.

Using clonogenic assays described in the methods, the cell survival fraction (SF) shown in Fig. 2 demonstrated > 90% cell kill when LNCaP C4-2B prostate cancer cells were treated with 20 Gy ^{18}F β^+ . Furthermore, Fig. 2 shows the relationship between the absorbed dose of ^{18}F β^+ emission and its impact on cell clonogenicity compared to X-ray external beam radiotherapy (EBRT) using the small animal radiation research platform (SARRP—See supplementary data). Both data have a linear quadratic (LQ) trend fitted for the purpose of visualization and to extrapolate the relative biological effectiveness (RBE). The SF data from ^{18}F are relatively linear up to 20 Gy, but trends towards an exponential decay at higher doses. SARRP X-ray irradiation induced an expected LQ trend in cell kill, with a shoulder at ~ 2.5 Gy, and a linear region at higher doses [α/β ratio = 3.66; Eq. (3)]. The calculated mean RBE from Fig. 2 was 0.42 for ^{18}F relative to the 0.5 SF for the SARRP.

Using Monte Carlo (MC) simulation of a linear DNA model in TOPAS-nBio we acquired the frequency of hits per one million primaries resulting in single strand breaks (SSBs) and double strand breaks (DSBs) from the emission of β^+ and β^- /electrons, presented as a function of the particle's kinetic energy in Fig. 3(i),(ii)^{10,11}. Both β^+ and β^- /electrons exhibited a similar trend for SSB with an exponential decrease for particles with higher kinetic energies (Fig. 3(i)). DSB frequency for both β^+ and electrons showed an exponential trend as their energy increased (Fig. 3(ii)). The high fluctuations in the recorded DSB for energies < 400 eV is primarily due to the limitations of the MC package PENELOPE, as the particle transport is not recorded below 100 eV. The spatial dependency of a DSB also contributes to the fluctuations, as variations in SSBs for a particular energy are significantly lower than that of DSBs. Observing the trend of the plot for the linear energy transfer (LET) (Fig. 3(iii)) for the primary particle simulated, the expected maximum SSB and DSB frequency for both β^+ and electron would have been at 250 eV for the simulated energies. Although the LET of β^+ and electrons significantly increase when they come to rest, β^+ have relatively higher LET ($\approx 7\%$ higher at $E = 250$ eV).

This is also consistent with the blob model where the β^+ creates an excess-electron/positive-ion pairs towards the end of its track. In addition to the β^+ annihilation ionization with outermost valence electrons (which is the predominant process), a fraction of sub-eV β^+ can tunnel through the repulsive nuclear potential and annihilate with core electrons (a phenomena used in Induced Auger Electron Spectroscopy)¹². This creates a vacancy in the inner electronic shell leading to an emission of high LET Auger electrons and, thus, a β^+ traversing the DNA

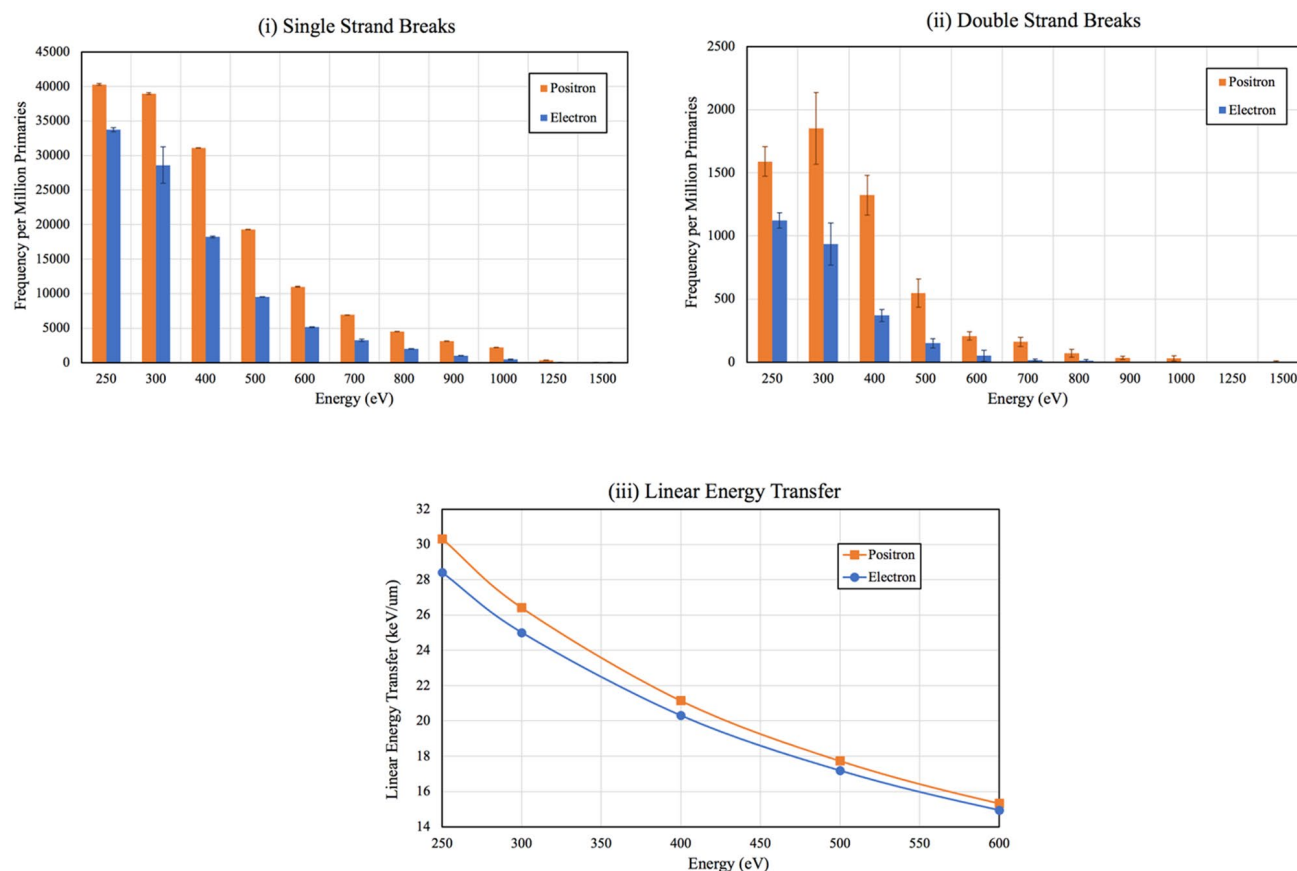


Figure 3. (i) SSB frequency and (ii) DSB frequency for β^+ and electrons from the TOPAS-nBio simulation. The highest difference in the frequency between β^+ and electrons occurred at 400 eV. (iii) Plot of the primary β^+ and electron LET (keV/μm) across the simulated energies.

has a higher probability of causing lethal damage when compared to electrons. This is again consistent with the trend observed for both the simulated SSB and DSB induced by β^+ tracks.

The greatest difference in SSBs and DBSs for the emission profiles occurred at 400 eV where β^+ demonstrated a 55% integral increase in SSBs compared to electrons, and a 117% increase in DSBs. These results are consistent with our previous study demonstrating the use of ^{89}Zr and ^{64}Cu for dose enhancement in nuclear medicine imaging compared to β^- therapeutic isotopes such as ^{67}Cu , by virtue of their higher ionization properties¹³. Another in vivo study demonstrated that both ^{64}Cu (with 38.5% β^-) and ^{67}Cu (with 100% β^-) were an effective targeted β^- RNT for subcutaneous human colon carcinoma carried in hamster thighs when radiolabeled with a mouse anti-human colorectal cancer¹⁴. Although in this study only the β^- emission of ^{64}Cu (with decay branching ratio: 38.5% β^- , 43.9% electron capture and 17.6% β^+ emission) was considered for therapy, our results suggest that there could be a noticeable contribution of β^+ emission in the therapeutic effectiveness of ^{64}Cu targeted RNT.

Cell survival curves of X-ray versus β^+ irradiation of LNCaP C4-2B cells revealed substantial cell kill ($\approx 70\%$ at 10 Gy) by ^{18}F β^+ emission. This may be therapeutically significant as previous in vitro studies have shown less than $\sim 30\%$ cell kill in a range of cancer cell lines by 10 Gy of β^- emitting radionuclides, ^{90}Y and ^{177}Lu ^{15,16}. This suggests that the RBE of ^{18}F β^+ emission may be three times higher than β^- emitters depending on the β^- and β^+ emitters being compared. Although these results suggest that other β^+ emitter isotopes such as ^{68}Ga , ^{64}Cu and ^{89}Zr could potentially be considered as a therapeutic isotope, due to their differences in positron energy spectrum, branching ratio and half-life, further in vitro experiments are required to quantify their therapeutic impact. The MC simulation demonstrated 1.5-fold and 2.2-fold more SSBs and DSBs, respectively, are induced by β^+ tracks, when compared to electron tracks. Thus, the direct interaction of a single β^+ with DNA results in more lethal (i.e., DSB) damage than for a single electron.

Based on the spur model (Fig. 1), a β^- /electron and β^+ have similar radiation tracks except at sub-keV energies^{7–9}. For a sub-keV electron, the mean separation between two energy deposition events and the formation of spurs (≈ 40 nm) is 20 times larger than the diameter of the DNA helix (≈ 2 nm)^{7,16}. However, for a sub-keV β^+ track (i.e., thermalized β^+), spurs are formed in a continuous distribution of radiation damage with a higher density of several electrons and ions. Thus, at sub-keV energies, β^+ have a higher LET compared to β^- /electrons (Fig. 3(iii))^{6,7}. In addition, the total number of ionizations per β^+ track is greater than per electron due to the additional ionization occurring at the terminal annihilation event. Moreover, in a β^+ spur the incident β^+ , secondary electrons and ions are within each other's electrostatic/Coulomb field resulting in greater localization of energy deposition and a higher probability of inducing direct DNA DSB.

As per convention, the radiobiological parameters of ^{18}F β^+ emission in our in vitro study were benchmarked against X-ray therapy. The low mean β^+ RBE of 0.42 compared to X-ray (SARRP) calculated from the cell survival curve can be attributed to the >200-fold difference in dose rate between the two modalities (i.e. $\approx 7\text{--}21$ mGy/min and 4.5×10^3 mGy/min respectively). For X-rays, the main mechanism of cell kill is through indirect methods primarily via the production of free radical species.

X-ray EBRT cell survival fraction curves often have a shoulder [quadratic region of LQ model; Eq. (3)] at 2 Gy contributed by the reconciliation of DNA damage and DNA repair^{1,2}. In LNCaP C4-2B cells this was observed at 2.5 Gy (Fig. 2) using the SARRP^{1,2}. While the lower initial slope observed for ^{18}F β^+ irradiation at this similar dose region may be due to dose rate differences between SARRP and ^{18}F irradiation, at the higher doses, the clonogenic survival approaches a deterministic process where the relative difference in number of surviving clonogenic cells is proportional to the increase in dose [linear region of LQ; Eq. (3)], and thus the corresponding survival curve becomes exponential¹⁷.

Current administration of β^+ emitting radionuclides in the clinic ensures that the doses absorbed to the patients in diagnostic nuclear medicine are minimized and reported values for mean effective doses for ^{18}F -FDG range from 3.4 to 13.4 mSv¹⁸. In RNT, therapeutic doses vary significantly due to differences in tumor type, burden and administration method. For example, absorbed doses range from 1.2 to 540 Gy in thyroid cancer patients, whereas for prostate cancer reported doses range from 3.4 to 92.5 Gy^{19,20}. The large differences between diagnostic and therapeutic doses in part explains the lack of investigation into β^+ emitters as a therapeutic agent. The abundance of currently available targeting ligands for imaging using β^+ emitters allow for a “theranostic” application with the same radionuclide to be feasible using this approach²¹.

Our study presents the first in vitro evidence of therapeutic effect of β^+ emitters in cancer medicine, with an attractive prospective for future studies. Clinically, the highly penetrating, low ionizing properties of the emitted 0.511 MeV photons mean that the safety and logistics behind the administration of therapeutic doses of β^+ emitters must be reconsidered. Although a specialized form of automated/remote injection in a shielded room will be required, the relatively short half-life, cost, availability and reduced imaging time is justifiable to find the means of overcoming the limitations presented.

Methods

Cell culture. The human prostate cancer cell line LNCaP C4-2B, (ATCC, Catalogue no. CRL-3315), was cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% v/v fetal bovine serum (FBS) and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

SARRP X-ray irradiation. LNCaP C4-2B cells were grown in T-25 flasks in 5 mL of medium for 3 days prior to the irradiation to ensure approximately 80% confluence of the cells in the flask. Using the SARRP (Xstrahl Inc, Atlanta, Georgia, USA), the EBRT open-field setup ($20 \times 20 \text{ cm}^2$) was utilized. Flasks were placed at the isocenter of the beam on top of a 3.6 cm Perspex backscatter base at a source-to-axis distance (SAD) of 33.4 cm to give a dose rate of 4.45 ± 0.06 Gy/min at the flask with the default X-ray beam energy spectrum of 225 kVp. Doses using this setup were confirmed using EBT3 GAFchromic™ films. Doses ranging from 0 to 7.5 Gy in increments of 2.5 Gy were delivered by changing the exposure time according to the dose rate. The cells were then harvested for seeding post-irradiation.

^{18}F irradiation. ^{18}F has a half-life of 109.77 min with a branching ratio of 96.73% β^+ emission and 3.27% electron capture²². The emitted β^+ has a mean energy of 249.8 keV and a maximum energy of 633.5 keV, corresponding to mean ranges of approximately 0.6 and 2.4 mm in water, respectively^{22,23}. The sodium fluoride (^{18}F) solution was obtained from Cyclotek NSW (batch number: F181362001) with an activity concentration of 4.14 GBq/mL.

LNCaP C4-2B cells were grown in an identical manner for the SARRP and ^{18}F irradiations. Doses were calculated analytically using Eqs. (1) and (2) and were verified in silico using the sphere model (2 cm^3 volume) in the OLINDA/EXM software Version 1.1 (Table 1)²⁴.

$$D = \frac{\tilde{A} \times \bar{E} \times \kappa}{m} \quad (1)$$

$$\tilde{A} = A_0 \int_0^T e^{-\lambda t} dt \quad (2)$$

where D (Gy) is the dose delivered, $\tilde{A}$ (Bq) is the cumulative activity, $\bar{E}$ (J) is the average energy of the emitted particle, κ is the branching ratio (0.9673), m (kg) is the mass of the target volume (2 mL), A_0 (Bq) is the initial activity, T (s) is the irradiation time and λ (s^{-1}) is the decay constant.

The initial activity concentrations to achieve 10–30 Gy after 10 half-lives (≈ 18 h) ranged from 26.2 to 78.6 MBq/mL. Stock activity of ^{18}F was diluted in an appropriate volume of medium to achieve the required initial activity for each analytical dose including a buffer time, confirmed using a dose calibrator with identical setup. Dose calibration confirmed the specific initial activity concentration of the radionuclide solution (at pH ≈ 7) and approximately 1% of the initial activity remaining after 18 h. For irradiation, the ^{18}F medium solution was passed through a $0.22 \mu\text{m}$ filter to remove biological contaminants and then administered to the T-25 flasks once solutions reached the desired initial activity including buffer time. After 18 h, the radioactive medium was removed, and cells were washed with phosphate buffered saline (PBS) before harvesting for seeding post-irradiation.

Measured A_0 (MBq)	Analytical positron dose (Gy)	OLINDA dose (Gy)
52.4 ± 0.5	9.7 ± 0.1	10.1 ± 0.1
104.9 ± 1.1	19.4 ± 0.2	20.1 ± 0.2
157.3 ± 1.6	29.1 ± 0.3	30.2 ± 0.3

Table 1. Dose calculations for the administered ^{18}F activities as determined from experimental (analytical) and in silico data (OLINDA/EXM).

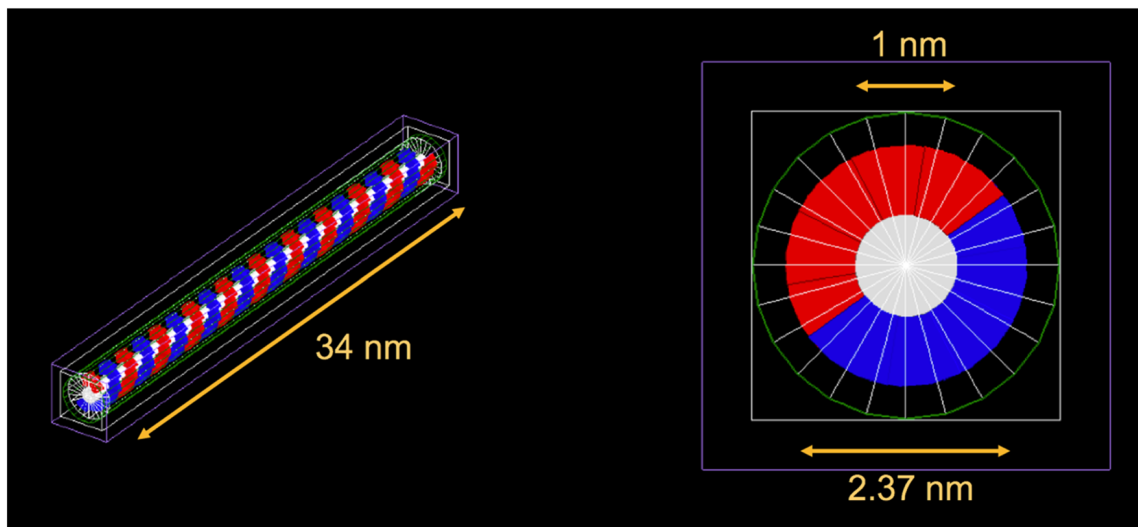


Figure 4. Linear DNA model in TOPAS-nBio, consisting of the sugar phosphate backbone (blue and red), bases (white) and volumetric source (green). 100 bases are simulated, with a total DNA length of 34 nm, outer and base diameter of 2.37 and 1 nm, respectively.

Clonogenic assay. Cell survival was determined using the clonogenic assay post-irradiation delayed plating (DP) method for both the SARRP and ^{18}F experiments.²⁵ The protocol used is further detailed in the “Supplementary Methods”.

