Application of the Linear-Quadratic Model to Targeted Radionuclide Therapy

A thesis submitted to the University of London for the degree of Doctor of Philosophy

by

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Declaration

This report is a result of the independent work of Iain Murray. I certify that this report does not incorporate without the acknowledgement, any material previously submitted for a degree or diploma in any university, and that to the best of my knowledge and belief it does not contain any material previously published or written by another person where due reference is not made clear.
Abstract

Title: Application of the Linear Quadratic Model to Targeted Radionuclide Therapy

The principal aim of this work was to test the hypothesis that the Linear-Quadratic (LQ) model of cell survival, developed for external beam radiotherapy (EBRT), could be extended to targeted radionuclide therapy (TRT) in order to predict dose-response relationships. The secondary aim was to establish the relevance of particular radiobiological phenomena to TRT and relate these results to any deviations from the response predicted by the LQ Model.

Methods: Cancer cell lines were treated with either EBRT or an in-vitro model of TRT. Dosimetry for the TRT was calculated using radiation transport simulations with the Monte Carlo PENELOPE code. Clonogenic as well as functional biological assays were used to assess cell response.

Results: Accurate dosimetry for in-vitro exposures of cell cultures to radioactivity was established. LQ parameters of cell survival were established for cancer cell lines reported to be prone to apoptosis, low dose hypersensitivity (LDH) or the bystander effect.

For apoptotic cells and cells exhibiting a bystander effect in response to EBRT, LQ parameters were found to be predictive of cell response to TRT. Apoptosis was not found to be a mode of cell death more specific to TRT than to EBRT. Bystander effects could not be demonstrated in cells exposed to TRT. Exposure to low doses of radiation may even protect against the bystander effect.

The LQ model was not predictive of cell response in cells previously shown to exhibit LDH. This led to a development of the LQ model based upon a threshold dose-rate for maximum repair. However, the current explanation of LDH may not explain the inverse dose-rate response.

Conclusion: The LQ model of cell survival to radiation has been shown to be largely predictive of response to low dose-rate irradiation. However, in cells displaying LDH, further adaptation of the model was required.
Acknowledgments

This thesis would not have been possible without the help and support of a number of people. First and foremost I would like to thank my supervisor, Professor Stephen Mather, not only for his help and advice but also for the opportunity in the first place.

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<th>Description</th>
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<tbody>
<tr>
<td>$^{111}$In</td>
<td>Indium-111</td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>Iodine 131</td>
</tr>
<tr>
<td>$^{137}$Cs</td>
<td>Caesium-137</td>
</tr>
<tr>
<td>$^{153}$Sm-EDTMP</td>
<td>Samarium-153-ethylene diamine tetramethylene phosphonate</td>
</tr>
<tr>
<td>$^{186}$Re-HEDP</td>
<td>Rhenium-186 hydroxyethylidene diphosphonate</td>
</tr>
<tr>
<td>$^{188}$Re-HEDP</td>
<td>Rhenium-188 hydroxyethylidene diphosphonate</td>
</tr>
<tr>
<td>$^{210}$Po</td>
<td>Polonium-210</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>$^{89}$SrCl</td>
<td>Strontium-89 Chloride</td>
</tr>
<tr>
<td>$^{90}$Y</td>
<td>Yttrium-90</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BED</td>
<td>Biologically Effective Dose</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CHK2</td>
<td>Human Checkpoint Kinase 2</td>
</tr>
<tr>
<td>CLDR</td>
<td>Continuously irradiating low dose-rate</td>
</tr>
<tr>
<td>CR</td>
<td>Complete Remission</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>D</td>
<td>Absorbed Dose</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA dependent protein kinase</td>
</tr>
<tr>
<td>DOTA</td>
<td>Tetraazacyclododecanetetraacetic</td>
</tr>
<tr>
<td>DTC</td>
<td>Differentiated Thyroid Carcinoma</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriamine-penta-acetic Acid</td>
</tr>
<tr>
<td>E</td>
<td>Energy</td>
</tr>
<tr>
<td>EBRT</td>
<td>External Beam Radiotherapy Treatment</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGSNRC</td>
<td>Electron Gamma Showers (National Research Council of Canada)</td>
</tr>
<tr>
<td>EUD</td>
<td>Equivalent Uniform Dose</td>
</tr>
<tr>
<td>eV</td>
<td>Electron Volt</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>GEANT</td>
<td>GEometry ANd Tracking</td>
</tr>
<tr>
<td>GJC</td>
<td>Gap junctional intercellular communication</td>
</tr>
<tr>
<td>Gy</td>
<td>Grays</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>ICRP</td>
<td>International Commission on Radiation Protection</td>
</tr>
<tr>
<td>IG2A</td>
<td>Immunoglobulin 2A</td>
</tr>
<tr>
<td>IR</td>
<td>Induced Repair</td>
</tr>
<tr>
<td>J</td>
<td>Joules</td>
</tr>
<tr>
<td>LDH</td>
<td>Low Dose Hypersensitivity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LET</td>
<td>Linear Energy Transfer</td>
</tr>
<tr>
<td>LQ</td>
<td>Linear Quadratic</td>
</tr>
<tr>
<td>m</td>
<td>Mass</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated Protein Kinase</td>
</tr>
<tr>
<td>MCNP</td>
<td>Monte Carlo N-Particle Transport Code</td>
</tr>
<tr>
<td>MDR</td>
<td>Monoexponentially decaying radiation</td>
</tr>
<tr>
<td>MIBG</td>
<td>Metaiodobenzylguanidine</td>
</tr>
<tr>
<td>MIRD</td>
<td>Medical Internal Radiation Dose</td>
</tr>
<tr>
<td>MR</td>
<td>Minor Response</td>
</tr>
<tr>
<td>NAT</td>
<td>Noradrenalin Transporter</td>
</tr>
<tr>
<td>NET</td>
<td>Neuroendocrine Tumour</td>
</tr>
<tr>
<td>NIS</td>
<td>Sodium Iodide Symporter</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP Ribose Polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Solution</td>
</tr>
<tr>
<td>PD</td>
<td>Progressive Disease</td>
</tr>
<tr>
<td>PENELOPE</td>
<td>PENetration and Energy Loss of Positrons and Electrons</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PK13</td>
<td>Protein Kinase 13</td>
</tr>
<tr>
<td>Poly-HEMA</td>
<td>Poly-hydroxyethyl-methacrylate</td>
</tr>
<tr>
<td>PR</td>
<td>Partial Remission</td>
</tr>
<tr>
<td>RBE</td>
<td>Relative Biological Efficiency</td>
</tr>
<tr>
<td>RE</td>
<td>Relative Effectiveness</td>
</tr>
<tr>
<td>RIT</td>
<td>Radioimmunotherapy</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SD</td>
<td>Stable Disease</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Suplhte</td>
</tr>
<tr>
<td>SF</td>
<td>Surviving Fraction</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single Photon Emission Computed Tomography</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>TRT</td>
<td>Targeted Radionuclide Therapy</td>
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1 Introduction