Radiobiological parameters. Using PRISM8 (GraphPad, v8.4.0, San Diego, California, USA), cell survival data from the SARRP irradiation were analyzed, and the LQ model [Eq. (3)] was fitted to calculate the radiobiological linear α (Gy^{-1}) and quadratic components β (Gy^{-2}) from the dose delivered D (Gy).¹⁷

$$SF = e^{-(\alpha D + \beta D^2)} \quad (3)$$

To calculate the RBE of ^{18}F irradiation, the cell survival data from the radionuclide experiments were plotted against the EBRT SARRP data, and the RBE determined at the point of 0.5 cell survival fraction. The protocol used is further detailed in the “Supplementary Methods”.

TOPAS-nBio simulation. An MC simulation model was developed in the TOPAS-nBio toolkit to investigate the sub-cellular damage mechanism at DNA level by β^+ and electron irradiations. All simulations were performed using TOPAS-nBio (version 1.0), which uses GEANT4 (Version 10.03.3)^{10,11}. A standard linear DNA model (a half cylinder base and a quarter cylinder backbone) was simulated on this platform (Fig. 4) and isotropically irradiated with a volume source of β^+ and electrons using energies ranging from 250 to 1500 eV to score the single and double strand breakages (SSBs and DSBs respectively)¹¹.

Particles with energies above 1500 eV were not simulated as the cross-section for those energies is significantly small at DNA level. The number of primary incident particles (primaries) for the β^+ and electrons was set to 10^6 , with 3 different seed numbers to gauge the spread in acquired values. The results are reported in frequency per million primaries for each energy. The strand breaks were scored by assuming an energy deposition threshold resulting in ionization of 17.5 eV, with a maximum of 10 bases between two SSBs to define a DSB¹¹. The LET for the primary β^+ and electron were simulated (GEANT4–NIST Database) in water for a range of sub-keV energies (250–600 eV).

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References

- Chaffer, C. L. & Weinberg, R. A. A Perspective on cancer cell metastasis. *Science* **331**, 1559–1564 (2011).
- Durante, M., Orecchia, R. & Loeffler, J. S. Charged-particle therapy in cancer: Clinical uses and future perspectives. *Nat. Rev. Clin. Oncol.* **14**, 483–495 (2017).

3. Nuhn, P. *et al.* Update on systemic prostate cancer therapies: Management of metastatic castration-resistant prostate cancer in the era of precision oncology. *Eur. Urol.* **75**, 88–99 (2019).
4. Frey, B. *et al.* Antitumor immune responses induced by ionizing irradiation and further immune stimulation. *Cancer Immunol. Immunother.* **63**, 29–36 (2014).
5. Pouget, J.-P. *et al.* Clinical radioimmunotherapy—The role of radiobiology. *Nat. Rev. Clin. Oncol.* **8**, 720–734 (2011).
6. Champion, C. & Loirec, C. L. Positron follow-up in liquid water: I. A new Monte Carlo track-structure code. *Phys. Med. Biol.* **51**, 1707–1723 (2006).
7. Mogensen, O. E. *Positron Annihilation in Chemistry* Vol. 58 (Springer, Berlin, 1995).
8. Stepanov, S. V. *et al.* Positronium in a liquid phase: Formation, Bubble state and chemical reactions. *Adv. Phys. Chem.* **2012**, 1–17 (2012).
9. *Clinical radiation oncology.* (Elsevier, Amsterdam, 2016).
10. Perl, J., Shin, J., Schumann, J., Faddegon, B. & Paganetti, H. TOPAS: An innovative proton Monte Carlo platform for research and clinical applications: TOPAS: An innovative proton Monte Carlo platform. *Med. Phys.* **39**, 6818–6837 (2012).
11. Schuemann, J. *et al.* TOPAS-nBio: An extension to the TOPAS Simulation toolkit for cellular and sub-cellular radiobiology. *Radiat. Res.* **191**, 125 (2018).
12. Chirayath, V. A. *et al.* Auger electron emission initiated by the creation of valence-band holes in graphene by positron annihilation. *Nat. Commun.* **8**, 16116 (2017).
13. Gholami, Y. H., Maschmeyer, R. & Kuncic, Z. Radio-enhancement effects by radiolabeled nanoparticles. *Sci. Rep.* **9**, 14346 (2019).
14. Connett, J. M. *et al.* Radioimmunotherapy with a ⁶⁴Cu-labeled monoclonal antibody: A comparison with ⁶⁷Cu. *Proc. Natl. Acad. Sci.* **93**, 6814–6818 (1996).
15. Chan, H. S. *et al.* In Vitro comparison of ²¹³Bi- and ¹⁷⁷Lu-radiation for peptide receptor radionuclide therapy. *PLoS ONE* **12**, e0181473 (2017).
16. Gholami, Y. H. *et al.* Comparison of radiobiological parameters for ⁹⁰Y radionuclide therapy (RNT) and external beam radiotherapy (EBRT) in vitro. *EJNMMI Phys.* **5**, (2018).
17. Jones, L., Hoban, P. & Metcalfe, P. The use of the linear quadratic model in radiotherapy: A review. *Australas. Phys. Eng. Sci. Med.* **24**, 132–146 (2001).
18. Quinn, B., Dauer, Z., Pandit-Taskar, N., Schoder, H. & Dauer, L. T. Radiation dosimetry of ¹⁸F-FDG PET/CT: Incorporating exam-specific parameters in dose estimates. *BMC Med. Imaging* **16**, 41 (2016).
19. Sgouros, G. *et al.* Patient-Specific Dosimetry for ¹³¹I Thyroid Cancer Therapy Using ¹²⁴I PET and 3-Dimensional-Internal Dosimetry (3D-ID) Software. **8**.
20. Violet, J. *et al.* Dosimetry of ¹⁷⁷Lu-PSMA-617 in metastatic castration-resistant prostate cancer: Correlations between pretherapeutic imaging and whole-body tumor dosimetry with treatment outcomes. *J. Nucl. Med.* **60**, 517–523 (2019).
21. Chen, K. & Chen, X. Positron emission tomography imaging of cancer biology: Current status and future prospects. *Semin. Oncol.* **38**, 70–86 (2011).
22. Tilley, D. R., Weller, H. R., Cheves, C. M. & Chasteler, R. M. Energy levels of light nuclei A = 18–19. *Nucl. Phys. A* **595**, 1–170 (1995).
23. Conti, M. & Eriksson, L. Physics of pure and non-pure positron emitters for PET: A review and a discussion. *EJNMMI Phys.* **3**, 8 (2016).
24. Stabin, M. G., Sparks, R. B. & Crowe, E. OLINDA/EXM: The Second-Generation Personal Computer Software for Internal Dose Assessment in Nuclear Medicine. **6**.
25. Franken, N. A. P., Rodermond, H. M., Stap, J., Haveman, J. & van Bree, C. Clonogenic assay of cells in vitro. *Nat. Protoc.* **1**, 2315–2319 (2006).

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Author contributions

T.H. designed and performed the experiment, developed the method and obtained the results. T.H. analyzed the data and wrote the manuscript. All the authors were participants in the discussion and interpretation of results, determination of the conclusions and revision of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Materials

Overlooked Potential of Positrons in Cancer Therapy

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Supplementary Methods

Clonogenic Assay

The DP method requires the harvesting and plating of cells after the initial DNA/cell repair processes are complete, typically > 6 hours post-irradiation.²⁵ The time between irradiation and cell harvesting kept constant at 18 hours post-irradiation. Plated cells were then incubated for 7 days to allow for 6 to 7 cell cycles (LNCaP C4-2B doubling time is 24 hours) to form colonies of minimum 50 cells.²⁵ After 7 days the medium was gently removed, cells washed with PBS and stained using 0.5 % w/v crystal violet in 50 % v/v methanol. After 15 minutes, the crystal violet stain was removed, cells washed with deionized water twice and dried overnight before imaging on a vSpot Spectrum EliSpot reader and analyzed using the colony counting software (Autoimmun Diagnostika GmbH, Straßberg, Germany). SF was calculated using equation (4).²⁵

$$SF = \frac{\text{No. Colonies: Irradiated Cells}}{\text{No. Colonies: Control Cells}} \quad (4)$$

Radiobiological Parameters

Doses delivered by SARRP were at a constant rate while ¹⁸F doses delivered with a constant Lea-Catcheside, G factor. Since the ¹⁸F dose rate mono-exponentially decays over the irradiation time, different ¹⁸F doses needed to be delivered with a constant G factor for RBE and SF comparison studies.¹⁶ A constant G factor assures an average constant DNA damage and repair rate for different doses of ¹⁸F. Therefore, different ¹⁸F doses with constant G factor can be comparable to doses delivered by X-rays at a constant dose rate, and the initial activities of ¹⁸F were dictated by keeping the G factor a constant. The G factor was calculated to be 0.35 using equation (5).¹⁶

$$G = \frac{2}{\lambda - \mu} \left(\frac{\lambda}{1 - e^{-\lambda T}} \right)^2 \left(\frac{1 - e^{-(\lambda + \mu)T}}{\lambda + \mu} - \frac{1 - e^{-2\lambda T}}{2\lambda} \right) \quad (5)$$

where the DNA repair time constant $\mu = \frac{\ln(2)}{\tau}$ and τ is the DNA repair half-life (30 minutes). Our previous paper discussed the relevance of using the extended LQ model for irradiation using radionuclides.¹⁶ The doses delivered were therefore calculated from the initial activity minus any residual activity. Due to the short half-life of ¹⁸F, the dose delivery was able to mimic a clinical administration of the radionuclide where the full decay of the radionuclide contributes to the total dose delivered.

Lea-Catcheside G-Factor Manipulation for the Optimisation of Positron Emission Radionuclide Therapy

This chapter details the *in vitro* experiments manipulating the Lea-Catcheside G-factor and explores the novel concept of fractionating dose delivery in RNT. The radiobiology of EBRT both clinically and experimentally has been well established due to its ability to target large lesions and the dosimetry is independent of any systemic influences when compared to RNT.^{104,106,121} Although immunological responses from EBRT have been gathering attention in the past two decades, the treatment planning and delivery methods for RNT has not been standardised.^{19,122}

The radiobiology of RNT has been developed from EBRT, and thus the modelling of RNT dose responses is still a hotly debated topic as it does not incorporate the complexities of radionuclide dose delivery.^{77,123,124} Additionally to the systemic effects of uptake, retention and excretion, the unique characteristics of the radionuclides themselves including its half-life, modes of decay and energies of emissions, intermediate daughter nuclides with their recoil and branching ratios, make the collective impact on the clonogenicity of tumours immensely difficult to model.^{105,125,126}

The model utilised throughout this thesis is the extended linear quadratic model (ELQM); the simplest modification for RNT from the gold standard LQ model used in EBRT dose modelling.^{84,108} Here, the Lea-Catcheside G-factor is used as a base to improve the cell kill response by manipulating irradiation time.

Although the definition of fractionation is not the same as that used in the EBRT, positron emission radionuclide therapy (PERT) fractionation discussed in this chapter attempts to take advantage of the high dose-rate regime of radionuclide decay through multiple administrations of activity. This methodology can only be realised through short

half-life radionuclides, making the β^+ emitters a prime candidate as a pilot in developing this novel concept of realising the theranostic implementation of post-therapy follow-up PET imaging in the clinic.

6.1. Background

The impact that the dose-rate of the low LET radiation has on the efficacy of tumor kill had been recognized as early as the 1970s, establishing itself within the relatively new field of radiobiology.^{84,127} With the recently reignited interest in RNT for cancer treatment, the vital role of radiobiology in the therapeutic arm of nuclear medicine has become apparent.² Due to the limitations in the dosimetry of RNT, the use of “absorbed dose” is not as pertinent in nuclear medicine when compared to the absorbed doses that EBRT treatment planning systems rely on.^{47,128–130} This is directly related to the concept of dose rate in RNT, a component that is constricted by the production and the half-life of the radionuclide, along with the pharmacodynamics and pharmacokinetics of the targeting agent and limitations in hardware.^{44,105,131}

The dose-rate plays an important role when attempting to improve TCP and limit NTCP, and thus is crucial for both EBRT and RNT.^{76,104,106,121} With EBRT, fractionation of dose delivery has shown a sparing of the healthy tissue, whilst effectively delivering larger total doses to the tumor.¹³² RNT, on the other hand, deals with the standard limitations faced with the administration and off-target effects when increasing the dose-rate or activity.^{2,56,105,133,134} Thus, a more sophisticated treatment planning system needs to be implemented into RNT dose delivery. The standard LQ model (Eq. 6.1) does not address the effects that the dose-rate has on the survival but attempts to encapsulate the overall impact on the survival to output the radiobiological parameters α and β .^{84,85,135}

$$SF = e^{-(\alpha D + \beta D^2)} \quad (6.1)$$

where SF is the survival fraction, D is the total absorbed dose, α and β are the radiobiological linear and quadratic parameters of the cell line, respectively.

The simplest of the extended LQ models available (Eq.6.2) is becoming more commonly utilized when incorporating the dose-rate effect to the radiobiological parameters of tumors.^{84,125}

$$SF = e^{-(\alpha D + \beta G D^2)} \quad (6.2)$$

$$\begin{aligned} G &= 2 \left(\frac{\dot{D}_0}{D} \right)^2 \int_0^T e^{-\lambda t} dt \int_{-\infty}^t e^{-\lambda t'} e^{-\mu(t-t')} dt' \\ &= \frac{2}{\lambda - \mu} \left(\frac{\lambda}{1 - e^{-\lambda T}} \right)^2 \left(\frac{1 - e^{-(\lambda+\mu)T}}{\lambda + \mu} - \frac{1 - e^{-2\lambda T}}{2\lambda} \right), \end{aligned} \quad (6.3)$$

where G is the Lea-Catcheside G-factor, D is the dose rate, μ is the DNA repair constant and t' and t are the times for two different SSBs that could result in a DSB, provided the difference between the times is less than the DNA repair time. The key difference between the two models is the Lea-Catcheside G-factor; this takes into account the total absorbed dose, as well as the changing dose rate and the time between the two SSBs that can lead to a DSB.^{84,108} In EBRT, the G-factor converges to unity as the dose rate is constant and the irradiation time is relatively very short when compared to RNT.⁷⁶ Theoretically, as the G-factor for therapeutic radionuclides is much less than that of EBRT, the key to optimisation of RNT may be in manipulating this factor for dose delivery.

In our recently published article, our team was able to show the first *in vitro* evidence of the therapeutic effect of β^+ emitters in cancer medicine, utilising the clonogenic assay to quantify a cell kill of > 90% when the metastatic castrate resistant prostate cancer cell line LNCaP C4-2B was irradiated with 20 Gy of fluorine-18 (^{18}F) β^+ .¹¹⁴ With radiobiological studies especially with β^- emitters, a relatively long half-life brings about difficulties when attempting to safely and efficiently observe its effects, particularly in the early

stages of cellular damage. By using β^+ emitters, the relatively shorter half-life allows for cleaner experimental protocols with a higher dose rate, with the benefit of observing the radiobiological effects on the cells at an earlier time point.

Lately, considerations of the radiobiological parameters and assays have become a standard when performing cellular studies utilizing γ , MV X-rays and particles (e.g., β , α^{2+} , Auger electron) in cancer therapy.^{25,74,134,136,137} Despite this crucial shift, majority of the focus in RNT has been on the development of the conjugated targeting agent or looking for novel β^- or α^{2+} emitting radionuclides without much insight into investigating clinically relevant radiobiological studies.

This study aimed to expand on the use of the β^+ emitter ^{18}F performed in **Chapter 5** by manipulating the Lea-Catcheside G-factor in the irradiation and performing various assays *in vitro* to elucidate the possible damaging pathways to improve the radiobiological understanding and efficacy of this new modality in RNT.

We also address the limitations that are brought about in translating the β^+ emission radionuclide therapy (PERT) into the clinic by developing a dose-rate effect rationale for the “fractionated” administration of the β^+ emitters in the therapeutic setting.

6.2. Methods

There are two main experimental setups in this chapter: the G-factor manipulation, and the acute versus fractionated irradiation, which is not described in **Chapter 4** as it extends on the standard protocol. All experiments were performed using the mCRPC cell line LNCaP C4-2B and the general protocol for cell culture, detailed below, was followed. The clonogenic assay was performed on all experiments, but the cell cycle and oxidative stress assays were only performed on the G-factor manipulation irradiation.

6.2.1. General Protocol for Cell Culture: Cell Count and Viability

The cell line was cultured in either T175 or T75 flasks in Roswell Park Memorial Institute (RPMI, Life Technologies) medium supplemented with 10% v/v foetal bovine serum (FBS, Sigma Aldrich). Cells were maintained in an incubator at 37°C with 5% CO₂.

Cells were sub-cultured (*"passaged"*) approximately twice a week, ranging from a ratio of 1:4 to 1:20 into the new flasks. For the experiment setup, a predetermined cell number was seeded into a T25 flask containing 5 mL of growth medium to reach the optimal cell density for the irradiation after 2 days.

Cells were harvested by washing with phosphate buffered saline (PBS, Astral Scientific), then adding approximately 1 mL of trypsin/EDTA (Life Technologies), before placing into the incubator for up to 5 minutes. Roughly 9 mL (aiming for a 1:1 of trypsin/EDTA to FBS) of culture medium (RPMI with 10% v/v FBS) was then added to inhibit the trypsin from acting further. The detached cells were then transferred to a tube and pelleted by centrifuging at 300 g for 3 minutes. The supernatant (culture medium and trypsin/EDTA solution) was removed before tapping the tube to break the pellet up. Finally, the cells were resuspended in the growth medium, free from trypsin, before a cell count and viability assay was performed.

25 to 50 μ L of cells resuspended in medium were added to the Count and Viability reagent (making up a total volume of 200 μ L), incubating for 5 minutes at room temperature. Cells were then resuspended by pipetting before counting on the Guava[®] Muse[®] Cell Analyser (Luminex) following the vendor's general protocol. If the number of cells present in the small aliquot used was too high, the accuracy of both the cell count and viability was compromised. This was avoided by diluting the resuspended cell stock with more growth medium or taking out less volume to add to the reagent. Viability of the cells were confirmed to be approximately 90% or higher to qualify for the experiments. If the viability fell below this before any administration of activity, the cells were passaged further and the steps were repeated again before proceeding.

6.2.2. G-factor Manipulation

The G-factor manipulation irradiation setup was based off of the procedures described in **Chapter 4**. As the cell cycle had to be incubated for an extra 24 hours, the number of flasks and activity required in the stock was doubled.