The aim of this work was to analyse the biological response of tumour cell lines that have been irradiated by prolonged low-dose rate beta irradiation as used in targeted radionuclide therapy (TRT).

There are a number of alternative methods of delivering a localised toxic dose of radiation in the treatment of cancer patients. Firstly, a focussed dose of radiation can be delivered using overlapping beams of radiation delivered from external sources such as linear accelerators. This is the most common form of radiation delivery in oncology and will be referred to as External Beam Radiotherapy Treatment (EBRT). Secondly, brachytherapy techniques may be used, whereby a localised radiation dose is delivered by inserting (either temporarily or permanently) a sealed radioactive source in situ within the patient. The third option is to deliver multi-site doses of radiation using TRT.

Although it is also possible to directly inject a radiopharmaceutical into a single target, the principle of TRT is that a radiopharmaceutical is incorporated or bound onto a biological target or targets, and delivers a localised prolonged dose of ionising radiation to each target. It is hoped that, due to specific binding of the radiopharmaceutical to the target only, other parts of the body are not targeted in the same way and that therefore unwanted radiation dose to those areas is avoided. One of the major advantages of this technique is that by relying on a biological process for localising the radiation source, it may be possible to effectively treat metastatic disease. This is not the case with EBRT or brachytherapy treatments.

Hence the aim of the therapy is that clinically significant doses of radiation can be delivered to multiple sites of metastatic disease, without the limiting side-effects that can occur with other systemic treatments such as chemotherapy.
1.1 TRT in the Treatment of Cancer

1.1.1 Radioiodine and Differentiated Thyroid Cancer
The use of radionuclide therapy as it is understood today, whereby an unsealed source is incorporated into a radiopharmaceutical that “seeks out” its biological target, dates back to the 1940s when the first treatments using iodine-131($^{131}$I) were carried out on patients with thyrotoxicosis, a benign thyroid disease [1]. $^{131}$I remains widely used today in the treatment of thyrotoxicosis and has also become established as a routine tool used in the clinical management of differentiated thyroid carcinoma (DTC). Iodide is taken up in both papillary and follicular carcinoma cells via the sodium / iodide symporter (NIS). Following diagnosis and subsequent thyroidectomy, $^{131}$I is used to target and destroy any remaining thyroid cells, including any sites of metastases.

Overall long term survival rates for thyroid cancer are close to 90% [2, 3]. In the case of follicular thyroid cancer, 10 year survival is 85% whilst for papillary thyroid cancer it is 93% [4]. Although this relatively high survival rate is also due to improved diagnostic and surgical techniques, radionuclide therapy for differentiated thyroid carcinoma using $^{131}$I is widely perceived as a highly successful form of cancer therapy [5, 6].

1.1.2 TRT in Treatment of Neuroendocrine Tumours
More recently, radionuclide therapy has established a role in the treatment of neuroendocrine tumours (NETs). Neuroendocrine cells are specialised hormone producing nerve cells that occur within the hormone secreting set of organs known as the endocrine system. Again $^{131}$I was the first radionuclide to be used in this field, in the context of radiolabelled metaiodobenzylguanidine (MIBG). In this case, the $^{131}$I is incorporated into the MIBG molecule which is an analogue of noradrenalin and is actively taken up by NETs via the Noradrenalin Transporter (NAT).

$^{131}$I-MIBG has been used therapeutically since the 1980s in the treatment of neuroblastoma [7,8], malignant phaeochromocytomas [9] as well as other NETs. However in contrast with the success of radioiodine for treatment of metastatic DTC, the use of $^{131}$I-MIBG has generally resulted in a palliative
effect only and is not seen as a treatment that will result in a complete response [10, 11, 12].

More recently, NETs have also been imaged and treated using radiolabelled peptides that bind to specific somatostatin receptor targets which are overexpressed on the surface of the NET cancer cells. As an administered drug, native somatostatin has a relatively short half-life in blood. Consequently, a number of more stable somatostatin analogues have been developed and radiolabelled [13].

Initial therapeutic treatments were carried out using $^{111}$In-DTPA Octreotide, on the assumption that the radiopharmaceutical was internalised within the cell and that Auger electrons (see section 1.2), emitted as part of the $^{111}$In radioactive decay were responsible for any therapeutic effect. Further developments in radiopharmacy resulted in the labelling of the octreotide analogue with yttrium-90 ($^{90}$Y), a pure beta emitter. The use of $^{90}$Y is a logical development, since the lack of a gamma emission should in theory allow a higher radiation dose to be delivered to the tumour burden whilst reducing the radiation dose to the rest of the patient. More recent advances have included the development of a new analogue octreotate, labelled with the beta emitting lutetium-177 ($^{177}$Lu) [14].

A review of peptide therapy using somatostatin analogues by Kwekkeboom et al showed the following range of responses to such radionuclide therapies [15]. As with $^{131}$I-MIBG, complete responses are a rare outcome.