The volume used to administer the activity was reduced from 5 to 1 mL, as the activity required for the irradiation decreased proportionally using this optimisation. Equation 6.3 was used to determine the G-factor of ^{18}F , provided in Table 6.1. After confirming that the irradiation times were feasible, three G-factor values were chosen for this experiment: 0.7, 0.8 and 0.9, corresponding to 103, 61 and 28 minutes of irradiation, respectively.

Irradiation Time (min)	Irradiation Time (s)	G-Factor
0.17	1	0.99
28	1680	0.90
61	3660	0.80
103	6180	0.70
160	9600	0.60
240	14400	0.50

TABLE 6.1. Calculated G-factors assuming a μ of 1 hour using Eq 6.3

Three doses were chosen for this experiment: 5, 10 and 15 Gy. This was determined by the dose response achieved in **Chapter 5**, as > 90% cell kill was achieved from 20 Gy over a similar 18 hour irradiation. Theoretically, the increase in G-factor will improve cell kill, thus a conservative approach was taken for the dose range.

Once the cells were ready for irradiation, the radionuclide solutions were prepared so that the initial activity required was set to a staggered administration time, creating a buffer time between the different aliquots when administered. This step was crucial to make the dose delivery as consistent as possible and maximise reproducibility. When the administration time was near, the growth medium was taken out from the flask before the radioactive

solution was pipetted in. After the irradiation time noted had passed, the activity was removed and washed thoroughly at least 3 times using PBS to prevent further irradiation from any residual activity. The clonogenic assay was performed on the batch of cells harvested approximately 2 hours from the initial irradiation. This is well within 6 hours, a time frame commonly used to distinguish immediate (< 6 hours) and delayed (> 6 hours) plating, seeding into 6-well plates before analysing the survival data after 7 days. For the remaining two assays, the cells were harvested 24 hours post-irradiation.

To ensure that the activity required for each batch of experiments was minimised, one absorbed dose target was attempted per run with the three G-factors, i.e., 15 Gy target dose with irradiation times and activities corresponding to 0.7, 0.8 and 0.9 G-factors. The initial experiment was performed with the 5 Gy target dose to allow for any potential optimisation and corrections to be implemented using the lowest amount of activity required.

6.2.3. *Cell Seeding Optimisation*

When harvesting cells post-irradiation to perform assays, an accurate cell count must be determined for the clonogenic assay seeding. The optimal seeding number was determined by plating a range of cell numbers in a 6-well plate and monitoring the confluence (surface coverage) over 7 days. The colonies formed were stained using the procedure in the section below, before determining the optimal seeding number qualitatively.

6.2.4. *Clonogenic Assay*

6-well plates were prepared with 5 mL of medium in each well. After performing the cell count, a specific volume of cells corresponding to the number of cells required to be seeded was gently pipetted into the well. The plates were then maintained in an incubator at 37°C with 5% CO₂ undisturbed for 7 days. Medium from each well was removed after the incubation time, gently washed with PBS, before 1 mL of 0.5% w/v crystal violet in 50% v/v methanol was added. The stain was removed after 15 to 30 minutes of staining

and was gently immersed in a water bath to remove the residual stain. The plates were then dried overnight before being imaged and analysed on the vSpot Spectrum EliSpot reader (Autoimmun Diagnostika GmbH, Straßberg, Germany). The survival fraction (SF) was then be calculated using equation 6.4.

$$SF = \frac{\text{Number of Colonies in a Well with Treated Cells}}{\text{Number of Colonies in a Well with Untreated Cells}} \quad (6.4)$$

There are two variations to the clonogenic assay: the immediate plating (IP) and delayed plating (DP). The definition of IP and DP varies with the time between the treatment and plating steps outlined above, where IP is less than 6 hours and DP is more than 6 but no later than 24 hours post-treatment. Radiobiological studies are recommended to be IP, and is therefore used throughout this experiment.¹¹⁵

6.2.5. *Cell Cycle Assay*

An appropriate volume of 70% v/v of ethanol was prepared, and stored in the freezer at -20 °C. Due to the stability of the solution, the ethanol was prepared on the day of the experiment. After performing the cell count, the cells were centrifuged at 300 g for 5 minutes. The supernatant was then discarded and the cells were resuspended in 1 mL of PBS per 10⁶ cells and centrifuged again at 300 g for 5 minutes. The supernatant was discarded again, but roughly 50 µL of PBS per 10⁶ cells was left in the vial. 1 mL of the ice-cold ethanol solution prepared at the beginning was then added drop-wise whilst on a light vortex. The sample was left for at least 3 hours in the freezer at -20 °C before 200 µL of the frozen cells were transferred to a 1.8 mL vial. The cells were centrifuged at 300 g for 5 minutes and the supernatant discarded before re-suspending in 250 µL of PBS per 5x10⁵ cells. Again, the cells were centrifuged at 300 g for 5 minutes and the supernatant discarded before re-suspending the cells in 200 µL of the Cell Cycle Reagent. The samples

were incubated in the dark at room temperature for 30 minutes and resuspended by pipetting before the readout on the Guava[®] Muse[®] Cell Analyser (Luminex) following the vendor protocol.

6.2.6. *Oxidative Stress Assay*

After performing the cell count, an intermediate solution (IS) was prepared by combining 1 μ L of the Oxidative Stress Reagent with 99 μ L of Assay Buffer. A working solution (WS) was made by diluting the IS by 1:80 ratio with the Assay Buffer. From the sample solution, 10 μ L was taken out and combined with 190 μ L of the WS and placed in the incubator at 37°C with 5% CO₂ for 30 minutes. The sample was resuspended by pipetting before readout on the Guava[®] Muse[®] Cell Analyser (Luminex) following the general protocol.

6.2.7. *Acute versus Fractionated Irradiation*

The cells in a T25 flask were irradiated with 16.7 ± 0.5 MBq of ¹⁸F in 0.5 mL of growth medium. After 28 minutes, 2.7 ± 0.1 MBq of ¹⁸F in 0.5 mL was administered to raise the total activity in the flask back to the initial activity in 1 mL. This was repeated once more, with the total radioactive growth medium totalling 1.5 mL. Two “top-up” activities were administered before the flask was incubated to irradiate. For the comparative study, the acute irradiation administered 28.9 ± 0.7 MBq of ¹⁸F in 1.5 mL before incubating. The activities corresponded to absorbed doses of 2, 1 then 4.11 Gy for the three fractions in order, compared to 7.11 Gy in one administration for the acute irradiation, both delivered over a total of 18 hours.

6.3. Results

The survival fraction data from the G-factor manipulation experiment is tabulated below in Table 6.2 and plotted in Figure 6.1. As G-factor approached unity, the impact on cell

survival increased, although a convergence between the 0.8 and 0.9 G-factors at 10 Gy was observed.

Dose	G Factor	Mean Colony Count	SF
Control	-	292.3 ± 45.5	100.0 ± 15.6
5 Gy	0.7 G	314.7 ± 16.6	107.6 ± 5.7
	0.8 G	199.7 ± 36.0	68.3 ± 12.3
	0.9 G	188.3 ± 49.1	64.4 ± 16.8
10 Gy	0.7 G	100.3 ± 31.6	34.3 ± 10.8
	0.8 G	59.1 ± 10.7	20.2 ± 3.7
	0.9 G	67.8 ± 15.7	23.2 ± 5.4
15 Gy	0.7 G	63.4 ± 26.6	21.7 ± 9.1
	0.8 G	35.1 ± 10.4	12.0 ± 3.6
	0.9 G	28.2 ± 16.3	9.6 ± 5.6

TABLE 6.2. Mean colony count and survival fraction data

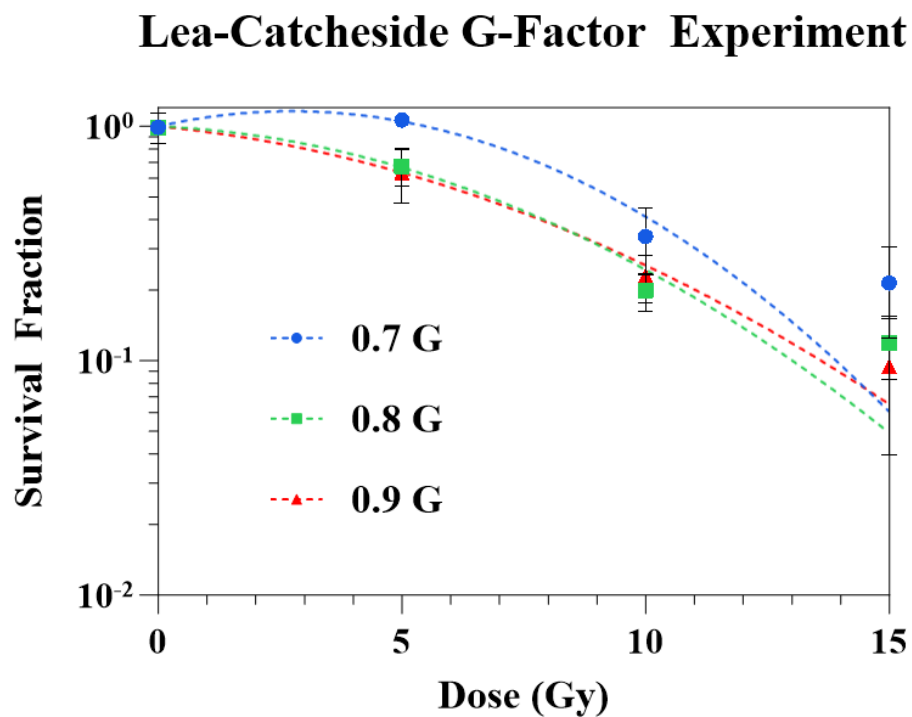


FIGURE 6.1. Survival curve from the G-factor experiment

The cell cycle assay results are tabulated in Table 6.3 and graphically depicted in Figure 6.2. With the exception of set of 0.7 G-factor irradiation, as the absorbed dose increased, there was a shift in the distribution from the G0/G1 to the G2/M phase, indicating a cell cycle arrest. The 15 Gy irradiation at 0.7 G-factor on the other hand showed a S phase arrest. The most prominent G2/M phase cell cycle arrest was observed for the cells irradiated 15 Gy at 0.8 and 0.9 G-factors.

Dose	G Factor	G0/G1 (%)	S (%)	G2/M (%)
Control	-	52.4 ± 3.6	11.3 ± 3.2	35.2 ± 1.6
5 Gy	0.7 G	50.9 ± 7.3	9.4 ± 1.8	39.5 ± 5.5
	0.8 G	54.5 ± 0.8	7.7 ± 0.3	37.8 ± 0.8
	0.9 G	55.3 ± 2.1	7.4 ± 0.2	37.2 ± 1.9
10 Gy	0.7 G	48.5 ± 1.8	7.8 ± 0.4	40.6 ± 1.4
	0.8 G	45.9 ± 2.6	8.1 ± 0.5	42.8 ± 2.1
	0.9 G	43.5 ± 2.8	7.2 ± 0.8	45.5 ± 3.2
15 Gy	0.7 G	43.0 ± 0.4	11.8 ± 0.8	45.0 ± 0.8
	0.8 G	35.1 ± 0.1	10.8 ± 1.4	53.9 ± 1.4
	0.9 G	35.1 ± 2.4	10.5 ± 1.3	54.1 ± 3.8

TABLE 6.3. Percentage of population in each phase of the cell cycle

The oxidative stress assay results are tabulated in Table 6.4 and visualised in Figure 6.3. An increase in intracellular ROS was observed with increasing total absorbed dose, but the impact of the dose rate was not evident over the three doses.

The clonogenic assay results from the fractionation of activity administration is tabulated in table 6.5. The colony counts were noted to be significantly higher due to the stability of the cells post-administration in the experimental setup, and the SF difference between the acute and fractionated was observed to be 7%.

Note that for all statistical analyses performed, the significance level α was set to 0.05. A one-way analysis of variance (ANOVA) was performed on all untreated samples in the same experiment to compare the impact of biological and technical replicates on the colony

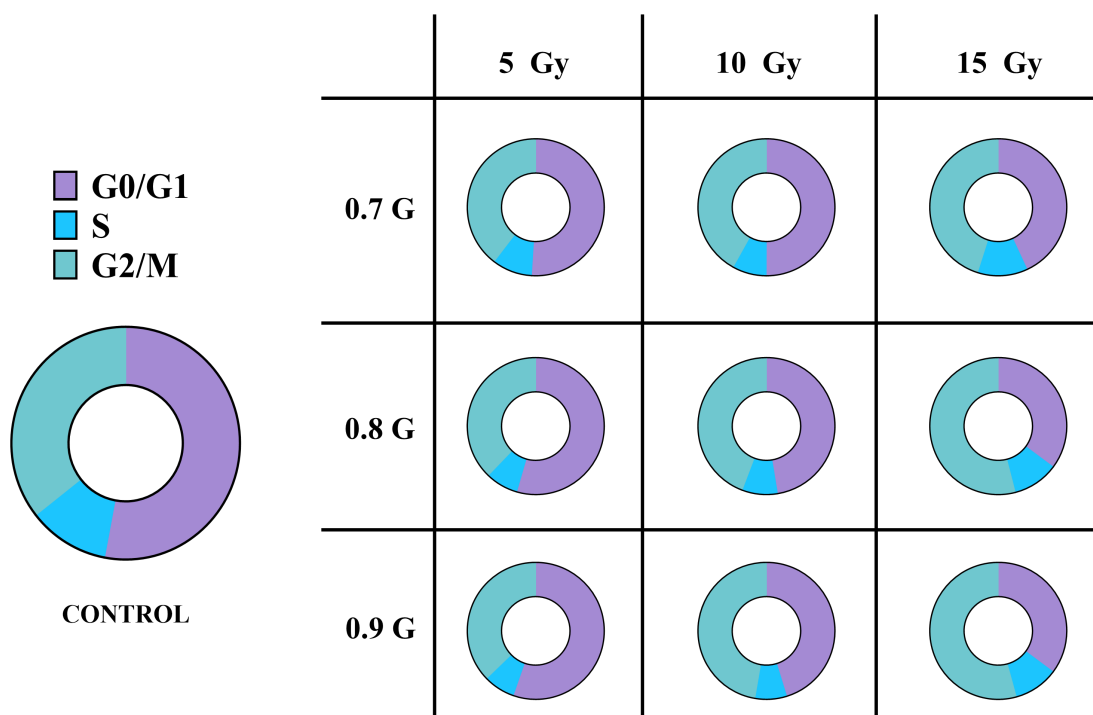


FIGURE 6.2. Cell cycle assay results from the G-factor experiment

Dose	G Factor	ROS Positive (%)	ROS Negative (%)
Control	-	12.9 ± 2.4	85.5 ± 1.3
5 Gy	0.7 G	12.9 ± 0.9	86.2 ± 0.3
	0.8 G	16.7 ± 0.2	82.2 ± 0.3
	0.9 G	14.7 ± 3.5	84.4 ± 3.9
10 Gy	0.7 G	19.0 ± 0.8	79.9 ± 0.6
	0.8 G	18.8 ± 3.4	80.1 ± 3.6
	0.9 G	19.0 ± 2.2	80.3 ± 2.3
15 Gy	0.7 G	26.6 ± 0.7	71.3 ± 0.4
	0.8 G	22.3 ± 5.9	76.4 ± 5.9
	0.9 G	28.3 ± 5.3	70.5 ± 4.8

TABLE 6.4. Percentage of population with and without intracellular ROS

formation across multiple days. There was no statistically significant difference in the mean colony counts between all of the controls performed as replicates for both the G-factor comparison ($F(8, 18) = [0.79]$, $p = 0.62$) and the fractionation ($F(5, 12) = [0.92]$, $p =$

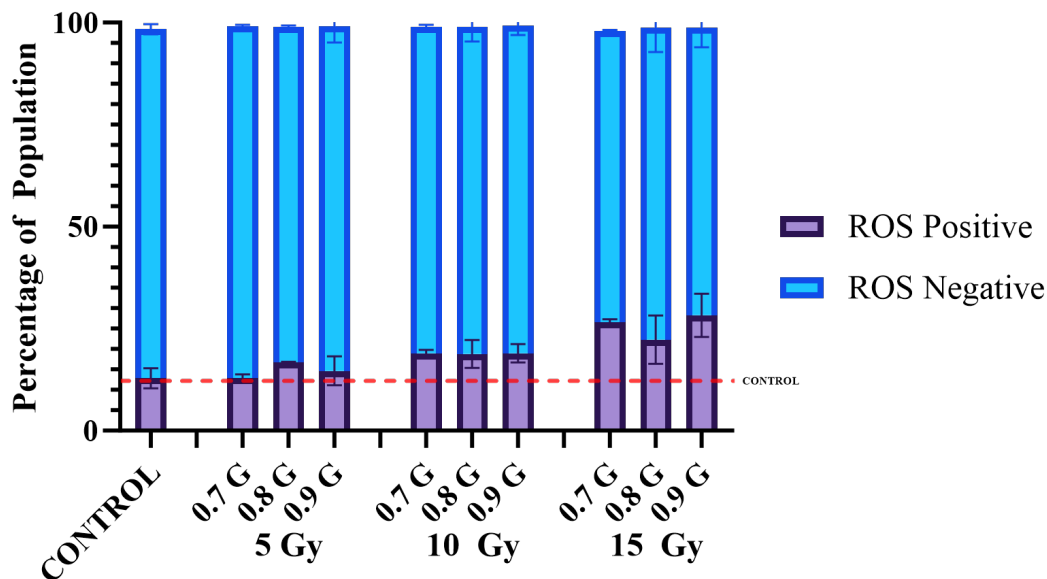


FIGURE 6.3. Oxidative stress assay results from the G-factor experiment

7.11 Gy Irradiation	Mean Colony Count	SF
Control	402.2 ± 36.4	100 ± 9.1
Acute	133.1 ± 14.0	33.1 ± 3.5
Fractionated	163.3 ± 24.4	40.6 ± 6.1

TABLE 6.5. Survival from the acute versus fractionated irradiation with a total absorbed dose of 7.11 Gy

0.50) experiments from the ANOVA. The results indicate that from the methods performed, regardless of whether they were biological or technical replicates, the untreated cells behaved consistently across the experiments performed.