<table>
<thead>
<tr>
<th>Radiopharmaceutical</th>
<th>Patients</th>
<th>%CR</th>
<th>%PR</th>
<th>%MR</th>
<th>%SD</th>
<th>%PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{111}$In-DTPA Octreotide</td>
<td>26</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>42</td>
<td>38</td>
</tr>
<tr>
<td>$^{111}$In-DTPA Octreotide</td>
<td>26</td>
<td>0</td>
<td>2</td>
<td>NA</td>
<td>81</td>
<td>12</td>
</tr>
<tr>
<td>$^{90}$Y-DOTA Octreotide</td>
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<td>0</td>
<td>6</td>
<td>NA</td>
<td>52</td>
<td>19</td>
</tr>
<tr>
<td>$^{90}$Y-DOTA Octreotide</td>
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<td>4</td>
<td>15</td>
<td>NA</td>
<td>65</td>
<td>11</td>
</tr>
<tr>
<td>$^{90}$Y-DOTA Octreotide</td>
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<td>6</td>
<td>17</td>
<td>NA</td>
<td>57</td>
<td>9</td>
</tr>
<tr>
<td>$^{90}$Y-DOTA Octreotide</td>
<td>54</td>
<td>0</td>
<td>7</td>
<td>13</td>
<td>61</td>
<td>19</td>
</tr>
<tr>
<td>$^{177}$Lu-DOTA Octreotide</td>
<td>76</td>
<td>1</td>
<td>29</td>
<td>12</td>
<td>39</td>
<td>18</td>
</tr>
</tbody>
</table>

CR: Complete Remission PR: Partial Remission MR: Minor Response SD: Stable Disease PD: Progressive Disease
1.1.3 Radio-immunotherapy
Radiolabelled somatostatin analogues are an example of using peptide receptors as a biological target. An alternative targeting mechanism is the use of radio-labelled antibodies that are associated with specific antigens produced by a particular cancer type.

As with peptide therapy, the first use of this technique was in cancer imaging rather than therapeutic applications [16]. However, during the past thirty years the technique has been developed culminating in the approval of two commercially available radioimmunotherapy (RIT) agents for the treatment of Non-Hodgkin's Lymphoma – $^{90}$Y-ibritumomab (Zevalin; IDEC Pharmaceuticals Corporation) and $^{131}$I-tositumomab (Bexxar; Corixa and GlaxoSmithKline Corporations).

In a randomised control trial, patients were treated with either a combination of Zevalin and the unlabelled antibody, or the cold antibody alone. An overall response rate of ~80% was demonstrated in patients receiving Zevalin compared to an overall response rate of 56% for the group treated with cold antibody only [17]. Complete responses were observed in 30% of Zevalin treated patients compared to 15% of non-Zevalin treated patients. Similar results have been observed with Bexxar [18].

1.1.4 Palliative Treatment of Bone Metastases
Another example of TRT that has become a routine clinical tool is the palliative treatment of multiple sites of bone metastases, primarily in breast, myeloma and prostate cancer patients [19]. A variety of different radiopharmaceuticals exist, such as $^{89}$SrCl$_2$, $^{186}$Re-HEDP, $^{188}$Re-HEDP and $^{153}$Sm-EDTMP which all target bone metastases. Despite covering a wide spectrum of radiation emissions and half-lives, no definitive advantage of one over another has been established.

1.1.5 Novel Developments in TRT
The examples of TRT described have become routinely applied tools in the treatment of various cancers. Although the lack of complete responses, particularly in the case of NETs, can be viewed as disappointing, TRT options are often considered as the most effective treatments available in a particular setting. Therefore research into new potential targets continues, whilst a
number of methods for enhancing the use of current radiopharmaceuticals are also under evaluation.

Advances in gene therapies mean that it is now possible to transfec a cell in order that it expresses receptors or targets that would not normally occur. This approach has also been used in combination with radionuclide therapy by transfecting malignant cells, with a transgene encoding the noradrenalin transporter (NAT) such that non-NET cells will take up $^{131}$I-MIBG. This approach has been applied to both gliomas [20, 21] and adenocarcinoma of the prostate [22]. However, it is worth noting that to date, this approach has only been used on an in-vitro basis.

Consideration has also been given to carrying out TRT in combination with other therapeutic modalities such as external beam radiotherapy [23, 24] or chemotherapy [25]. The hypothesis of such studies is that the therapeutic effect of combining treatment is greater than the sum of the benefits when such treatments are carried out individually. Again, it should be noted that clinical validation of such schemes has not yet been established.
1.2 Treatment Planning and Dosimetry in TRT

1.2.1 Absorbed Dose
Ionising radiation is a form of energy which, when delivered to a target volume, results in the deposition of some or all of that energy within that target. The fundamental measure of the energy deposited is the absorbed dose, defined as the energy per unit mass.

\[ D = \frac{E}{m} \]

Equation 1.1

E is the energy measured in Joules, m is the mass in kg and D is measured in Grays (Gy). Alternatively absorbed dose may be expressed in eV/g.

1.2.2 Radionuclide Emissions
In the context of radiation therapy, including both EBRT and brachytherapy, a number of different types of radiation emissions have been used or are under investigation.

**Alpha Particles:**

\[ zX \rightarrow (z-2)Y + 2^+He \]

Equation 1.2

An alpha particle is a helium nucleus consisting of two neutrons and two protons ejected from the unstable parent nucleus. The kinetic energy of such particles may range from 4 to 9MeV. Typical ranges in tissue are 10^{-6} m.

**Beta Particles:**

\[ zX \rightarrow (z+1)Y + e^{-} \]

Equation 1.3

Beta particles are negatively charged electrons and are emitted with a spectrum of kinetic energy, the maximum energy of which depends on the radionuclide. As these electrons interact with the material through which they travel, they cause ionisations, leading in turn to further electrons. The majority of radionuclides used for TRT are beta emitters.
**Gamma Rays:**
Gamma ray photons are non-particulate emissions with much longer ranges in human tissue and are consequently not desired in TRT, although high energy photon beams (typically 6MeV) are used in external beam radiotherapy. To date, the bulk of radiobiology research has concerned the use of such photon beams.

**Electron Capture, Internal Conversion and Auger Electrons:**
Energy is not always dissipated by ejecting particles from the atomic nucleus. In a process known as electron capture, proton rich nuclei may absorb an electron from those orbiting around the nucleus, resulting in conversion of a proton to a neutron and emission of a neutrino.