A second ANOVA was performed between the controls and treated samples to determine if the treatment had a statistically significant effect on the clonogenicity of the cells. The analysis revealed that there was a statistically significant difference in mean colony counts between the samples ($F(11, 96) = [112.49]$, $p = 9.18 \times 10^{-50}$). A Dunnett's test post-hoc was performed to determine if the clonogenicity of each treated sample group was significantly different from the control. This was done by comparing the difference between the mean of

each treated sample group ($\bar{x}_j$) and the mean of the control group ($\bar{x}_0$) with Dunnett's test statistic when the group means are not equal, given in equation 6.5.

$$|\bar{x}_j - \bar{x}_0| = t_d \sqrt{W \frac{1}{n_j} + \frac{1}{n_0}}, \quad (6.5)$$

where Dunnett's critical value t_d is 2.797 from Dunnett's Table (sample size of 12 and 96 degrees of freedom), W is the mean squares within the sample groups obtained from the ANOVA and n is the sample size of the groups.

Three controls were independently examined between the groups, and with the exception of one sample group (5 Gy irradiation at 0.7G), all p-values were given as 0. This indicated that the treatments were statistically significant in impacting the clonogenicity of the cell lines.

Finally, to determine if the variation in G-factor impacted the clonogenicity with the same absorbed dose, an ANOVA was performed. For the three total absorbed doses 5, 10 and 15 Gy, the output was ($F(2, 24) = [35.09]$, $p = 7.51 \times 10^{-8}$), ($F(2, 24) = [9.56]$, $p = 8.85 \times 10^{-4}$) and ($F(2, 24) = [8.99]$, $p = 1.22 \times 10^{-3}$), respectively. As the results indicated a statistically significant difference with varying G-factors over all absorbed doses, a post-hoc Tukey HSD (honestly significant difference) was performed to determine which groups were responsible for this observation. This was done by comparing the q-statistic for Tukey's test (given in equation 6.6 with the critical q value q_{crit} of 3.532, taken from the Studentised range q table.

$$q = \frac{\bar{x}_{max} - \bar{x}_{min}}{\sqrt{W/n}}, \quad (6.6)$$

where W is the mean squares within the sample groups obtained from the ANOVA and n is the sample size of the groups. For all three total absorbed doses, there was a statistically significant difference between 0.7 and 0.8 G, and 0.7 and 0.9 G, giving q statistics of 10.07 and 10.43, 5.97 and 4.90, and 4.65 and 5.60 for the two G-factor comparisons at 5, 10 and 15 Gy, respectively. The difference between 0.8 and 0.9 G were not statistically significant

for all three absorbed doses, giving q statistics of 0.36, 1.58 and 0.95 at 5, 10 and 15 Gy, respectively.

6.4. Discussion

As hypothesised, the increasing impact on cell survival with the higher dose rate irradiation was evident at each of the three total absorbed doses. Comparing the 0.8 and 0.9 G-factors, this effect was less accentuated. This can possibly indicate a threshold at which cell kill effects are diminished with increasing initial activity at given absorbed doses.

Although direct DSB generation is the ideal trigger resulting in cell death, as β particles are low LET, it is more likely that a cluster of SSBs leading to misrepair induced by NHEJ is the pathway responsible for the loss of clonogenicity. The irradiation times of 0.8 and 0.9 G-factors were below or around the assumed DNA half-life of an hour, whereas the 0.7 G-factor was nearly double. To increase the efficiency of the treatment, an accurate measurement of the DNA repair half-life that leads to a proper calculation of the G-factor must be performed. For example, a DNA repair half-life of 30 and 120 minutes at the 0.9 G-factor would lead to a decrease (0.82) and increase (0.95) in G-factors for the same 28 minute irradiation time, respectively. Therefore, it can be hypothesised that the saturation point of damage is dictated by the repair half-life more than the actual G-factor value itself, implying that radiation resistance of particular cell lines can only be overcome at extremely high dose rates, comparable to that of EBRT.

Observing the shift in distribution across the cell cycle phases with increasing total absorbed dose, 24 hours post-irradiation displayed an evident cell cycle arrest at the G2/M phases. Despite the assay not being able to distinguish between the G2 and M phases, this shift was concordant with the survival fraction results implying that the G2/M checkpoint prevented cells from entering the mitotic phase, and led to apoptosis or necrosis.

The difference between the 5 and 10 Gy to the 15 Gy was not only the percentage of cells in the G2/M phase, but also for those in the S phase. The S phase population for all

three 15 Gy exposures were comparable to the control, suggesting either a S phase arrest compared to the lower doses were observed, or the transition to the G2/M had not fully completed. The assay performed at the 24 hour time-point is merely one snapshot of the cell population transitioning through the cell cycle. The delays in this progression appears to be more prominent at higher total absorbed doses, and therefore multiple time points post-irradiation may be required to understand the dose-rate responses to the cell cycle arrests in different phases.¹²⁴

In the oxidative stress assay, an expected result of increasing ROS positive cells with total absorbed dose was observed. Although the therapeutic effect under investigation was from the β^+ particles, the emitted γ were increasingly available to generate additional intracellular ROS, leading to a maximum increase in ROS of 15.4% at 15 Gy (0.9 G-factor). The variation in the G-factor did not affect the production of the ROS, hinting that the impact of the increasing dose-rate on the survival was due to the direct strand breakages from the β^+ particles. Multiple time-points should also be investigated for the oxidative stress assay to monitor the time required to reach the maximum ROS generation possible, which may reveal a correlation with the DNA repair half-life of the cell line.

A previous study by *Gholami et al.* calculated the intrinsic limitation to the G-factor for a selection of commonly used clinical radionuclides, where the radionuclide is allowed to decay infinitely (G_∞).⁷⁶ It was shown that shorter half-life radionuclides have a higher G_∞ , and therefore therapeutic efficacy, as their higher initial dose rate allows for the DNA damage to overcome or compete with its repair and proliferation. Regardless, the G-factor manipulation has allowed for the increase in cell kill. This indicates that if a more targeted agent can be developed, it can allow for a higher dose rate to be delivered with the same activity delivered currently to the lesion of interest, effectively increasing the G-factor of the radionuclide in the clinical setting.

The concept of fractionating the irradiation in this experiment was brought about by attempting to manipulate the irradiation time to ensure a high G-factor dose delivery in the

first two fractions, as shown in figure 6.4. The activity after the final fraction administration was not removed intentionally to mimic a clinical scenario as close as possible, where the decay cannot be “switched off”. The total absorbed dose was identical, but the total activity delivered was less for the fractionation due to the changing volumes with the additional fractions.

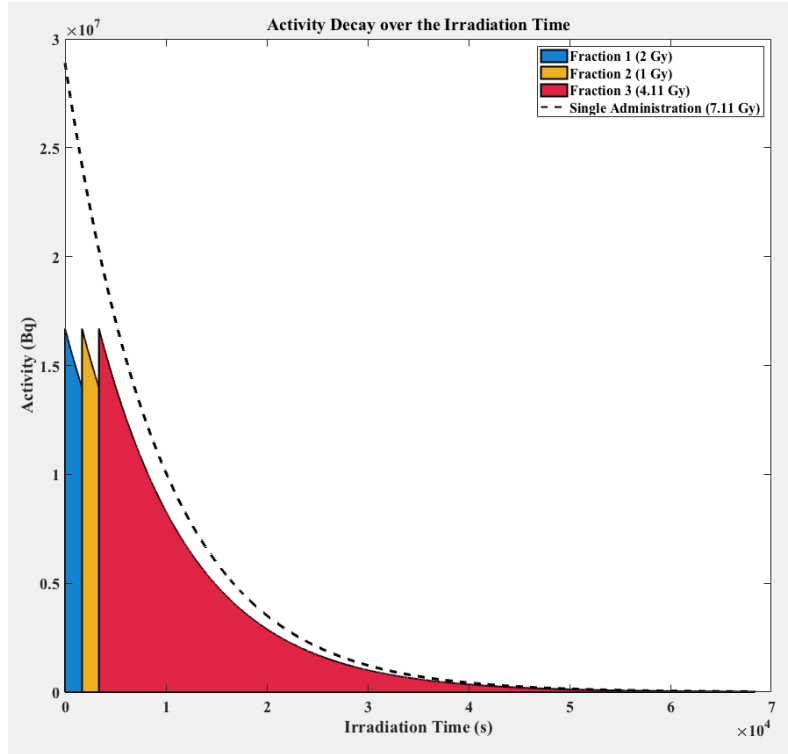


FIGURE 6.4. Fractionation of activity in the *in vitro* setup

From the considerations of the dose-rate effects, Dale (1996) notes the competition of repair and proliferation with damage that leads to a certain dose rate or time, coined as “critical dose rate” ($\dot{D}_{crit}$) and “critical time” (T_{crit}) where any additional dose deposited becomes “wasted”.¹⁰⁶ Although this concept is oversimplified, it forms a valid theoretical baseline of how total absorbed dose should be used in the observation of cellular response. The critical time and dose rate for the cell line was calculated for the fractionated administration using equation 6.7 and 6.8 and plotted in figure 6.5. The theoretical absorbed dose that would impact the cell kill was calculated to be 5.44 and 5.49 Gy for acute

and fractionated irradiation, respectively with a critical time of under 4 hours. Further investigations should be performed where the activity is removed from the flask at this time point to compare the survival with the theoretically saturated point of damage.

$$\dot{D}_{crit} = \frac{0.693}{\alpha T_{doubling}} \quad (6.7)$$

$$T_{crit} = -\frac{1}{\lambda} \ln\left(\frac{0.693}{\alpha \dot{D}_0 T_{doubling}}\right) \quad (6.8)$$

where, α is the linear radiobiological component of the cell line, $T_{doubling}$ is the doubling time of the cell line, λ is the decay constant and $\dot{D}_0$ is the initial dose rate.

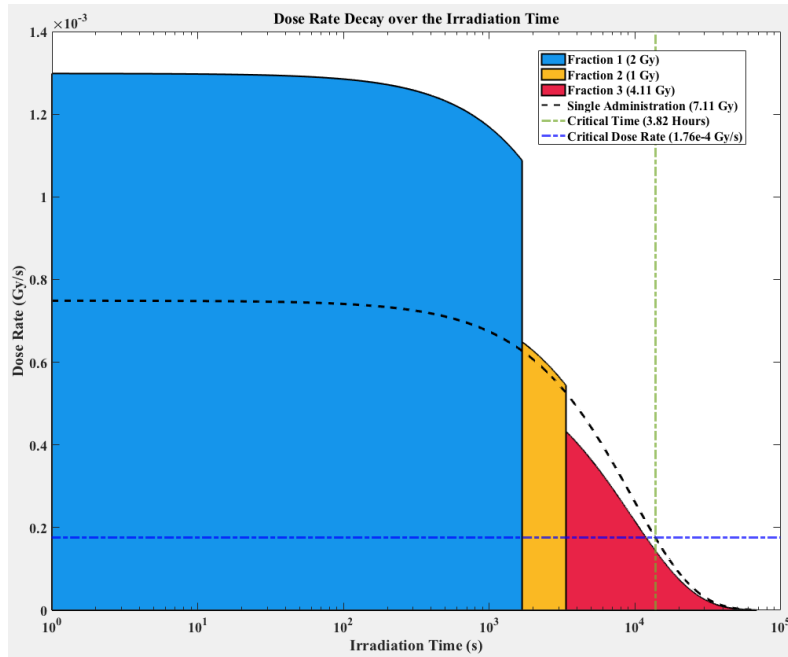


FIGURE 6.5. Dose rates in the individual fractions

The survival results indicated that although acute administration had higher efficacy of an average of 7% cell kill, fractionation had comparable impact on the cell survival with less activity. There are obvious limitations with the administration of fractionated activities in the clinical setting; primarily, the selectivity, uptake and retention cannot be guaranteed in the

same precision as an *in vitro* setup. The clinically synonymous application of fractionation currently is the dosing schedules in RNT, for example, every 6 weeks for Lu-PSMA therapy, but the proof-of-concept that less activity can be used for substantial clonogenic effects show promising results when the implementation of PERT can be incorporated into the treatment pipeline.¹¹⁴

6.5. Conclusion

Therapeutic radionuclides with relatively shorter half-lives are still uncommon. Through the investigation into the therapeutic effect of β^+ emitters, if the manipulation of the G-factor can be translated in a clinically feasible method, already available diagnostic radionuclides can be incorporated into the theranostic setting.

The impact of the dose rate on the cellular response, in particular the G2/M cell cycle arrest, indicates that RNT still has unexplored potential of radiobiological optimisation in the context of the theranostic radionuclides in use. An ideal radiobiological assay that was not incorporated due to the limitations of time would be the γ H2AX assay to identify SSBs and DSBs. This can further pinpoint the modes of damage and repair in the arrested phases of the cell cycle, completing the full characterisation of the damage profile in altering the G-factor. The results would assist in potentially elucidating the most efficacious interval to deliver the fractionated doses, by monitoring the various magnitudes of the SSBs and DSBs post-irradiation.

By manipulating this G-factor, the significantly shorter irradiation allowed for the isolated effects of β^+ on the survival to be observed compared to the results in the previous chapter. Despite this, to clinically implement the removal of the activity once the desired dose is delivered is currently not possible. Although to identify the isolated therapeutic effects that β^+ particles possess in the clinic is difficult with the current technologies, it would be impractical to utilise β^+ emitters as a stand-alone theranostic agent. Regardless, this does pave way for the targeting of micrometastatic lesions and circulating tumour

cells, which would effectively act as a secondary form of adjuvant radionuclide therapy. Further investigation on the effects would need to be performed to fully consolidate the incorporation of β^+ in the therapeutic context of nuclear medicine, detailed in *Chapter 8*.

Prescriptive Dosimetry of Positron Emitters in PERT using a Theranostic Radionuclide Surrogate

This chapter details a proof-of-concept dosimetric analysis required for the clinical implementation of β^+ emitters as a theranostic agent for PERT.

The effective clearance of the theranostic agent in nuclear medicine is determined by both the physical half-life of the radionuclide, along with the biological clearance rate of the targeting ligand to which the radionuclide is bound. Time activity curves (TACs), generated by the analysis of multiple time-point imaging, provide data on the cumulated activity of the tracer in the organs of interest, allowing for dosimetric analysis that can lead to treatment response evaluation, along with prediction of radiotoxicity related complications for dose-limiting organs.^{138–141}

Multiple time-point imaging for a single administration of a theranostic agent is not often performed clinically; the personalisation of RNT is still an emerging field in standard clinical nuclear medicine. Sites that perform such imaging protocols implement them for the purpose of dosimetry in research or clinical trials, which allows for the collection of data to be utilised in various analyses.^{71,138} In this analysis, three time-point imaging of a neuroendocrine tumour patient administered with ^{177}Lu -DOTATATE was analysed to perform prescriptive dosimetry for β^+ emitters, to act as a surrogate to determine the activities required to deposit an equivalent dose to the organs of interest. The 3, 24 and 120 hours post-injection images of the patient allowed for the longer half-life of ^{177}Lu to act as a surrogate for the shorter half-life radionuclides, ^{18}F , ^{68}Ga and ^{64}Cu , that is the subject of PERT. The results were generated from two dosimetry software, OLINDA/EXM and MIM Encore, and also compared for the purpose of correspondence between an adult body phantom and patient image derived dosimetry data.^{45,142}

7.1. Background

The prospective use of a predictive β^+ radionuclide-based dosimetry in the treatment planning for RNT has recently been explored by our group in the publication *⁶⁴Cu Treatment Planning and ⁶⁷Cu Therapy with Radiolabelled SARTATE ([⁶⁴Cu/ ⁶⁷Cu]MeCOSAR-Octreotate) in Subjects with Unresectable Multifocal Meningioma – Initial Results for Human Imaging, Safety, Biodistribution and Radiation Dosimetry.*¹⁴³ This is the first time that ⁶⁴Cu and ⁶⁷Cu have been used in humans as a theranostic pair. The use of both copper radionuclides in the planning and therapy, allows for the simplification of the potentially intricate biodistribution variabilities that are encountered when using different elements in the treatment pipeline, as the chelation chemistry involved is not dissimilar. The use of β^+ emitters as a theranostic agent, the main concept explored in this thesis, intrinsically bypasses the potential shortcomings of the differences in uptake, retention and clearance rates between the diagnostic treatment planning radioligands and its theranostic counterpart.

The comparatively shorter half-life of β^+ emitters is a clinically favourable characteristic in initial staging or follow-up scans, but becomes a limitation when attempting to perform a personalisation of treatment through pre-treatment dosimetry. To improve the understanding of the individuals' variabilities in the radioligand uptake, whether it be due to the innate patient differences or disease burden, multiple time-point imaging must be performed for both improved lesion detection and accurate biodistribution data. To harness the potential advantage of dose-rate effects in PERT, shorter half-life radionuclides are more appropriate. Alternatively, to produce and administer the increased amount of radioactivity to patients with a target of matching the dose to that delivered by current therapeutic and theranostic radionuclides the production of these PERT-suitable radionuclides may not be feasible.

The current treatment schedule of ¹⁷⁷Lu-PSMA in the clinic has been arbitrarily defined as every 6 to 8 weeks. Although not explicitly stated, this can stem from multiple rationales: ensuring the clearance of the relatively longer-lived radionuclide in highly avid lesions from the previous administration does not interfere with reporting, matching the frequency of

standard of care tests (e.g., blood tests, PSA) and therefore, allowing time for noticeable changes to these levels, to promote the recovery of normal tissue from potential radiation damage, and even potentially to prevent the development of treatment failure due to developing radioresistance.^{138–141} Purely considering the total dose delivery and physical half-life of the radionuclide, PERT would allow for the implementation of a shortened interval between each cycle, provided that the dose to the normal tissues can be managed.

Although the data presented in this chapter is extracted from a ^{177}Lu -DOTATATE patient who had three imaging time-points at 3, 24 and 120 hours post-injection, it aimed to demonstrate a proof-of-concept study, using the clearance data to model a TAC for the shorter-lived β^+ emitters. This allowed for the quantification of radioactivity required for administration, using two dosimetry software platforms, OLINDA/EXM and MIM Encore, to generate dosimetry results with the intention of matching the absorbed doses from ^{177}Lu therapy.^{45,142}

Limitations of variability in methods and software exist in current radionuclide dosimetry, addressed comprehensively in *An International Study of Factors Affecting Variability of Dosimetry Calculations, Part 1: Design and Early Results of the SNMMI Dosimetry Challenge*.¹³¹ The article is for ^{177}Lu SPECT imaging, but the steps involved in the generation of dosimetric data is synonymous in the field of nuclear medicine with PET imaging data for PERT. To briefly summarise, the analysis begins with the collection of quantitative images, followed by a segmentation process to define volumes of interest (VOI). The activities in the VOIs are then generated from standard phantom data or voxelised activity maps, and a curve-fitting regression is performed to compute time-integrated activity values to generate cumulated activity or absorbed dose maps. Finally, the computed data are combined to output the absorbed dose at the organ specific or voxelised level. Even with such a structured approach, sources of variability exist at each step, contributing to the challenges faced in clinical dosimetry. Figure 7.1 shows the MIM Encore workflow output of the auto-segmented VOI and the associated dose maps for the dosimetry calculations.