**Figure 1.1 Schematic of characteristic X-ray / auger electron production**

Internal conversion is a process whereby the energy of an excited nucleus is transferred to an orbital electron which is subsequently ejected from the atom. Thus the electrons may have a similar energy to beta emissions but are distinguished by their origin from the orbital rather than the nucleus.
Both electron capture and internal conversion lead to a vacancy in the electron orbit. Higher valence orbital electrons will move to fill the vacancy, thus moving from a higher energetic state to a lower state. The difference in energy is generally released in the form of an X-ray, but a proportion of this energy may be transferred to other orbiting electrons which are subsequently emitted from the atom (see figure 1.1). Such electrons are called Auger electrons and have a much lower energy spectrum than beta emissions. These energies are usually of the order of eV and consequently have much shorter ranges in tissue than beta particles. They have been used to some extent in TRT, e.g. in the case of $^{111}$In-somatostatin analogs, tumour toxicity is due to Auger emissions.

**1.2.3 Linear Energy Transfer and Cross-Fire**

The Linear Energy Transfer (LET) is a measure of the energy transferred to a medium per unit path length by ionising radiation particles. Alpha particles and Auger electrons are classified as high-LET particles, whilst beta particles are classified as low-LET particles.

Despite the low-LET of their emissions, the principal radionuclides used at present for TRT are all beta emitters. Although the energy imparted across the whole path length of the particle is relatively low, the energy is not distributed evenly along the path. As the beta particle loses energy, the probability of interaction with the tissue medium in which it travels increases, and hence the pathway of the particle will end in a cluster of ionisation events. In addition, there is also the possibility that excited orbital electrons will be released with sufficient energy to cause further ionisation events themselves.

This deposition of energy may be simulated using Monte Carlo radiation transport codes, as illustrated in figure 1.2. Simulated interactions for a beam of 978keV electrons representative of Low LET beta particles, and a beam of 500eV electrons representing High LET Auger electrons are shown.

Although the ranges of the Auger electrons are much lower, the high density distribution of the high LET radiation means that, for a given dose of radiation the energy deposition is more concentrated within the cell and that therefore
high LET radiation is considered to have a higher relative biological efficiency (RBE) than that of low LET radiation.

Figure 1.2 PENELOPE simulation of electron shower for A) 978keV monoenergetic electrons, illustrative of $^{90}$Y emissions, LET = 0.26keV/µm and B) 500eV monoenergetic electrons, illustrating Auger electrons emitted by $^{125}$I, LET = 25keV/µm

In fact, when a cluster of cells is considered, it is likely that any response to ionising radiation from a particular cell will be due to a radiation particle originating from a radionuclide attached to another cell a particular distance
away, rather than any radionuclide attached to the affected cell. This effect is termed cross-fire and is considered to be one of the advantages of using beta irradiation.

1.2.4 Treatment Planning
As previously stated, the aim of any radiotherapy is to deliver a high dose of radiation to a particular target or set of targets, whilst delivering minimal doses to the rest of the body. When a patient receives external beam radiotherapy, it is standard clinical practice to prescribe a particular dose of radiation to the tumour volume, and to subsequently plan the way in which this treatment can be delivered. This relies on a well established understanding of the behaviour of radiation beams in human tissue and a sophisticated methodology for calculating the dose delivered throughout the patient.

In the case of TRT, the standard clinical practice that has evolved, is to prescribe all patients a fixed amount of radioactivity at regular intervals (typically every 6 months). Routinely little, if any, consideration is given to calculating the absorbed dose to either the tumour volumes or to critical organs. This may be explained by the technically challenging nature of such dosimetric calculations, coupled with the high success rate of these empirical protocols using radioiodine in DTC.

However, in order to maximise the potential of other radiopharmaceuticals, there has been a renewed interest in dosimetry and treatment planning in TRT. Firstly, establishing dosimetry of critical organs, such as bone marrow in the case of $^{131}$I-MIBG, or the kidneys in the case of $^{90}$Y-Octreotate, has been used to establish the maximum level of radioactivity that can be safely administered on an individual patient basis [26, 27]. Furthermore there is also a desire to calculate the radiation dose to the tumour, and attempt to relate this to a likely outcome and prognosis [28].

For EBRT, the absorbed dose is determined by considering the position and intensity of the various radiation beams directed through a patient. In the case of TRT, the absorbed dose is a combination of the amount of radioactivity initially administered to the patient as well as the biodistribution and biokinetics...
of the radiopharmaceutical within that patient. Hence, the challenge of TRT dosimetry is firstly to quantify the distribution of radioactivity, as a function of both space and time, and secondly to use this data to calculate the absorbed dose to a particular target. A number of different methods exist to meet these aims and are discussed below.

1.2.5 Medical Internal Radiation Dose (MIRD) Internal Dosimetry
Initial assessments of internal radiation dosimetry from radiopharmaceuticals date back to the 1960s when assessments were made of exposures from radiopharmaceuticals designed for diagnostic use. Recall from equation 1.1 that the total absorbed dose to a target is the sum of the ionising radiation energy absorbed within that target, divided by its mass. Since the absorbed energy originates from the radionuclide emissions, it is first necessary to calculate the total number of decays over the treatment time considered. This is referred to as the cumulative activity and is defined as

\[ \tilde{A} = \int_0^T A dt \]

Equation 1.4

\( T \) is the treatment time and \( A \) is the radioactivity at time \( t \).

Under the Medical Internal Radiation Dose (MIRD) system [29, 30], a “reference man” was constructed from a series of phantoms that could be used to represent the different organs of the body filled with varying concentrations of a particular radionuclide. The principle of MIRD is to simulate radioactivity in one organ at a time and to calculate the absorbed dose to all other organs in the body, as well as to the source organ itself. Since absorbed dose is defined as the energy absorbed per unit mass, the dose from any organ acting as a source of radioactivity to a given target (which may or may not be the source) is given as

\[ D_{T\leftarrow S} = \frac{\tilde{A} \times \Delta \times \phi_{T\leftarrow S}}{m} \]

Equation 1.5
where $\Delta$ is the average energy emitted by the radionuclide, $\phi$ is the fraction of energy absorbed in terms of a specific target and organ pair and $m$ is the mass of the target organ.