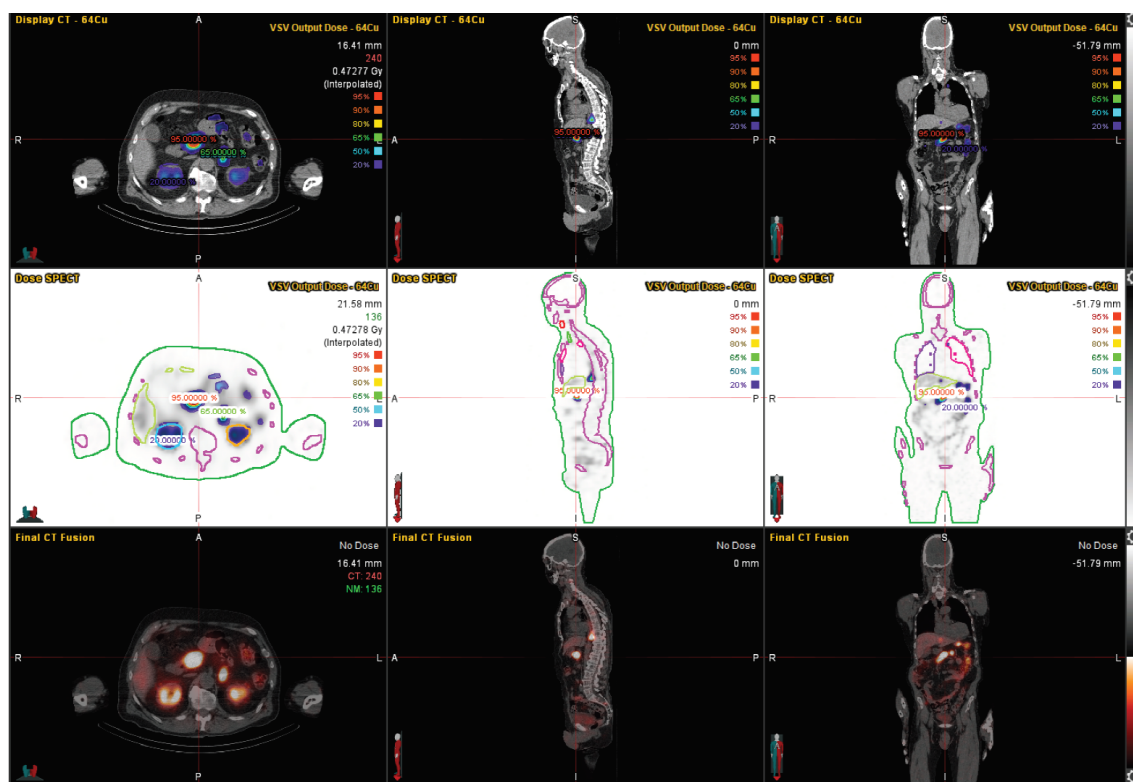


FIGURE 7.1. MIM Encore - Processing window in the surrogate radionuclide dosimetry workflow

7.2. Methods

7.2.1. MIM Organ Dosimetry: Integrated Activity

Quantified SPECT images at approximately 3, 24 and 120 hours post-injection from a patient administered with a single dose of 7.6 GBq of ^{177}Lu -DOTATATE was loaded into the MIM Encore program.¹⁴² An artificial intelligence, auto-segmentation of the organs was performed on the low dose CT image at the 24 hour time-point and processed with the quantified images using the in-house dosimetry workflow. The output of the data, given as the integrated total activity concentration per unit volume ($\text{Bq/mL} \times \text{mL}$), was taken to be the non-decay corrected data; the effective clearance of the radioligand was therefore obtained for the organs of interest: kidneys, liver, lungs, red bone marrow, spleen, thyroid and the remainder of the body in the field of view of the scan. The output dose values are all density corrected with a CT-based density map.

7.2.2. OLINDA Regression: Dosimetry Analysis

The integrated activity at the three time points for the ^{177}Lu data was converted into percentage of the injected dose (% ID), used as input for the OLINDA/EXM software.⁴⁵ Multiple iterations of regression curve fitting were performed to achieve $R^2 \approx 1$ on a triple-exponential decay relation, given in equation 7.1. The A , B and C values are collectively the initial % ID for each organ, the a , b and c are collectively the decay constants in hours^{-1} , and t is the time in hours. This output TAC generated the cumulated activity ($\text{Bq} \times \text{hr/Bq}$) of the radionuclide at each organ of interest, along with the absorbed dose per unit activity (mGy/MBq) and effective dose per unit activity (mSv/MBq).

$$\% \text{ ID}(t) = Ae^{-a \times t} + Be^{-b \times t} + Ce^{-c \times t} \quad (7.1)$$

To generate the effective clearance of the β^+ emitting radionuclides, the ^{177}Lu data was decay corrected to produce the theoretical biological clearance values of % ID. The decay factors for ^{18}F , ^{68}Ga and ^{64}Cu were applied to the biological clearance data, and effective clearance values of % ID were obtained for the three radionuclides. An identical process of regression curve fitting was performed for the β^+ emitters on OLINDA, with the same dosimetric outputs as ^{177}Lu generated. Each organ absorbed dose was then matched to that of ^{177}Lu , to quantify the activity required by the β^+ emitters to deposit the same amount of energy.

7.2.3. MIM Encore: Surrogate Dosimetry Workflow

An in-house workflow in MIM Encore was utilised to generate the TAC and absorbed dose to organs for comparison with the OLINDA data.¹⁴² This surrogate dosimetry workflow extends on the analysis performed in section 7.2.1., and incorporates the user input radionuclide decay factor automatically, correcting for the physical decay and changes in the TAC fit. The process performed through OLINDA along with the manual removal and application of the decay factor was done within the single workflow. The output data

was collected in the same format as OLINDA, with the exception of cumulated activity and effective dose which were omitted.

7.3. Results

The results from section 7.2.1. are given in table 7.1. Out of the nine organs that were defined by the artificial intelligence auto-segmentation of the low dose CT, six organs were converted to % ID.

The parameters resulting from the regression curve fitting of the clearance data to equation 7.1 for section 7.2.2. are given in table 7.3, where A and B are the effective and biological clearances of ^{177}Lu , respectively, and C, D and E are the effective clearances of ^{64}Cu , ^{68}Ga and ^{18}F , respectively. The OLINDA outputs of the cumulated activity and dose deposition are given in table 7.4. The MIM surrogate radionuclide dosimetry results from section 7.2.3. are given in table 7.7

Time Passed From Injection (hr)	2.93	25.60	118.40
Kidney (% ID)	2.10	1.89	0.68
Liver (% ID)	3.25	2.78	1.70
Lung (% ID)	1.92	0.51	0.28
Bone (% ID)	4.82	1.92	1.15
Spleen (% ID)	0.40	0.26	0.11
Thyroid (% ID)	0.03	0.01	0.00
Remainder of Body (% ID)	46.3	15.1	9.4

TABLE 7.1. Percentage injected activity of ^{177}Lu -DOTATATE in 6 organs of interest and remainder of body at three time points, generated by MIM Encore

A

¹⁷⁷ Lu - Effective Clearance % ID Regression Parameters						
	A	B	C	a	b	c
Kidney	2.22E+00	2.68E+00	6.20E-05	1.31E-02	7.64E+00	1.91E+00
Liver	1.59E+00	1.42E+00	3.11E-01	8.11E-03	1.75E-02	2.57E-03
Lung	3.71E-01	5.99E-01	3.57E+00	2.65E-01	1.07E-02	3.87E-01
Red Marrow	1.93E+00	2.22E+00	2.03E+00	8.72E-03	1.06E-01	1.68E-01
Spleen	2.96E-01	9.86E-02	3.86E-02	1.26E-02	7.02E-02	1.29E-01
Thyroid	2.05E+00	9.40E-01	7.59E-03	1.69E+00	1.92E+00	7.93E-03
Remainder of Body	1.40E+01	3.20E+01	1.21E+01	1.03E-01	1.04E-01	6.44E-03

B

¹⁷⁷ Lu - Biological Clearance % ID Regression Parameters						
	A	B	C	a	b	c
Kidney	2.24E+00	2.68E+00	6.20E-05	9.44E-03	7.64E+00	1.91E+00
Liver	1.75E+00	1.15E+00	4.20E-01	3.77E-03	1.53E-02	6.40E-04
Lung	3.17E-01	6.00E-01	3.76E+00	3.27E-01	6.42E-03	3.87E-01
Red Marrow	2.11E+00	2.21E+00	2.17E+00	5.14E-03	1.58E-01	1.64E-01
Spleen	3.26E-01	7.41E-02	6.81E-02	9.13E-03	1.83E-01	2.15E-01
Thyroid	2.15E+00	8.29E-01	7.59E-03	1.69E+00	1.92E+00	3.65E-03
Remainder of Body	1.44E+01	3.20E+01	1.46E+01	1.39E-01	1.22E-01	3.70E-03

C

⁶⁴ Cu - Effective Clearance % ID Regression Parameters						
	A	B	C	a	b	c
Kidney	1.04E+00	1.03E+00	6.45E-02	6.55E-02	6.09E-02	8.71E-02
Liver	6.38E-01	8.25E-01	1.86E+00	5.34E-02	5.53E-02	6.83E-02
Lung	2.32E-03	2.05E+00	3.31E-01	4.40E+00	1.09E-01	2.69E-01
Red Marrow	3.82E-01	4.82E+00	3.68E-01	2.12E-01	9.09E-02	2.05E-01
Spleen	5.65E-02	1.56E-01	2.08E-01	7.73E-02	7.22E-02	7.36E-02
Thyroid	8.46E-03	1.18E-02	1.04E-02	1.35E-01	1.01E-01	1.15E-01
Remainder of Body	4.75E+01	5.27E+00	1.35E+00	1.10E-01	7.02E-02	1.80E-01

D

⁶⁸ Ga - Effective Clearance % ID Regression Parameters						
	A	B	C	a	b	c
Kidney	2.65E-01	2.79E-01	3.20E-01	3.09E-01	3.15E-01	3.02E-01
Liver	3.83E-01	5.10E-01	4.88E-01	3.63E-01	3.02E-01	3.07E-01
Lung	2.72E-01	2.79E-01	2.45E-01	3.21E-01	3.03E-01	3.09E-01
Red Marrow	6.09E-01	9.77E-01	3.64E-01	3.19E-01	2.83E-01	3.59E-01
Spleen	7.43E-02	4.82E-02	5.13E-02	3.33E-01	3.19E-01	3.34E-01
Thyroid	3.45E-03	3.09E-03	4.17E-03	3.21E-01	3.04E-01	3.13E-01
Remainder of Body	5.39E+00	9.62E+00	4.70E+00	3.41E-01	2.92E-01	3.55E-01

E

¹⁸ F - Effective Clearance % ID Regression Parameters						
	A	B	C	a	b	c
Kidney	4.96E-01	5.03E-01	7.34E-01	3.35E-01	3.02E-01	3.07E-01
Liver	5.50E-01	8.18E-01	1.40E+00	3.50E-01	3.66E-01	2.93E-01
Lung	4.81E-01	5.23E-01	6.53E-01	3.34E-01	3.66E-01	2.97E-01
Red Marrow	1.41E+00	1.44E+00	1.11E+00	3.20E-01	3.00E-01	3.14E-01
Spleen	1.10E-01	1.00E-01	1.21E-01	3.40E-01	3.11E-01	3.01E-01
Thyroid	8.30E-03	7.46E-03	6.63E-03	3.14E-01	3.36E-01	3.53E-01
Remainder of Body	2.06E+01	5.13E+00	1.25E+01	3.20E-01	3.48E-01	2.87E-01

TABLE 7.3. OLINDA analysis: regression parameters for TAC fitting for the A) effective and B) biological clearance of ¹⁷⁷Lu, C) effective clearance of ⁶⁴Cu, D) effective clearance of ⁶⁸Ga and E) effective clearance of ¹⁸F

A

¹⁷⁷ Lu	Bq*hr/Bq	mGy/MBq
Kidney	1.70E+00	4.95E-01
Liver	3.98E+00	1.87E-01
Lung	6.66E-01	6.06E-02
Red Marrow	2.54E+00	1.32E-01
Spleen	2.52E-01	1.23E-01
Thyroid	2.65E-02	1.12E-01
Remainder of Body	2.32E+01	4.08E-02

B

⁶⁴ Cu	Bq*hr/Bq	mGy/MBq
Kidney	3.59E-01	9.95E-02
Liver	5.41E-01	2.81E-02
Lung	2.00E-01	1.87E-02
Red Marrow	5.66E-01	2.68E-02
Spleen	5.72E-02	2.93E-02
Thyroid	2.70E-03	1.29E-02
Remainder of Body	5.14E+00	1.03E-02

C

⁶⁸ Ga	Bq*hr/Bq	mGy/MBq
Kidney	2.80E-02	4.52E-02
Liver	4.33E-02	1.34E-02
Lung	2.56E-02	1.35E-02
Red Marrow	6.38E-02	1.61E-02
Spleen	5.28E-03	1.56E-02
Thyroid	3.40E-04	8.87E-03
Remainder of Body	6.20E-01	6.65E-03

D

¹⁸ F	Bq*hr/Bq	mGy/MBq
Kidney	5.54E-02	3.82E-02
Liver	8.58E-02	1.39E-02
Lung	5.07E-02	1.24E-02
Red Marrow	1.27E-01	1.53E-02
Spleen	1.05E-02	1.55E-02
Thyroid	6.70E-04	9.01E-03
Remainder of Body	1.23E+00	7.30E-03

TABLE 7.4. OLINDA cumulated activity and dosimetry output for A) ¹⁷⁷Lu, B) ⁶⁴Cu, C) ⁶⁸Ga and D) ¹⁸F

	⁶⁴ Cu (GBq)	⁶⁸ Ga (GBq)	¹⁸ F (GBq)
Kidneys	37.79	83.19	98.43
Liver	50.53	105.97	102.16
Lungs	24.60	34.07	37.10
Red Marrow	37.31	62.11	65.36
Spleen	31.88	59.87	60.26
Thyroid	66.05	96.05	94.56
Remainder of Body	30.00	46.47	42.33

TABLE 7.5. Activities required to match 7.585 GBq ¹⁷⁷Lu dose to organs for ⁶⁴Cu, ⁶⁸Ga and ¹⁸F

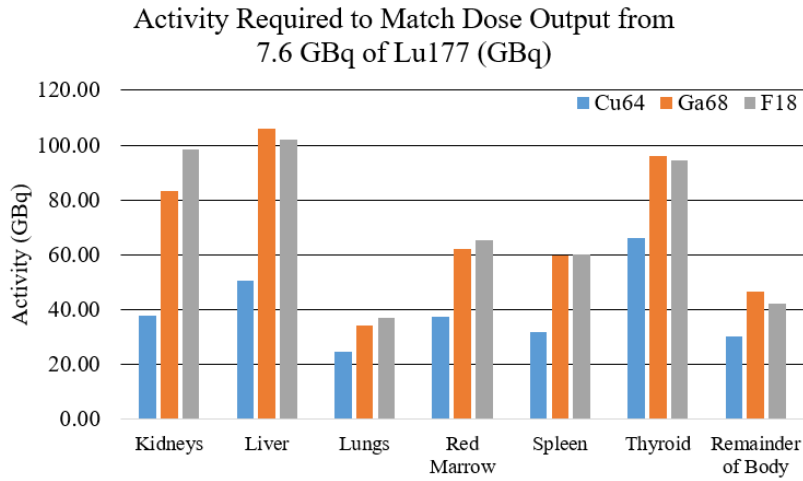


FIGURE 7.2. Graphical representation of activities required to match 7.6 GBq ^{177}Lu dose

	^{64}Cu (mGy/MBq)	^{68}Ga (mGy/MBq)	^{18}F (mGy/MBq)
Kidneys	1.26E-01	5.83E-02	5.12E-02
Liver	4.42E-02	2.31E-02	2.39E-02
Lungs	2.96E-02	2.70E-02	2.76E-02
Red Marrow	1.44E-02	8.33E-03	7.87E-03
Spleen	9.99E-02	2.94E-02	3.48E-02
Thyroid	1.74E-02	1.38E-02	1.17E-02
Remainder of Body	1.26E-02	8.33E-03	9.92E-03

TABLE 7.6. MIM surrogate dosimetry workflow output

	^{64}Cu	^{68}Ga	^{18}F
Kidneys	1.27	1.29	1.34
Liver	1.57	1.72	1.72
Lungs	1.58	2.00	2.22
Red Marrow	0.54	0.52	0.51
Spleen	3.41	1.88	2.24
Thyroid	1.35	1.56	1.29
Remainder of Body	1.23	1.25	1.36

TABLE 7.7. MIM/OLINDA activity ratio for 6 organs and remainder of body

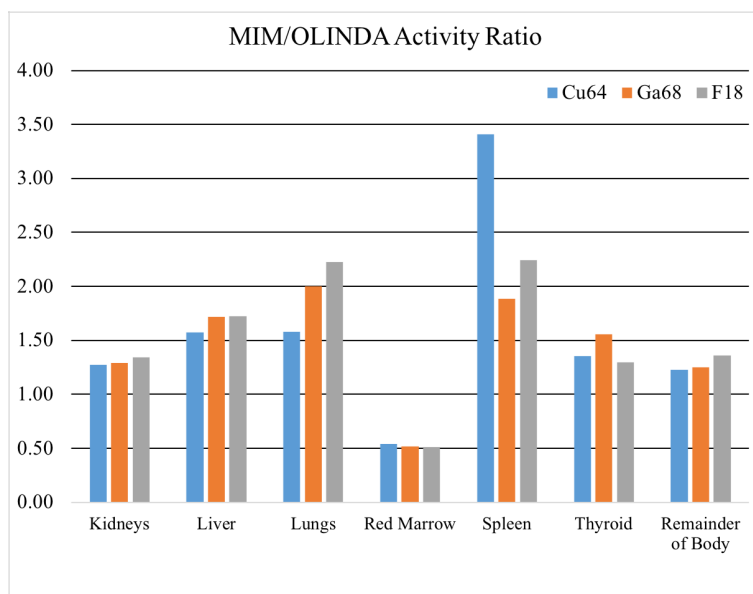


FIGURE 7.3. Graphical representation of the activity ratio between MIM and OLINDA outputs

7.4. Discussion

The results given in this chapter are based purely on organ dose, and were performed to provide an insight into the safety and biodistribution of the shorter half-life tracers. Optimisation is required to take the *treatment efficacy* into account, with tumour lesion dosimetry analysis in particular, but the initial values of the *one-dimensional* concept of absorbed dose are all presented in this chapter. Although the activities required for the three β^+ emitters to match the organ dose of ^{177}Lu is relatively high, the concept of using what are conventionally diagnostic radionuclides at therapeutically equivalent levels rationalises this. The dose could be delivered, for example, weekly over four weeks.