It is common to group these parameters into a single factor. Thus, in the equation below, the $S$-factor accounts for the average energy of the various radionuclide emissions in the source organ, and the fraction of that energy absorbed by a particular target organ.

$$D = S \times A$$

Equation 1.6

By repeating this exercise for all organs, it is possible to build a look-up table linking the absorbed dose to all organs within the body, with the cumulative activity measured for each organ.

Although widely adopted for dosimetry of diagnostic radiopharmaceuticals, the method suffers from several disadvantages when applied to a patient undergoing TRT.

The physical distribution of an individual patient is not precisely represented by an average “reference man”, both in terms of the size of the various organs and the distance between them. Both of these factors affect the accuracy of the derived $S$-factors.

In addition, the model also suffers from the assumption that organs have homogenous uptake of the radionuclide. In the case of a cancer patient with a metastatic disease burden this is often not the case and indeed it is not possible to define a tumour within the context of “reference man”.

1.2.6 Voxel Based Internal Dosimetry with Point Kernels

When using MIRD, time-activity curves for various organs would typically be measured using a sequence of 2D planar images. However, modern methods of imaging, using either Single Photon Emission Computed Tomography (SPECT) or Positron Emission Tomography (PET), mean that accurately quantifying the distribution of a radionuclide in 3D is increasingly possible.

These developments in imaging technology have led to more accurate and appropriate techniques for treatment planning of TRT patients. The space-time
distribution of the radiopharmaceutical is measured in four dimensions using sequential PET or SPECT images, using either a positron or gamma emitting version of the radiopharmaceutical. The physical distribution of tumour and organs within the patient is established through the use of a CT scan. By registering the sequential images, it is then possible to calculate the cumulative activity on a voxel by voxel basis, rather than considering the whole organ. Thus these scans provide a basis for calculating the dose distribution (see figure 1.3). Although sets of S-factors relating the cumulative activity in one voxel to the absorbed dose in all voxels are available, an alternative technique known as dose point kernel convolution may also be used. A dose point kernel is a function describing the dose distribution around a unit activity, located in a single voxel. By convolving this function (defined as a function of the spatial parameter $\rho$) with the distribution of cumulative activity (defined as a function of the spatial parameter $r$) a patient specific absorbed dose distribution is derived.

\[
D(r) = \int_{-\infty}^{\infty} \tilde{A}(r - \rho) d(\rho) d\rho
\]

Equation 1.7 Convolution of cumulative activity with dose point kernel

The disadvantage of this technique is the fact the dose point kernel is normally simulated in water. Hence, the convolution technique will not take account of significant tissue inhomogeneities such as bone or lung tissue.
1.2.7 Monte Carlo Radiation Transport Simulation

The deposition of energy by ionising radiation is a result of a series of energy losses by the radiation particles emitted. The laws of physics, which govern these interactions are precisely known, and probability distributions of particular outcomes may be calculated for given input data: i.e. given an initial energy, direction and a description of the material through which it moves, it is possible to a) simulate the potential path of a single particle and all the secondary particles to which it gives rise, and b) track the deposition of energy within that material. For a single particle, this is a stochastic process for which repeating the simulation is unlikely to provide the same answer. If however, this simulation is repeated for millions of particles then it is possible to build up an accurate and precise measure of the macroscopic pattern of interactions and dosimetry that would occur in a simulated situation.

Such Monte Carlo simulations find widespread use in dosimetry and are used to provide data for MIRD S-factors, as well as generating the point dose kernels described above. Furthermore it is possible to overcome the disadvantage of the point dose kernel technique regarding inhomogeneities, by including a CT scan of the patient in the Monte Carlo simulation. The distribution of the radiopharmaceutical is provided by either a SPECT or PET scan.

In addition the use of radiation transport codes can be extended to the microscopic scale, in order to assess dosimetry at the cellular level. In the various models used to consider the dosimetry of TRT, an assumption is often made of a uniform distribution of radiopharmaceutical, whether that is on the macroscopic scale in the case of the MIRD system for organ dosimetry, or at the scale of voxel based dosimetry still of the order of several millimetres. However, it is known that at the cellular level this will not be the case, even with tumours that appear uniform on imaging [31]. It is expected that this will have a significant effect on the cellular dose distribution and subsequent response of the cell population, particularly in the case of low range emissions such as Alpha particles or Auger electrons [32, 33].
1.2.8 Dose-Response Relationships in Clinical TRT

The emergence of these ever more sophisticated dosimetry techniques has led to a number of evaluations of the correlation between dose and response. Early assessments of dose-response in patients treated for differentiated thyroid carcinoma, suggested an absorbed dose of 300Gy was necessary for successful ablation [34, 35]. More recently, Flux et al showed that successful ablation could occur at much lower doses [36]. Although they showed a statistically significant difference in the absorbed dose to the thyroid remnant between successful and unsuccessful treatments, there remained a substantial overlap between the failed ablations (7-49Gy) and complete eradication of the thyroid (12-470Gy). Thus absorbed dose alone was shown not to predict treatment outcome.

Analysis of patient outcome in the context of treating Non-Hodgkin’s Lymphoma with $^{131}$I-tositumomab (Bexxar) showed no correlation with tumour dose [37]. However, a significant correlation was found by Dewaraja et al (in the same clinical setting) when they calculated Equivalent Uniform Dose (see 1.3.5) instead of absorbed dose only [38].

It is not just important to establish dose-response relationships in tumours. It is often more important to be able to predict the tolerance in critical organs at risk from the administered radioactivity. For example, in radiopeptide therapy, it is usually the kidneys which are the dose-limiting organ. A review of 18 patients by Barone et al showed no correlation between kidney absorbed dose and renal toxicity. When Biologically Effective Doses (see 1.3.5) were calculated to take account of differences in dose-rate a much clearer relationship was observed ($r=0.93$) [39].