Addressed throughout this thesis, there is still a common misconception that the absorbed dose from RNT is equivocal to that of EBRT. Furthermore, the determination of the relative biological effectiveness (RBE) of the procedure is crucial, especially if the treatment planning is to be implemented clinically using the methods described in this chapter. The RBE takes into account the modes of DNA damage, particle v.s. ionisation and the formation of reactive oxygen species, biologically effective dose, dose rate and overall treatment regimen, all which impact the efficacy of the dose absorbed to the target. Thus, this deceptive concept of absorbed dose implies that the activities required to deliver an equivalent dose of ^{177}Lu to the organs is still difficult to accurately quantify in relation to its radiobiological effects, even between radionuclides, and also neglects any immunological responses.

The linear energy transfer of Auger electrons are orders of magnitude higher than both β^- and β^+ particles (0.2 keV/ μm v.s. 4 to 25 keV/ μm). Those that have multiple modes of decay such as ^{64}Cu with a *cocktail* of emitted particles ($\approx 18\% \beta^+$, $\approx 39\% \beta^-$ and $\approx 43\%$ Auger electrons from electron capture (EC)) will introduce further inaccuracies associated with the treatment efficacy of the absorbed dose in the calculations performed in this chapter. This, compounding with the higher dose rates, both from the higher activities required and

the shorter half-life, makes the predictive off-target effects immensely difficult to manage from the absorbed dose values.

Although the tumour lesion dosimetry is not performed here, if an identical quantity of dose as ^{177}Lu could be deposited into a volume of interest, but in a drastically shorter amount of time, the probability of creating complex, irreparable DNA damage is significantly higher; this was addressed in **Chapter 6**, regarding the intrinsic limitations of certain clinical radionuclides, as their G-factor in systemic treatment is heavily restricted by the half-life. Further investigation identifying the retention time of the tracer within the tumour across a longer time frame will lower the activities required to deposit the target dose, effectively resulting in a lowered normal organ dose.

As with any radiobiological studies that are being integrated into therapeutic nuclear medicine, the concepts of tumour control probability (TCP) and normal tissue complication probability (NTCP) utilised in fractionated EBRT must be taken into consideration, especially with the higher dose rates of radionuclides for use in PERT. The radiobiological justification between the hypofractionation schedules (higher doses per fraction and a shortened overall schedule) that are implemented in EBRT, should be incorporated when determining the upper limit of activity in the administration of shorter half-life β^+ emitters. Current *fractionation* schedules of RNT, as mentioned above, are arbitrarily defined as 6 to 8 weeks between administration. If the recovery and repair of the dose-limiting organs can be determined, by taking NTCP into account, the interval between the PERT administration can be shortened.

Discrepancies between the OLINDA and MIM Encore results stem predominantly from the definition of the organ volumes and the density correction methods when converting the cumulated activity or integral activity to dose.¹⁴² CT-based density correction maps take into account any heterogeneity in organ density; a necessary feature in personalisation of treatment planning. Highest disparity was observed for the ^{18}F results of red marrow and ^{64}Cu results of spleen, with ratios of 0.51 and 3.41, respectively. The MIM contour of the

bones did not isolate the red marrow, resulting in a ratio of <1 . The fluctuations in variability across organs display the importance of the personalisation of therapy; for the analysed patient, the actual mass of the spleen was smaller than the average used in OLINDA (82 g v.s. 183 g), which would have led to a significant overdosing if standard phantom masses were used to prescribe the activities.

The computed dosimetry results from both OLINDA and MIM Encore of ^{68}Ga , despite having a shorter half-life and higher β^+ emission energy than ^{18}F , show that the dose deposition is equally efficient per unit of activity. This is attributed to the energy absorption from the low energy x-rays and Auger electrons produced in the EC process - a branching percentage of approximately 10.5%. The decay chains of both ^{64}Cu and ^{68}Ga , from the contributions of Auger electrons with the β particles, can allow for a potential improvement in the treatment efficacy that may be realised in PERT, compared to ^{18}F with a smaller fraction of Auger electron emission from EC ($\approx 3.3\%$).

7.5. Conclusion

This chapter demonstrated a proof-of-concept biodistribution dosimetry study of utilising therapeutic radionuclide clearance data to prescribe the tolerable activity of β^+ emitters. Variability of using different software involved in dosimetry is a clinically recognised limitation.^{45,131} The standardisation of the type of scan, whether it be a planar or tomographic, along with the dosimetric analysis, must be normalised for the purpose of consistent pre-treatment evaluation for therapy.

The underlying concept behind using β^+ emitters for therapy takes into account both the shorter half-life of the radionuclide to intrinsically increase the dose rate, but also to harness the therapeutic effect of the particle itself. With radionuclides such as ^{64}Cu , a combination therapy of β^+ and β^- with low energy x-rays and Auger electrons should be explored further to improve the treatment outcome for patients with metastatic disease.

Conclusion and Future Work

8.1. Conclusion

The work presented in this thesis highlighted the importance of interdisciplinary collaboration toward the goal of personalised treatment in RNT. Current means of determining the prescribed dose is based on parameters such as the tumour size, extent of metastasis and the general condition of the patient to handle potential side effects from the off-target effects of the treatment.^{7,53} Although personalisation is achieved to a certain extent, it does not differentiate one patient's cancer characteristics from another or the variations in uptake between patients, thus is not capable of extracting the full benefits of the prescribed dose. As the data obtained from *in silico*, *in vitro* and *in vivo* studies can collectively begin to reveal the essential parameters needed to improve the efficacy of treatment in the clinic, the importance of fundamental research is exemplified, and is reflected throughout this thesis.

No gold standard exist for *in vitro* radionuclide irradiation investigations. This is predominantly due to the difficulties faced if one were to take into account different half-lives, modes of decay and branching ratios coupled with a spectrum of emitted photon energies. While **Chapter 4** detailed a protocol for short half-life radionuclides, this methodology was created to act as a template, adaptable to *most* therapeutic or theranostic radionuclide, provided that the cells irradiated are suitable for a clonogenic assay. The protocol aimed to suit the needs and experimental outcomes of any radiobiological project, with key notes and points embedded to minimise optimisation time.

In the midst of developing a standardised protocol for *in vitro* radiobiological RNT studies, a thought experiment on the therapeutic potential along the thermalisation track of β^+ before positronium formation and annihilation was brought about. These discussions

led to the investigation of the strand breaks on a simple, linear DNA model in TOPAS-nBio, eventually allowing for the protocol “in-development” to be tested with a novel modality in RNT, coined as PERT, explored in **Chapter 5**. High activities of β^+ emitters described would currently be difficult to implement into the clinic, but the impact on the clonogenicity of the cancer cells demonstrated that investigation into the fundamental physics can lead to the unexpected development of novel concepts.

To address and overcome some of the limitations posed by the escalated doses required in the possible PERT implementation in the clinic, the manipulation of the Lea-Catcheside G-factor performed in **Chapter 6** demonstrated that cells may exhibit a certain limit to their dose response when a high dose rate is achieved, similar to the recombination of the ROS or free radicals in EBRT. The fact that higher LET particles causes irreparable DNA damage more efficiently would imply that no ceiling should exist, and therefore an intrinsic limitation may potentially exist in this experimental structure. The small pilot study “fractionating” dose delivery did not improve the dose response when compared to the “acute”, but it demonstrated that the impact on the clonogenicity can still be achieved by partitioning the activity. Clinical implementation of fractionated dose delivery in PERT therefore would become more feasible whilst maintaining the treatment efficacy through the development of a highly specific targeting agent.

8.2. Future Work

8.2.1. Radiobiological Assays

A large collection of assays are available to further characterise the dose-response from a radiobiological perspective. Some assays which were not included in this thesis due to the difficulties in optimisation were the γ -H2AX and apoptosis assays. Once these results can be collected, an effort to standardise the radiobiological assays should be made to allow for a catalogue of radiobiological parameters for various cancer cell lines available.

Furthermore, multiple time-points should be investigated for the assays due to the delayed effects of radiation as well as cellular response. This will complete the full picture of the radiobiological characterisations attempted throughout this thesis.

8.2.2. *Extending the in vitro Experiments*

Before the progression along the translational research pipeline, the efficacy of the β^+ emitter ^{18}F when labelled to targeting agents such as PSMA-11 (PSMA-HBED-CC) must be explored. As PSMA ligands are internalised into PSMA receptor expressing PCa cells, the source-to-target geometry and temporal variability of the site of the β^+ emission changes dramatically. Further optimisation must be performed to determine the ratio of uptake to determine the efficacy of the internalised radionuclide. This must be accompanied by a more sophisticated *in silico* dosimetry platform to estimate the difference between fractions of internalisation, and results will be compared with the unlabelled PERTs results in **Chapters 5 and 6**.

8.2.3. *in silico Experiments*

Expanding on the use of the two dosimetry software platforms in **Chapter 7**, investigation into the tumour lesion retention must be performed. Increasing the number of patient scans will also allow for a detailed statistical analysis on variability of organ and lesion volumes, along with improved biodistribution data for prescriptive dosimetry. Dose response from patients can also be used to explore the clinical relative biological effectiveness between the surrogate radionuclide and any novel tracers that are currently being developed.

References

- [1] Douglas Hanahan and Robert A. Weinberg. Hallmarks of Cancer: The Next Generation. *Cell*, 144(5):646–674, March 2011.
- [2] An Aerts, Uta Eberlein, Sören Holm, Roland Hustinx, Mark Konijnenberg, Lidia Strigari, Fijs W.B. Van Leeuwen, Gerhard Glatting, and Michael Lassmann. EANM position paper on the role of radiobiology in nuclear medicine. *European Journal of Nuclear Medicine and Molecular Imaging*, 48(11):3365–3377, October 2021.
- [3] Simon R. Cherry, James A. Sorenson, and Michael E. Phelps. *Physics in Nuclear Medicine*. Elsevier, 2012.
- [4] M Ervik, F Lam, J Ferlay, L Mery, I Soerjomataram, and F Bray. Cancer Today, 2016.
- [5] Douglas Hanahan and Robert A Weinberg. The Hallmarks of Cancer. *Cell*, 100(1):57–70, January 2000.
- [6] Michael Baumann, Mechthild Krause, Jens Overgaard, Jürgen Debus, Søren M. Bentzen, Juliane Daartz, Christian Richter, Daniel Zips, and Thomas Bortfeld. Radiation oncology in the era of precision medicine. *Nature Reviews Cancer*, 16(4):234–249, April 2016.
- [7] Gerhardt Attard, Chris Parker, Ros A Eeles, Fritz Schröder, Scott A Tomlins, Ian Tannock, Charles G Drake, and Johann S de Bono. Prostate cancer. *The Lancet*, 387(10013):70–82, January 2016.
- [8] Claire L Vale, David Fisher, Andrew Kneebone, Christopher Parker, Maria Pearse, Pierre Richaud, Paul Sargos, Matthew R Sydes, Christopher Brawley, Meryem Brihoum, Chris Brown, Sylvie Chabaud, Adrian Cook, Silvia Forcat, Carol Fraser-Browne, Igor Latorzeff, Mahesh K B Parmar, and Jayne F Tierney. Adjuvant or

early salvage radiotherapy for the treatment of localised and locally advanced prostate cancer: a prospectively planned systematic review and meta-analysis of aggregate data. *The Lancet*, 396(10260):1422–1431, October 2020.

- [9] Michael Yan, Andre G. Gouveia, Fabio L. Cury, Nikitha Moideen, Vanessa F. Bratti, Horacio Patrocinio, Alejandro Berlin, Lucas C. Mendez, and Fabio Y. Moraes. Practical considerations for prostate hypofractionation in the developing world. *Nature Reviews Urology*, 18(11):669–685, November 2021.
- [10] J F Fowler. 21 years of Biologically Effective Dose. *The British Journal of Radiology*, 83(991):554–568, July 2010.
- [11] H. Rodney Withers. The Four R’s of Radiotherapy. In John T. Lett and Howard Adler, editors, *Advances in Radiation Biology*, volume 5 of *Advances in Radiation Biology*, pages 241–271. Elsevier, 1975. ISSN: 0065-3292.
- [12] G. Gordon Steel, T.J. McMillan, and J.H. Peacock. The 5Rs of Radiobiology. *International Journal of Radiation Biology*, 56(6):1045–1048, January 1989.
- [13] Sophie Le Caër. Water Radiolysis: Influence of Oxide Surfaces on H₂ Production under Ionizing Radiation. *Water*, 3(1):235–253, February 2011.
- [14] Leo E. Gerweck and Sergey V. Kozin. Relative biological effectiveness of proton beams in clinical therapy. *Radiotherapy and Oncology*, 50(2):135–142, February 1999.
- [15] Yoshitaka Matsumoto, Nobuyoshi Fukumitsu, Hitoshi Ishikawa, Kei Nakai, and Hideyuki Sakurai. A Critical Review of Radiation Therapy: From Particle Beam Therapy (Proton, Carbon, and BNCT) to Beyond. *Journal of Personalized Medicine*, 11(8):825, August 2021.
- [16] Anna A. Friedl, Kevin M. Prise, Karl T. Butterworth, Pierre Montay-Gruel, and Vincent Favaudon. Radiobiology of the FLASH effect. *Medical Physics*, 49(3):1993–2013, March 2022.

- [17] Anthony E. Mascia, Emily C. Daugherty, Yongbin Zhang, Eunsin Lee, Zhiyan Xiao, Mathieu Sertorio, Jennifer Woo, Lori R. Backus, Julie M. McDonald, Claire McCann, Kenneth Russell, Lisa Levine, Ricky A. Sharma, Dee Khuntia, Jeffrey D. Bradley, Charles B. Simone, John P. Perentesis, and John C. Breneman. Proton FLASH Radiotherapy for the Treatment of Symptomatic Bone Metastases: The FAST-01 Nonrandomized Trial. *JAMA Oncology*, 9(1):62, January 2023.
- [18] Willi Kreis. Current Chemotherapy and Future Directions in Research for the Treatment of Advanced Hormone-Refractory Prostate Cancer. *Cancer Investigation*, 13(3):296–312, January 1995.
- [19] Ira Mellman, George Coukos, and Glenn Dranoff. Cancer immunotherapy comes of age. *Nature*, 480(7378):480–489, December 2011.
- [20] Benjamin Frey, Yvonne Rubner, Lorenz Kulzer, Nina Werthmöller, Eva-Maria Weiss, Rainer Fietkau, and Udo S. Gaipl. Antitumor immune responses induced by ionizing irradiation and further immune stimulation. *Cancer Immunology, Immunotherapy*, 63(1):29–36, January 2014.
- [21] Jean-Pierre Pouget, Isabelle Navarro-Teulon, Manuel Bardiès, Nicolas Chouin, Guillaume Cartron, André Pèlegri, and David Azria. Clinical radioimmunotherapy—the role of radiobiology. *Nature Reviews Clinical Oncology*, 8(12):720–734, December 2011.
- [22] Laurent Schwartz, Thomas Seyfried, Khalid O. Alfarouk, Jorgelindo Da Veiga Moreira, and Stefano Fais. Out of Warburg effect: An effective cancer treatment targeting the tumor specific metabolism and dysregulated pH. *Seminars in Cancer Biology*, 43:134–138, April 2017.
- [23] Øyvind S. Bruland, Sten Nilsson, Darrell R. Fisher, and Roy H. Larsen. High-Linear Energy Transfer Irradiation Targeted to Skeletal Metastases by the alpha-Emitter ²²³Ra: Adjuvant or Alternative to Conventional Modalities? *Clinical Cancer Research*, 12(20):6250s–6257s, October 2006.