The outcome of these results overall, has been the recognition that absorbed dose alone may not predict response to treatment, and that in future phase III trials designed to appraise the potential benefits of dosimetry and treatment planning, radiobiological modelling appropriate to TRT should be utilised [40, 41].
1.3 Radiobiology for TRT
Despite the advances made in internal dosimetry, the actual dose necessary to elicit a favourable response in a patient is not well known [42]. For example, investigations into palliative treatment of bone metastases have shown no correlation between dose and response [43-45]. The vast majority of radiobiology research has been carried out at dose-rates typical of external beam radiotherapy, given over minutes as opposed to the days that can be taken to deliver a TRT dose. In order to further improve the design of trials of new therapeutic approaches to TRT, it is essential to have an accurate description of the energy deposition both within tumour and normal cells. However, that information must be complemented by an understanding of the radiobiological responses relating to such methods of radiation dose delivery.

1.3.1 Interactions of radiation with cells and DNA
The interaction of radiation with cells can be considered as either direct or indirect. In the first situation the radiation directly causes the ionisation of a particular atom or group of atoms that make up part of a biological target, e.g. DNA. The ionisation may initiate a chemical change that may in turn lead to a biological change.

In the case of indirect interaction, the radiation will ionise other atoms or molecules within a cell (water being the most likely example) leading to the creation of highly reactive free radical species. Although free radicals are short lived, they may react with the biological target, thus leading to a biological change.

Although ionisation events will occur throughout the cell, the most sensitive target when irradiating cells has long been established as the DNA within the cell nucleus. For example, short range high LET alpha particles emitted by polonium-210 ($^{210}$Po) were shown to be lethal at a dose of 1Gy when localised to the nucleus. When localised to the cell membrane, a dose of 250Gy was required to reduce the rate of cell growth [46]. The damage that can occur to DNA ranges from single and double strand breaks in the backbone of DNA,
damage to the base subunits of DNA, to cross-links between the DNA strands as well as DNA and nuclear proteins. Single strand breaks are breaks in the DNA chain and are thought to occur as a result of free radical attacks on the chemical bonds along the DNA chain. A double strand break describes a similar situation, although the break has occurred on both opposing chains. These latter are considered to be more lethal and harder to repair since a single strand break may be repaired with reference to the corresponding complementary base. It is single and double strand breaks that are traditionally thought to have the greatest correlation with cell death.

1.3.2 Response of Cells to Radiation
Free radicals within the cell do not occur solely as a result of irradiation and are in fact an everyday occurrence. It is estimated that between 10,000 and 150,000 oxidative lesions are produced each day in each cell, as a result of reactive oxygen species (ROS) produced as by-products of common cellular processes [47]. Consequently a number of DNA repair processes normally exist and these will also be activated in response to ionising radiation. The response of cells in dealing with damaged DNA may be divided into three components: Recognition of damage, a period of damage assessment, followed by either repair or cell death [48].

Initial DNA damage results in the increased activity of proteins such as ataxia telangiectasia mutated (ATM) and DNA dependent protein kinase (DNA-PK) As damage is “detected” via these mechanisms a number of proteins may be subsequently activated in order to promote repair of the cell DNA. It is common to observe cell cycle arrest following irradiation and a number of different checkpoints have been described [49].

Under normal circumstances, cells may move through the cell cycle as illustrated in figure 1.4. During mitosis (M phase) a cell will divide to produce two daughter cells. The remaining three phases are collectively known as interphase. The S phase is the phase during which DNA transcription activities occur. G1 and G2 are so called gap phases which occur between S phase and M phase as shown.
In order to progress from phase to another, a particular set of proteins known as cyclins must form complexes with enzymes called cyclin-dependent kinases (CDKs). The diagram below illustrates the pathways initiated by ATM that can lead to cell cycle arrest in response to DNA damage.

As can be seen, ATM activates the human checkpoint kinase 2 (Chk2) which in turn phosphorylates Cdc25A and Cdc25C. These proteins are necessary in order to activate the CDK1 kinase associated with the G2/M transition. Thus their phosphorylation renders them inactive and therefore blocks the cell cycle.
ATM also activates the p-53 protein, which will in turn activate a further protein p-21. These will bind to and therefore inactivate the cyclin-E-CDK2 complex that governs the passage from G1 to S phase. The subsequent delay in cell cycle progression allows a period of DNA repair to proceed, either by homologous recombination whereby the corresponding section of the sister chromatid is used as a template for repair, or by non-homologous end-joining in which repair is carried out independently. Following cell cycle checkpoints, any unrepaired cells may either proceed with cell division (mitosis) or undergo programmed cell death (apoptosis). If a cell undergoes mitosis without having made necessary repairs and still contains DNA breaks or chromosome aberrations, then that cell will normally undergo a passive form of cell death termed necrosis. Necrotic cells are characterised by loss of cell membrane integrity, cell swelling, and random fragmentation of DNA. Apoptotic cells on the other hand, are observed to have more ordered DNA fragmentation and break down into small apoptotic bodies. Necrotic cell death is thought to be more likely following exposure to higher radiation doses, whereas apoptosis has been associated with lower levels of radiation dose [50] [51].

1.3.3 Clonogenic Cell Survival
A further alternative to both necrosis and apoptosis is accelerated senescence, whereby a cell does not die but does lose its ability to divide. It is important to note that the relevant end-point, when considering TRT of cancer cells, is not necessarily cell death but whether the irradiated cells retain the ability to reproduce. As such, it is common to characterise response to different levels of irradiation through the use of a clonogenic assay, in which individual cells are seeded throughout a culture medium and incubated. After a certain period of time, colonies of cells are counted and compared to the number of colonies formed by a non-irradiated negative control sample.

1.3.4 The Linear Quadratic Model
In describing the clonogenic response of cells to radiation, a number of different models have been proposed. Of these, the Linear Quadratic model
has become the most well established as a radiobiological model in external beam therapy, principally due to its ease of use and its successful fit to the experimental data [47, 52].

The surviving fraction (SF) of cells after a single instantaneous exposure to ionising radiation, resulting in an absorbed dose D, is predicted by the expression:

\[ SF = e^{-(\alpha D + \beta D^2)} \]

where \( \alpha \) and \( \beta \) are tissue specific parameters. The \( \alpha \) term has been linked conceptually to the creation of double strand breaks by a single ionisation event whilst the \( \beta \) term has been linked to the creation of a double strand break caused by adjacent single strand breaks, arising from two unrelated ionisation events. As the dose is increased, the probability of the second type of event increases and hence the quadratic term in the equation becomes more important. A typical cell survival curve is shown in the figure below \((\alpha=0.2, \beta=0.02)\).
Figure 1.6 Clonogenic Survival Curve

Such curves are plotted on a log-linear scale and tend to be characterised by a linear shoulder before the curve bends away.

In the context of EBRT, one of the main reasons that the LQ model has become particularly popular is the way in which it predicts the outcome of fractionating the delivery of radiotherapy. Fractionation involves the splitting of the delivery of the total dose into a number of smaller doses, delivered at regular intervals (normally 24 hours). The LQ model in its most simple form assumes that sub-lethal damage is repaired between fractions such that when the next fraction is delivered the damage is independent of any previous radiation events. For example if a dose of 20Gy was delivered in 4 fractions of 5Gy each, the survival curve for the cell line in the figure above would appear as shown in figure 1.7. Effectively the portion of the surviving fraction curve up to 5Gy is replicated 4 times, resulting in a less effective treatment. The LQ model predicts this effect according to the equation

$$SF = \left(\exp\left(-\alpha d + \beta d^2\right)\right)^n = \exp\left(-\alpha nd + \beta nd^2\right)$$

Equation 1.9

where $n$ is the number of fractions and $d$ is the dose per fraction.
Figure 1.7 The Effect of Dose Fractionation

The reason for applying this apparently less effective treatment is seen by considering the effect of radiation on normal tissues. These tissues tend to be characterised by a broader shoulder, such that the effect of fractionating the dose delivery is to spare normal tissues from unwanted side effects.
Figure 1.8 Dose Sparing Effect. Survival curves are shown for A) Acute delivery of radiation to malignant tissue ($\alpha=0.5$, $\beta=0.05$) and normal tissue ($\alpha=0.1$, $\beta=0.33$). B) Fractionated ($n=20$) delivery of radiation to the same hypothetical tissues.

This model for fractionating dose delivery has also been extrapolated by Dale to the modelling of dose-rate effects [104]. An increasingly lower dose-rate and protracted time for dose delivery can be considered equivalent to delivering the radiation dose in a number of fractions with an increasingly large value of $n$ and decreasing value of $d$.

1.3.5 Radiobiological Dosimetry
As the previous section makes clear, the measure of total absorbed dose alone does not provide an indication of outcome without being placed into context. For example, dose-rate or fractionation scheme, tumour radiosensitivity, as
well as dose distribution within a tumour, will all play a part in determining the response to radiation.

Therefore a number of further parameters have been defined which seek to aid comparison of alternative treatment plans. The first of these is the Biologically Effective Dose (BED) which is defined by the following equation.

\[ BED = -\ln(SF) / \alpha \]

Equation 1.10

The BED which has units of Gy is a conceptual term. It represents the dose that would be required if treatment was delivered such that there was complete repair of damage associated with the $\beta$ parameter, to match the effect of the treatment under consideration. This allows comparison of alternative treatment regimes including TRT.

The LQ model provides a means of calculating the surviving fraction of a collection of cells, assumed to have the same radiosensitivity. It can be seen that the higher the number of cells present, the higher the probability that some will survive. The Tumour Control Probability (TCP) is a concept that accounts for this factor and is defined as follows [24, 53]. Since complete tumour eradication is a rare outcome in TRT, the relevance of this parameter to this treatment modality is perhaps debatable.

\[ TCP = \exp(-N_0 \times SF) \]
\[ = \exp(-N_0 \exp(-\alpha BED)) \]

Equation 1.11

The Equivalent Uniform Dose (EUD) is a concept in considering the effectiveness of treatments in which absorbed dose is not evenly distributed over a target volume. It is defined as the BED which, if distributed uniformly over the same volume, would result in the same Surviving Fraction [54]. Although, it was originally defined as a concept to be applied to Intensity Modulated Radiotherapy (IMRT), it has nonetheless been applied by others to treatments with TRT [38].

\[ EUD = -\frac{1}{\alpha} \ln(SF(\alpha)) \]

Equation 1.12
1.3.6 Dose-rate, Apoptosis and TRT
There is a sizeable opinion expressed in the literature that models developed for EBRT may not translate directly to TRT where doses are delivered at dose-rates several orders of magnitude lower than is the case in EBRT.

Apoptosis, also known as “programmed cell death” was originally described by Kerr et al in 1972 [55]. Apoptotic response is characterised morphologically by an ordered set of changes such as cell shrinkage, the cleaving of DNA into regular subdivisions and the breakdown of the cell into smaller membrane bound apoptotic bodies.

A wide variety of pathways have been described that lead to a cell undergoing apoptosis. Essentially these can be subdivided into “intrinsic” pathways, where the process is initially triggered within the cell, or extrinsic pathways triggered by receptors in the cell membrane.

As has been described, apoptosis is a mechanism for cell death thought to be more predominant at lower doses in EBRT. Since TRT is a low dose-rate treatment, the hypothesis that apoptosis is the predominant pathway for cell death in this modality has been widely discussed and reported in the literature [27, 56, 57]. One group who measured apoptosis in gamma and beta-irradiated HL60 leukaemia cells, using both FACS analysis and Western Blot techniques, found that comparable levels of apoptosis were observed when either technique was used [58]. However, their results also suggested a higher rate of apoptosis when the dose-rate used to deliver the beta-irradiated dose was lowered. Another group also confirmed the induction of apoptosis in a HeLa Hep2 cell line using a low dose-rate gamma source as a surrogate for TRT treatment [59].