- [24] Chai-Hong Yeong, Mu-hua Cheng, and Kwan-Hoong Ng. Therapeutic radionuclides in nuclear medicine: current and future prospects. *Journal of Zhejiang University. Science. B*, 15(10):845–863, October 2014.
- [25] Cristina Müller, Nicholas P. van der Meulen, Martina Benešová, and Roger Schibli. Therapeutic Radiometals Beyond ^{177}Lu and ^{90}Y : Production and Application of Promising α -Particle, β^- -Particle, and Auger Electron Emitters. *Journal of Nuclear Medicine*, 58(Supplement 2):91S–96S, September 2017.
- [26] Brian F. Hutton. The origins of SPECT and SPECT/CT. *European Journal of Nuclear Medicine and Molecular Imaging*, 41(S1):3–16, May 2014.
- [27] Sten Carlson. A Glance At The History Of Nuclear Medicine. *Acta Oncologica*, 34(8):1095–1102, January 1995.
- [28] Terry Jones and David Townsend. History and future technical innovation in positron emission tomography. *Journal of Medical Imaging*, 4(1):011013, March 2017.
- [29] R Nutt. The History of Positron Emission Tomography. *Molecular Imaging & Biology*, 4(1):11–26, February 2002.
- [30] J. Cal-González, J.L. Herraiz, S. España, E. Vicente, E. Herranz, M. Desco, J.J. Vaquero, and J.M. Udías. Study of CT-based positron range correction in high resolution 3D PET imaging. *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment*, 648:S172–S175, August 2011.
- [31] Ottavia Bertolli, Afroditi Eleftheriou, Matteo Cecchetti, Niccolò Camarlinghi, Nicola Belcari, and Charalampos Tsoumpas. PET iterative reconstruction incorporating an efficient positron range correction method. *Physica Medica*, 32(2):323–330, February 2016.
- [32] Hunor Kertész, Maurizio Conti, Vladimir Panin, Jorge Cabello, Deepak Bharkhada, Thomas Beyer, Laszlo Papp, Walter Jentzen, Jacobo Cal-Gonzalez, Joaquín L. Herraiz, Alejandro López-Montes, and Ivo Rausch. Positron range in combination

- with point-spread-function correction: an evaluation of different implementations for [124I]-PET imaging. *EJNMMI Physics*, 9(1):56, August 2022.
- [33] K. F. Eckerman, R. J. Westfall, J. C. Ryman, and M. Cristy. Availability of Nuclear Decay Data in Electronic Form, Including Beta Spectra Not Previously Published:. *Health Physics*, 67(4):338–345, October 1994.
- [34] Hossein Jadvar. Targeted Radionuclide Therapy: An Evolution Toward Precision Cancer Treatment. *American Journal of Roentgenology*, 209(2):277–288, August 2017.
- [35] Sergey Gudkov, Natalya Shilyagina, Vladimir Vodeneev, and Andrei Zvyagin. Targeted Radionuclide Therapy of Human Tumors. *International Journal of Molecular Sciences*, 17(1):33, December 2015.
- [36] National Nuclear Data Center, information extracted from the NuDat database, <https://www.nndc.bnl.gov/nudat/>.
- [37] Sneha S. Kelkar and Theresa M. Reineke. Theranostics: Combining Imaging and Therapy. *Bioconjugate Chemistry*, 22(10):1879–1903, October 2011.
- [38] Amin I. Kassis. Therapeutic Radionuclides: Biophysical and Radiobiologic Principles. *Seminars in Nuclear Medicine*, 38(5):358–366, September 2008.
- [39] Edward M Wolin. The Expanding Role of Somatostatin Analogs in the Management of Neuroendocrine Tumors. *Gastrointestinal Cancer Research*, 5(5), 2012.
- [40] Alireza Aslani, Graeme M Snowdon, Dale L Bailey, Geoffrey P Schembri, Elizabeth A Bailey, Nick Pavlakis, and Paul J Roach. Lutetium-177 DOTATATE Production with an Automated Radio- pharmaceutical Synthesis System. *Asia Oceania Journal of Nuclear Medicine & Biology*, 3(2):107–115, 2015.
- [41] D.T. Goodhead. Initial Events in the Cellular Effects of Ionizing Radiations: Clustered Damage in DNA. *International Journal of Radiation Biology*, 65(1):7–17, January 1994.
- [42] Sirtex SIR-Spheres Pty Ltd. SIR-Spheres Y-90 resin microspheres.

- [43] The QUEST Investigator Team, Kathy P. Willowson, Michael Tapner, and Dale L. Bailey. A multicentre comparison of quantitative ^{90}Y PET/CT for dosimetric purposes after radioembolization with resin microspheres: The QUEST Phantom Study. *European Journal of Nuclear Medicine and Molecular Imaging*, 42(8):1202–1222, July 2015.
- [44] Kathy P. Willowson, Enid Eslick, Hyunju Ryu, Aurora Poon, Elizabeth J. Bernard, and Dale L. Bailey. Feasibility and accuracy of single time point imaging for renal dosimetry following ^{177}Lu -DOTATATE (‘Lutate’) therapy. *EJNMMI Physics*, 5(1):33, December 2018.
- [45] Michael G Stabin, Richard B Sparks, and Eric Crowe. OLINDA/EXM: The Second-Generation Personal Computer Software for Internal Dose Assessment in Nuclear Medicine. *Journal of Nuclear Medicine*, 46:1023–1027, 2005.
- [46] Paul E. Kinahan and James W. Fletcher. Positron Emission Tomography-Computed Tomography Standardized Uptake Values in Clinical Practice and Assessing Response to Therapy. *Seminars in Ultrasound, CT and MRI*, 31(6):496–505, December 2010.
- [47] Harry Marquis, Dale L. Bailey, and Kathy P. Willowson. PARTIAL VOLUME EFFECT IN SPECT & PET IMAGING AND IMPACT ON RADIONUCLIDE DOSIMETRY ESTIMATES. *Asia Oceania Journal of Nuclear Medicine and Biology*, 11(1):44–54, 2023.
- [48] J Ferlay, I Soerjomataram, M Ervik, R Dikshit, S Eser, C Mathers, M Rebelo, D. M. Parkin, D Forman, and F Bray. Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11, 2012.
- [49] Australian Institute of Health and Welfare. Cancer compendium: information and trends by cancer type, 2017.
- [50] Melissa M. Center, Ahmedin Jemal, Joannie Lortet-Tieulent, Elizabeth Ward, Jacques Ferlay, Otis Brawley, and Freddie Bray. International Variation in Prostate Cancer Incidence and Mortality Rates. *European Urology*, 61(6):1079–1092, June 2012.

- [51] Hans Lilja, David Ulmert, and Andrew J. Vickers. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. *Nature Reviews Cancer*, 8(4):268–278, April 2008.
- [52] Harry G. Rittenhouse, Judith A. Finlay, Stephen D. Mikolajczyk, and Alan W. Partin. Human Kallikrein 2 (hK2) and Prostate-Specific Antigen (PSA): Two Closely Related, but Distinct, Kallikreins in the Prostate. *Critical Reviews in Clinical Laboratory Sciences*, 35(4):275–368, January 1998.
- [53] Jan-Erik Damber and Gunnar Aus. Prostate Cancer. *Lancet*, 371:1710–1721, May 2008.
- [54] George T. Mellinger, Donald Gleason, and John Bailar. The Histology and Prognosis of Prostatic Cancer. *Journal of Urology*, 97(2):331–337, February 1967.
- [55] Peter A Humphrey. Gleason grading and prognostic factors in carcinoma of the prostate. *Modern Pathology*, 17(3):292–306, March 2004.
- [56] Kirsten Bouchelouche, Peter L Choyke, and Jacek Capala. Prostate Specific Membrane Antigen—A Target for Imaging and Therapy with Radionuclides. *Discovery medicine*, 9(44):55–61, January 2010.
- [57] Francesca Cutruzzolà, Giorgio Giardina, Marina Marani, Alberto Macone, Alessandro Paiardini, Serena Rinaldo, and Alessio Paone. Glucose Metabolism in the Progression of Prostate Cancer. *Frontiers in Physiology*, 8, February 2017.
- [58] Nicholas G. Zaorsky, Brian J. Davis, Paul L. Nguyen, Timothy N. Showalter, Peter J. Hoskin, Yasuo Yoshioka, Gerard C. Morton, and Eric M. Horwitz. The evolution of brachytherapy for prostate cancer. *Nature Reviews Urology*, 14(7):415–439, July 2017.
- [59] Thomas Van Den Broeck, Roderick C.N. Van Den Bergh, Nicolas Arfi, Tobias Gross, Lisa Moris, Erik Briers, Marcus Cumberbatch, Maria De Santis, Derya Tilki, Stefano Fanti, Nicola Fossati, Silke Gillissen, Jeremy P. Grummet, Ann M. Henry, Michael Lardas, Matthew Liew, Olivier Rouvière, Jakub Pecanka, Malcolm D. Mason, Ivo G.

- Schoots, Theo H. Van Der Kwast, Henk G. Van Der Poel, Thomas Wiegel, Peter-Paul M. Willemse, Yuhong Yuan, Thomas B. Lam, Philip Cornford, and Nicolas Mottet. Prognostic Value of Biochemical Recurrence Following Treatment with Curative Intent for Prostate Cancer: A Systematic Review. *European Urology*, 75(6):967–987, June 2019.
- [60] Paul J. Torren and Martin E. Gleave. Novel non-AR therapeutic targets in castrate resistant prostate cancer. *Translational Andrology and Urology*, 2(3):265–277, September 2013.
- [61] P. D. Ottewell, N. Wang, J. Meek, C. A. Fowles, P. I. Croucher, C. L. Eaton, and I. Holen. Castration-induced bone loss triggers growth of disseminated prostate cancer cells in bone. *Endocrine Related Cancer*, 21(5):769–781, September 2014.
- [62] Christopher Logothetis, Michael J. Morris, Robert Den, and Robert E. Coleman. Current perspectives on bone metastases in castrate-resistant prostate cancer. *Cancer and Metastasis Reviews*, 37(1):189–196, March 2018.
- [63] Cyril Barinka, Pavel Šácha, Jan Sklenář, Petr Man, Karel Bezouška, Barbara S. Slusher, and Jan Konvalinka. Identification of the N-glycosylation sites on glutamate carboxypeptidase II necessary for proteolytic activity. *Protein Science*, 13(6):1627–1635, June 2004.
- [64] Sam S Chang, Victor E Reuter, W D W Heston, Neil H Bander, Lana S Grauer, and Paul B Gaudin. Five Different Anti-Prostate-specific Membrane Antigen (PSMA) Antibodies Confirm PSMA Expression in Tumor-associated Neovasculature. *Cancer Research*, 59:3192–3198, July 1999.
- [65] Sam S Chang. Overview of Prostate-Specific Membrane Antigen. *Reviews in Urology*, 6(Supplementary 10), 2004.
- [66] Clemens Kratochwil, Ali Afshar-Oromieh, Klaus Kopka, Uwe Haberkorn, and Frederik L. Giesel. Current Status of Prostate-Specific Membrane Antigen Targeting in Nuclear Medicine: Clinical Translation of Chelator Containing Prostate-Specific

- Membrane Antigen Ligands Into Diagnostics and Therapy for Prostate Cancer. *Seminars in Nuclear Medicine*, 46(5):405–418, September 2016.
- [67] Ali Afshar-Oromieh, Uwe Haberkorn, Christian Zechmann, Thomas Armor, Walter Mier, Fabian Spohn, Nils Debus, Tim Holland-Letz, John Babich, and Clemens Kratochwil. Repeated PSMA-targeting radioligand therapy of metastatic prostate cancer with ¹³¹I-MIP-1095. *European Journal of Nuclear Medicine and Molecular Imaging*, 44(6):950–959, June 2017.
- [68] A. Afshar-Oromieh, J. W. Babich, C. Kratochwil, F. L. Giesel, M. Eisenhut, K. Kopka, and U. Haberkorn. The Rise of PSMA Ligands for Diagnosis and Therapy of Prostate Cancer. *Journal of Nuclear Medicine*, 57:79S–89S, October 2016.
- [69] Louise Emmett, Megan Crumbaker, Bao Ho, Kathy Willowson, Peter Eu, Lalith Ratnayake, Richard Epstein, Ashley Blanksby, Lisa Horvath, Alex Guminski, Kate Mahon, Craig Gedye, Charlotte Yin, Phillip Stricker, and Anthony M. Joshua. Results of a Prospective Phase 2 Pilot Trial of ¹⁷⁷Lu–PSMA-617 Therapy for Metastatic Castration-Resistant Prostate Cancer Including Imaging Predictors of Treatment Response and Patterns of Progression. *Clinical Genitourinary Cancer*, 17(1):15–22, February 2019.
- [70] Kambiz Rahbar, Axel Bode, Matthias Weckesser, Nemanja Avramovic, Michael Claesener, Lars Stegger, and Martin Bögemann. Radioligand Therapy With ¹⁷⁷Lu-PSMA-617 as A Novel Therapeutic Option in Patients With Metastatic Castration Resistant Prostate Cancer:. *Clinical Nuclear Medicine*, 41(7):522–528, July 2016.
- [71] R J S Calopedos, V Chalasani, R Asher, L Emmett, and H H Woo. Lutetium-¹⁷⁷-labelled anti-prostate-specific membrane antigen antibody and ligands for the treatment of metastatic castrate-resistant prostate cancer: a systematic review and meta-analysis. *Prostate Cancer and Prostatic Diseases*, 20(3):352–360, September 2017.

- [72] Pim J. van Leeuwen, Phillip Stricker, George Hruby, Andrew Kneebone, Francis Ting, Ben Thompson, Quoc Nguyen, Bao Ho, and Louise Emmett. 68Ga-PSMA has a high detection rate of prostate cancer recurrence outside the prostatic fossa in patients being considered for salvage radiation treatment. *BJU International*, 117(5):732–739, May 2016.
- [73] G. Sgouros, S. J. Knox, M. C. Joiner, W. F. Morgan, and A. I. Kassis. MIRD Continuing Education: Bystander and Low Dose-Rate Effects: Are These Relevant to Radionuclide Therapy? *Journal of Nuclear Medicine*, 48(10):1683–1691, October 2007.
- [74] Boon Q. Lee, Hooshang Nikjoo, Jörgen Ekman, Per Jönsson, Andrew E. Stuchbery, and Tibor Kibédi. A stochastic cascade model for Auger-electron emitting radionuclides. *International Journal of Radiation Biology*, 92(11):641–653, November 2016.
- [75] H Nikjoo, D Emfietzoglou, T Liamsuwan, R Taleei, D Liljequist, and S Uehara. Radiation track, DNA damage and response—a review. *Reports on Progress in Physics*, 79(11):116601, November 2016.
- [76] Yaser H. Gholami, Kathy P. Willowson, Nicholas J. Forwood, Rozelle Harvie, Nicholas Hardcastle, Regina Bromley, HyunJu Ryu, Samuel Yuen, Viive M. Howell, Zdenka Kuncic, and Dale L. Bailey. Comparison of radiobiological parameters for 90Y radionuclide therapy (RNT) and external beam radiotherapy (EBRT) in vitro. *EJNMMI Physics*, 5(1):18, December 2018.
- [77] Jac A. Nickoloff, Neelam Sharma, and Lynn Taylor. Clustered DNA Double-Strand Breaks: Biological Effects and Relevance to Cancer Radiotherapy. *Genes*, 11(1):99, January 2020.
- [78] C. Arena, V. De Micco, E. Macaeva, and R. Quintens. Space radiation effects on plant and mammalian cells. *Acta Astronautica*, 104(1):419–431, November 2014.
- [79] Luminex Corporation. Muse Cell Cycle Kit Instruction Manual, 2019.

- [80] Luminex Corporation. Muse Oxidative Stress Kit Instruction Manual, 2019.
- [81] Elzbieta Pawlowska, Joanna Szczepanska, Magdalena Szatkowska, and Janusz Blasiak. An Interplay between Senescence, Apoptosis and Autophagy in Glioblastoma Multiforme—Role in Pathogenesis and Therapeutic Perspective. *International Journal of Molecular Sciences*, 19(3):889, March 2018.
- [82] Cornelius A Tobias. The Repair-Misrepair Model in Radiobiology: Comparison to Other Models. *Radiation Research Society*, 104(2), 1985.
- [83] Stanley B. Curtis. Lethal and Potentially Lethal Lesions Induced by Radiation — A Unified Repair Model. *Radiation Research*, 106(2):252, May 1986.
- [84] David J. Brenner. The Linear-Quadratic Model Is an Appropriate Methodology for Determining Isoeffective Doses at Large Doses Per Fraction. *Seminars in Radiation Oncology*, 18(4):234–239, October 2008.
- [85] Dževad Belkić and Karen Belkić. Mechanistic description of survival of irradiated cells: repair kinetics in Padé linear-quadratic or differential Michaelis–Menten model. *Journal of Mathematical Chemistry*, 51(9):2572–2607, October 2013.
- [86] S. Agostinelli, J. Allison, K. Amako, J. Apostolakis, H. Araujo, P. Arce, M. Asai, D. Axen, S. Banerjee, G. Barrand, F. Behner, L. Bellagamba, J. Boudreau, L. Broglia, A. Brunengo, H. Burkhardt, S. Chauvie, J. Chuma, R. Chytracsek, G. Cooperman, G. Cosmo, P. Degtyarenko, A. Dell’Acqua, G. Depaola, D. Dietrich, R. Enami, A. Feliciello, C. Ferguson, H. Fesefeldt, G. Folger, F. Foppiano, A. Forti, S. Garelli, S. Giani, R. Giannitrapani, D. Gibin, J.J. Gómez Cadenas, I. González, G. Gracia Abril, G. Greeniaus, W. Greiner, V. Grichine, A. Grossheim, S. Guatelli, P. Gumplinger, R. Hamatsu, K. Hashimoto, H. Hasui, A. Heikkinen, A. Howard, V. Ivanchenko, A. Johnson, F.W. Jones, J. Kallenbach, N. Kanaya, M. Kawabata, Y. Kawabata, M. Kawaguti, S. Kelner, P. Kent, A. Kimura, T. Kodama, R. Kokoulin, M. Kossov, H. Kurashige, E. Lamanna, T. Lampén, V. Lara, V. Lefebure, F. Lei, M. Liendl, W. Lockman, F. Longo, S. Magni, M. Maire, E. Medernach,

- K. Minamimoto, P. Mora De Freitas, Y. Morita, K. Murakami, M. Nagamatu, R. Nartallo, P. Nieminen, T. Nishimura, K. Ohtsubo, M. Okamura, S. O’Neale, Y. Oohata, K. Paech, J. Perl, A. Pfeiffer, M.G. Pia, F. Ranjard, A. Rybin, S. Sadilov, E. Di Salvo, G. Santin, T. Sasaki, N. Savvas, Y. Sawada, S. Scherer, S. Sei, V. Sirotenko, D. Smith, N. Starkov, H. Stoecker, J. Sulkimo, M. Takahata, S. Tanaka, E. Tcherniaev, E. Safai Tehrani, M. Tropeano, P. Truscott, H. Uno, L. Urban, P. Urban, M. Verderi, A. Walkden, W. Wander, H. Weber, J.P. Wellisch, T. Wenaus, D.C. Williams, D. Wright, T. Yamada, H. Yoshida, and D. Zschiesche. Geant4—a simulation toolkit. *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment*, 506(3):250–303, July 2003.
- [87] J. Allison, K. Amako, J. Apostolakis, H. Araujo, P. Arce Dubois, M. Asai, G. Barrand, R. Capra, S. Chauvie, R. Chytrcek, G.A.P. Cirrone, G. Cooperman, G. Cosmo, G. Cuttone, G.G. Daquino, M. Donszelmann, M. Dressel, G. Folger, F. Foppiano, J. Generowicz, V. Grichine, S. Guatelli, P. Gumplinger, A. Heikkinen, I. Hrivnacova, A. Howard, S. Incerti, V. Ivanchenko, T. Johnson, F. Jones, T. Koi, R. Kokoulin, M. Kossov, H. Kurashige, V. Lara, S. Larsson, F. Lei, O. Link, F. Longo, M. Maire, A. Mantero, B. Mascialino, I. McLaren, P. Mendez Lorenzo, K. Minamimoto, K. Murakami, P. Nieminen, L. Pandola, S. Parlati, L. Peralta, J. Perl, A. Pfeiffer, M.G. Pia, A. Ribon, P. Rodrigues, G. Russo, S. Sadilov, G. Santin, T. Sasaki, D. Smith, N. Starkov, S. Tanaka, E. Tcherniaev, B. Tome, A. Trindade, P. Truscott, L. Urban, M. Verderi, A. Walkden, J.P. Wellisch, D.C. Williams, D. Wright, and H. Yoshida. Geant4 developments and applications. *IEEE Transactions on Nuclear Science*, 53(1):270–278, February 2006.
- [88] J. Allison, K. Amako, J. Apostolakis, P. Arce, M. Asai, T. Aso, E. Bagli, A. Bagulya, S. Banerjee, G. Barrand, B.R. Beck, A.G. Bogdanov, D. Brandt, J.M.C. Brown, H. Burkhardt, Ph. Canal, D. Cano-Ott, S. Chauvie, K. Cho,

- G.A.P. Cirrone, G. Cooperman, M.A. Cortés-Giraldo, G. Cosmo, G. Cuttone, G. Depaola, L. Desorgher, X. Dong, A. Dotti, V.D. Elvira, G. Folger, Z. Francis, A. Galoyan, L. Garnier, M. Gayer, K.L. Genser, V.M. Grichine, S. Guatelli, P. Guèye, P. Gumplinger, A.S. Howard, I. Hřivnáčová, S. Hwang, S. Incerti, A. Ivanchenko, V.N. Ivanchenko, F.W. Jones, S.Y. Jun, P. Kaitaniemi, N. Karakatsanis, M. Karamitros, M. Kelsey, A. Kimura, T. Koi, H. Kurashige, A. Lechner, S.B. Lee, F. Longo, M. Maire, D. Mancusi, A. Mantero, E. Mendoza, B. Morgan, K. Murakami, T. Nikitina, L. Pandola, P. Paprocki, J. Perl, I. Petrović, M.G. Pia, W. Pokorski, J.M. Quesada, M. Raine, M.A. Reis, A. Ribon, A. Ristić Fira, F. Romano, G. Russo, G. Santin, T. Sasaki, D. Sawkey, J.I. Shin, I.I. Strakovsky, A. Taborda, S. Tanaka, B. Tomé, T. Toshito, H.N. Tran, P.R. Truscott, L. Urban, V. Uzhinsky, J.M. Verbeke, M. Verderi, B.L. Wendt, H. Wenzel, D.H. Wright, D.M. Wright, T. Yamashita, J. Yarba, and H. Yoshida. Recent developments in Geant4. *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment*, 835:186–225, November 2016.
- [89] J. Perl, J. Shin, J. Schümann, B. Faddegon, and H. Paganetti. TOPAS: An innovative proton Monte Carlo platform for research and clinical applications. *Medical Physics*, 39(11):6818–6837, November 2012.
- [90] J. Schuemann, A. L. McNamara, J. Ramos-Méndez, J. Perl, K. D. Held, H. Paganetti, S. Incerti, and B. Faddegon. TOPAS-nBio: An Extension to the TOPAS Simulation Toolkit for Cellular and Sub-cellular Radiobiology. *Radiation Research*, 191(2):125, January 2018.
- [91] S. Turajlic and C. Swanton. Metastasis as an evolutionary process. *Science*, 352(6282):169–175, April 2016.
- [92] Terry L Riss, Richard A Moravec, Andrew L Niles, Hélène A Benink, Tracy J Worzella, and Lisa Minor. Cell Viability Assays. In *The Assay Guidance Manual*. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing

Translational Sciences, 2016.

- [93] Paul L. de Souza, Anne Louise Capp, Nana Chikhladze, Zaza Mezvrishvili, Marinella Messina, and Gisela Mautner. Phase I study of a novel S1P inhibitor, NOX66, in combination with radiotherapy in patients with metastatic castration-resistant prostate cancer. *Journal of Clinical Oncology*, 38(15_suppl):5533–5533, May 2020. Publisher: Wolters Kluwer.
- [94] Alisa Schnellbaecher, Dennis Binder, Stephanie Bellmaine, and Aline Zimmer. Vitamins in cell culture media: Stability and stabilization strategies. *Biotechnology and Bioengineering*, 116(6):1537–1555, June 2019.
- [95] Jesco Pfitzenmaier, Janna E Quinn, Austin M Odman, Jian Zhang, Evan T Keller, Robert L Vessella, and Eva Corey. Characterization of C4-2 Prostate Cancer Bone Metastases and Their Response to Castration. *Journal of Bone and Mineral Research*, 18(10):1882–1888, October 2003.
- [96] Pamela J Russell and Elizabeth A Kingsley. Human Prostate Cancer Cell Lines. In *Methods in Molecular Medicine: Prostate Cancer Methods and Protocols*, volume 81, pages 21–39. Humana Press Inc., 2003.
- [97] David A. Silver, Inmaculada Pellicer, Warren D Heston, and Carlos Cordon-Cardo. Prostate-specific Membrane Antigen Expression in Normal and Malignant Human Tissues. *Clinical Cancer Research*, 3:81–85, January 1997.
- [98] George N Thalmann, Ploutarchos E Anezinis, Shi-Ming Chang, Haiyen E Zhau, E Edmund Kim, Vicki L. Hopwood, Sen Pathak, Andrew C. von Eschenbach, and Leland W. K. Chung. Androgen-independent Cancer Progression and Bone Metastasis in the LNCaP Model of Human Prostate Cancer’. *Cancer Research*, 54:2577–2581, May 1994.
- [99] M. Verheijen, M. Lienhard, Y. Schrooders, O. Clayton, R. Nudischer, S. Boerno, B. Timmermann, N. Selevsek, R. Schlapbach, H. Gmuender, S. Gotta, J. Geraedts, R. Herwig, J. Kleinjans, and F. Caiment. DMSO induces drastic changes in human

- cellular processes and epigenetic landscape in vitro. *Scientific Reports*, 9(1):4641, March 2019.
- [100] Michael Timm, Lasse Saaby, Lise Moesby, and Erik Wind Hansen. Considerations regarding use of solvents in in vitro cell based assays. *Cytotechnology*, 65(5):887–894, October 2013.
- [101] John Wong, Elwood Armour, Peter Kazanzides, Iulian Iordachita, Erik Tryggestad, Hua Deng, Mohammad Matinfar, Christopher Kennedy, Zejian Liu, Timothy Chan, Owen Gray, Frank Verhaegen, Todd McNutt, Eric Ford, and Theodore L. DeWeese. High-Resolution, Small Animal Radiation Research Platform With X-Ray Tomographic Guidance Capabilities. *International Journal of Radiation Oncology*Biology*Physics*, 71(5):1591–1599, August 2008.
- [102] Philip A. Watson, Vivek K. Arora, and Charles L. Sawyers. Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. *Nature Reviews Cancer*, 15(12):701–711, December 2015.
- [103] David V. Becker and Clark T. Sawin. Radioiodine and thyroid disease: the beginning. *Seminars in Nuclear Medicine*, 26(3):155–164, July 1996.
- [104] Roger Dale and Alejandro Carabe-Fernandez. The Radiobiology of Conventional Radiotherapy and Its Application to Radionuclide Therapy. *Cancer Biotherapy and Radiopharmaceuticals*, 20(1):47–51, February 2005.
- [105] William T. Millar. Application of the linear-quadratic model with incomplete repair to radionuclide directed therapy. *The British Journal of Radiology*, 64(759):242–251, March 1991.
- [106] R G Dale. Dose-rate effects in targeted radiotherapy. *Physics in Medicine and Biology*, 41(10):1871–1884, October 1996.
- [107] Iain Murray and Glenn Flux. Applying radiobiology to clinical molecular radiotherapy. *Nuclear Medicine and Biology*, 100-101:1–3, September 2021.

- [108] D. E. Lea and D. G Catcheside. The mechanism of the induction by radiation of chromosome aberrations in tradescantia. *Journal of Genetics*, 44:216–245, December 1942.
- [109] Kevin M. Prise and Joe M. O’Sullivan. Radiation-induced bystander signalling in cancer therapy. *Nature Reviews Cancer*, 9(5):351–360, May 2009.
- [110] Igor Shuryak and David J Brenner. REVIEW OF QUANTITATIVE MECHANISTIC MODELS OF RADIATION-INDUCED NON-TARGETED EFFECTS (NTE). *Radiation Protection Dosimetry*, 192(2):236–252, December 2020.
- [111] David Cunningham and Zongbing You. In vitro and in vivo model systems used in prostate cancer research. *Journal of biological methods*, 2(1), 2015.
- [112] Peter M Smith-Jones, Shankar Vallabahajosula, Stanley J Goldsmith, Vincent Navarro, Catherine J Hunter, Diego Bastidas, and Neil H Bander. In Vitro Characterization of Radiolabeled Monoclonal Antibodies Specific for the Extracellular Domain of Prostate-specific Membrane Antigen. *Cancer Research*, 60:5237–5243, 2000.
- [113] Zbynek Novy, Pavel Barta, Jana Mandikova, Milan Laznicek, and Frantisek Trejtnar. A comparison of in vitro methods for determining the membrane receptor expression in cell lines. *Nuclear Medicine and Biology*, 39(7):893–896, October 2012.
- [114] Takanori Hioki, Yaser H. Gholami, Kelly J. McKelvey, Alireza Aslani, Harry Marquis, Enid M. Eslick, Kathy P. Willowson, Viive M. Howell, and Dale L. Bailey. Overlooked potential of positrons in cancer therapy. *Scientific Reports*, 11(1):2475, January 2021.
- [115] Nicolaas A P Franken, Hans M Rodermond, Jan Stap, Jaap Haveman, and Chris van Bree. Clonogenic assay of cells in vitro. *Nature Protocols*, 1(5):2315–2319, December 2006.
- [116] James M. Kelly, Alejandro Amor-Coarasa, Anastasia Nikolopoulou, Dohyun Kim, Clarence Williams, Shankar Vallabhajosula, and John W. Babich. Assessment of PSMA targeting ligands bearing novel chelates with application to theranostics:

- Stability and complexation kinetics of $^{68}\text{Ga}^{3+}$, $^{111}\text{In}^{3+}$, $^{177}\text{Lu}^{3+}$ and $^{225}\text{Ac}^{3+}$. *Nuclear Medicine and Biology*, 55:38–46, December 2017.
- [117] Teresa Neumaier, Joel Swenson, Christopher Pham, Aris Polyzos, Alvin T. Lo, PoAn Yang, Jane Dyball, Aroumougame Asaithamby, David J. Chen, Mina J. Bissell, Stefan Thalhammer, and Sylvain V. Costes. Evidence for formation of DNA repair centers and dose-response nonlinearity in human cells. *Proceedings of the National Academy of Sciences*, 109(2):443–448, January 2012.
- [118] Huichen Wang, Zhao-Chong Zeng, Tu-Anh Bui, Eiichiro Sonoda, Minoru Takata, Shunichi Takeda, and George Iliakis. Efficient rejoining of radiation-induced DNA double-strand breaks in vertebrate cells deficient in genes of the RAD52 epistasis group.
- [119] Stephen Joseph McMahon and Kevin M. Prise. A Mechanistic DNA Repair and Survival Model (Medras): Applications to Intrinsic Radiosensitivity, Relative Biological Effectiveness and Dose-Rate. *Frontiers in Oncology*, 11:689112, June 2021.
- [120] Donna L. Nile, Colin Rae, Iain J. Hyndman, Mark N. Gaze, and Robert J. Mairs. An evaluation in vitro of PARP-1 inhibitors, rucaparib and olaparib, as radiosensitisers for the treatment of neuroblastoma. *BMC Cancer*, 16(1):621, December 2016.
- [121] Silvia C Formenti and Sandra Demaria. Systemic effects of local radiotherapy. *The Lancet Oncology*, 10(7):718–726, July 2009.
- [122] Wilfred Ngwa, Omoruyi Credit Irabor, Jonathan D. Schoenfeld, Jürgen Hesser, Sandra Demaria, and Silvia C. Formenti. Using immunotherapy to boost the abscopal effect. *Nature Reviews Cancer*, 18(5):313–322, May 2018.
- [123] Stephen J. McMahon, Jan Schuemann, Harald Paganetti, and Kevin M. Prise. Mechanistic Modelling of DNA Repair and Cellular Survival Following Radiation-Induced DNA Damage. *Scientific Reports*, 6(1):33290, September 2016.

- [124] Anne Hansen Ree, Trond Stokke, Se Bratland, Sebastian Patzke, Ragnhild V Nome, Sigurd Folkvord, Leonardo A Meza-Zepeda, Kjersti Flatmark, Ystein Fodstad, and Yvonne Andersson. DNA Damage Responses in Cell Cycle G2 Phase and Mitosis – Tracking and Targeting. *ANTICANCER RESEARCH*, 2006.
- [125] Stephen J McMahon and Kevin M Prise. Mechanistic Modelling of Radiation Responses. *Cancers*, 11(2):205, February 2019.
- [126] Stephen Joseph McMahon. The linear quadratic model: usage, interpretation and challenges. *Physics in Medicine & Biology*, 64(1):01TR01, December 2018.
- [127] R.K. Sachs, P. Hahnfeld, and D.J. Brenner. The link between low-LET dose-response relations and the underlying kinetics of damage production/repair/misrepair. *International Journal of Radiation Biology*, 72(4):351–374, January 1997.
- [128] Marta Cremonesi, Mahila Ferrari, Lisa Bodei, Giampiero Tosi, and Giovanni Paganelli. Dosimetry in Peptide Radionuclide Receptor Therapy: A Review. *Journal of Nuclear Medicine*, 47(9):1467–1475, 2006.
- [129] Dale L. Bailey, Thomas M. Hennessy, Kathy P. Willowson, E. Courtney Henry, David L.H. Chan, Alireza Aslani, and Paul J. Roach. In vivo quantification of ^{177}Lu with planar whole-body and SPECT/CT gamma camera imaging. *EJNMMI Physics*, 2(1):20, December 2015.
- [130] J. Wevrett, A. Fenwick, J. Scuffham, and A. Nisbet. Development of a calibration protocol for quantitative imaging for molecular radiotherapy dosimetry. *Radiation Physics and Chemistry*, 140:355–360, November 2017.
- [131] Carlos Uribe, Avery Peterson, Benjamin Van, Roberto Fedrigo, Jake Carlson, John Sunderland, Eric Frey, and Yuni K Dewaraja. An International Study of Factors Affecting Variability of Dosimetry Calculations, Part 1: Design and Early Results of the SNMMI Dosimetry Challenge. *Journal of Nuclear Medicine*, 62(Supplement 3):36S–47S, December 2021.

- [132] N.E. Donlon, R. Power, C. Hayes, J.V. Reynolds, and J. Lysaght. Radiotherapy, immunotherapy, and the tumour microenvironment: Turning an immunosuppressive milieu into a therapeutic opportunity. *Cancer Letters*, 502:84–96, April 2021.
- [133] Amin I Kassis and S James Adelstein. Radiobiologic Principles in Radionuclide Therapy. *Journal of Nuclear Medicine*, 46(1), 2005.
- [134] Sadaf Aghevlian, Amanda J. Boyle, and Raymond M. Reilly. Radioimmunotherapy of cancer with high linear energy transfer (LET) radiation delivered by radionuclides emitting α -particles or Auger electrons. *Advanced Drug Delivery Reviews*, 109:102–118, January 2017.
- [135] Larry Bodgi, Aurélien Canet, Laurent Pujo-Menjouet, Annick Lesne, Jean-Marc Victor, and Nicolas Foray. Mathematical models of radiation action on living cells: From the target theory to the modern approaches. A historical and critical review. *Journal of Theoretical Biology*, 394:93–101, April 2016.
- [136] Franz Buchegger, Florence Perillo-Adamer, Yves M. Dupertuis, and Angelika Bischof Delaloye. Auger radiation targeted into DNA: a therapy perspective. *European Journal of Nuclear Medicine and Molecular Imaging*, 33(11):1352–1363, November 2006. WOS:000241522600016.
- [137] Jorgen Elgqvist, Oskar Vilhelmsson Timmermand, Erik Larsson, and Sven-Erik Strand. Radiosensitivity of Prostate Cancer Cell Lines for Irradiation from Beta Particle-emitting Radionuclide ^{177}Lu Compared to Alpha Particles and Gamma Rays. *Anticancer Research*, 36:103–110, 2016.
- [138] Louise Emmett, Kathy Willowson, John Violet, Jane Shin, Ashley Blanksby, and Jonathan Lee. Lutetium-177-PSMA radionuclide therapy for men with prostate cancer: a review of the current literature and discussion of practical aspects of therapy. *Journal of Medical Radiation Sciences*, 64(1):52–60, March 2017.
- [139] Wolfgang P. Fendler, Svenja Reinhardt, Harun Ilhan, Andreas Delker, Guido Böning, Franz J. Gildehaus, Christian Stief, Peter Bartenstein, Christian Gratzke, Sebastian

- Lehner, and Axel Rominger. Preliminary experience with dosimetry, response and patient reported outcome after 177Lu-PSMA-617 therapy for metastatic castration-resistant prostate cancer. *Oncotarget*, 8(2), January 2017.
- [140] Gefei Chen, Zhonglin Lu, Han Jiang, Ali Afshar-Oromieh, Axel Rominger, Kuangyu Shi, and Greta S. P. Mok. Lu-177-PSMA dosimetry for kidneys and tumors based on SPECT images at two imaging time points. *Frontiers in Medicine*, 10:1246881, November 2023.
- [141] Catherine Meyer, Laszlo Szidonya, Celeste Winters, Anna Mench, Nadine Mallak, and Erik Mittra. Quantitative imaging for 177Lu-PSMA treatment response monitoring and dosimetry. *Frontiers in Nuclear Medicine*, 3:1291253, December 2023.
- [142] A S Nelson, D Mirando, A Kruzer, R Niman, N M Cole, and T Stork. Dosimetry for Targeted Molecular Radiotherapy. *MIM White Paper*, 2023.
- [143] Dale L. Bailey, Kathy P. Willowson, Matthew Harris, Colin Biggin, Alireza Aslani, Nigel A. Lengkeek, Jon Stoner, M. Enid Eslick, Harry Marquis, Michelle Parker, Paul J. Roach, and Geoffrey P. Schembri. ^{64}Cu Treatment Planning and ^{67}Cu Therapy with Radiolabeled [$^{64}\text{Cu}/^{67}\text{Cu}$]MeCOSar-Octreotate in Subjects with Unresectable Multifocal Meningioma: Initial Results for Human Imaging, Safety, Biodistribution, and Radiation Dosimetry. *Journal of Nuclear Medicine*, 64(5):704–710, May 2023.