1.3.7 Hypersensitivity and Increased Radioresistance
A further phenomenon observed during in-vitro experiments on EBRT, is that of low dose hypersensitivity (LDH). When certain cell lines are exposed to acute radiation doses of the order of ~10cGy, clonogenic assays demonstrate a surviving fraction lower than that which would be predicted using the LQ
model. As the dose is increased, the surviving fraction then increases (i.e. radioresistance appears to increase) before then falling away as would be anticipated by the LQ model [60]. This hypersensitivity has been explained by low doses failing to trigger repair mechanisms that would normally repair DNA damage, and in particular a failure of cell cycle checkpoints preventing damaged cells passing into mitosis [61]. This has led to further investigations into the effect of dose-rate upon cell survival in the context of TRT. When cell lines that had been demonstrated to show LDH behaviour in response to acute doses of gamma radiation were exposed to protracted dose deliveries of the order of 10-100 cGy/hour, an inverse dose-rate effect was observed whereby lower surviving fractions resulted from lower dose-rate treatments [62]. In contrast, cell lines that did not demonstrate LDH failed to show this effect.

1.3.8 Alternative Targets / Bystander Effect
Although the cell nucleus of a cell is seen as the principal target in irradiation, evidence has accrued within the last twenty years for other mechanisms leading to cell death.

A number of responses have been classified as non-targeted responses [63] whereby biological effects are not thought to be specifically related to DNA damage. One of the most interesting of these has been the bystander effect. The bystander effect is a phenomenon whereby cells that have not been directly irradiated, may undergo the same response as neighbouring cells that have been irradiated. Clinical evidence for the bystander effect dates back as far as the 1950s [64] when plasma transferred from irradiated patients resulted in chromosome breakages in human lymphocytes. Further in-vitro evidence was demonstrated by irradiating cells in culture, spinning down the suspension and transferring the media to unirradiated cell culture, which then demonstrated a response. More recently, groups have used radiation micro-beams to target individual cells with alpha particles and then observed responses in neighbouring cells. Although interest in the bystander effect has been predominant amongst the radiation protection community, its role in the context of TRT has also been the subject of investigation. Boyd et al, experimenting with human glioma and bladder carcinoma cells, found that
when using gamma irradiation the cell killing effect of medium from irradiated cells reached a plateau after ~2Gy radiation dose. On the other hand, the cell killing effect of media from $^{131}$I-MIBG $\beta$ irradiated cells continued to increase as the dose administered was increased. In the case of alpha irradiation, the bystander effect was observed to reach a peak at ~5Gy before the effect then diminished as the dose was increased [65].
1.4 Aims and Objectives
The principal aim of this work was to test the hypothesis that the Linear Quadratic model developed and routinely applied to external beam radiotherapy can be extended to targeted radionuclide therapy.

In order to assess dose-response relationships, it was first necessary to establish and validate an accurate dosimetry technique that would provide the link between the amount of radioactivity to which cell cultures were exposed and the resulting absorbed dose. To this end, chapter 3 is a description of the use of the PENELOPE (PENetration and Energy Loss of Positrons and Electrons) Monte Carlo algorithm to meet these requirements.

The subsequent chapters make use of this dosimetry to investigate the dose response of both haematological and solid tumour cell cultures. In each instance, cell lines were first treated with different doses of single fraction EBRT in order to characterise the $\alpha$ and $\beta$ parameters describing cell survival with respect to the Linear Quadratic model. The same cell lines were then exposed to low dose-rate irradiation using $^{90}\text{Y}$. Measured cell survival was compared to the survival predicted using Dale’s version of the LQ model developed specifically for a monoexponentially decaying rate of irradiation [104].

In chapter 4 this approach is taken with two leukaemia cells lines, HL60 and MOLT-4. In addition, the hypothesis that cancer cells are more likely to enter apoptosis when treated with low dose-rate irradiation rather than EBRT was investigated.

In chapter 5, the application of the LQ model to cells displaying Low Dose Hypersensitivity was investigated. The first aim of this chapter was to explore modifications to the LQ model that could predict any observed increase in sensitivity to lower dose-rate treatment. Furthermore, an investigation was also made of the hypothesis that the biological explanation applied to Low Dose Hypersensitivity was the underlying mechanism to any observed increase in the sensitivity of cells to low dose-rate irradiation.

Chapter 6 concentrates on the response of cells which are able to effect a bystander response when treated with EBRT. Media transfer experiments were used to investigate whether such responses could be elicited when cells were
treated with low dose-rate irradiation, as well as whether the LQ model was an appropriate tool in predicting cell survival.
2 Materials and Methods

2.1 Materials

2.1.1 Reagents

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<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
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<tr>
<td>0.25% (w/v) Trypsin – 0.53mM EDTA solution</td>
<td>Cambrex</td>
</tr>
<tr>
<td>111InCl</td>
<td>Covidien</td>
</tr>
<tr>
<td>1M EDTA</td>
<td>BLT Pharmacy</td>
</tr>
<tr>
<td>3% Methycellulose in Iscove’s Modified Dulbecco’s Medium</td>
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<tr>
<td>59YCl</td>
<td>Perkin Elmer</td>
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<td>Ammonium Acetate</td>
<td>Prepared In House</td>
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<td>Abcam</td>
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<td>ATM-p1981 antibody</td>
<td>Cell Signalling</td>
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<td>BT-474</td>
<td>Barts Cancer Institute</td>
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<td>Promega</td>
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<td>Invitrogen</td>
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<td>BLT Chemotherapy Unit</td>
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<td>BDH Laboratory</td>
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<td>Mouse Secondary</td>
<td>Clare Hall, Cancer Research UK</td>
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<td>PBS</td>
<td>Dako</td>
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<td>Barts Cancer Institute</td>
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<td>Trypan Blue</td>
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<td>YAML568</td>
<td>Sigma-Aldrich</td>
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<td></td>
<td>Therapeutic Antibody Centre (Oxford)</td>
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### 2.1.2 Equipment

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<td>LKB Wallac</td>
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<tr>
<td>Allegra 21R centrifuge</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>Chromatography Column</td>
<td>Biorad</td>
</tr>
<tr>
<td>Cylcone plus storage phosphor system</td>
<td>Perkin Elmer</td>
</tr>
<tr>
<td>Developer G138i curix 60 processor</td>
<td>AGFA</td>
</tr>
<tr>
<td>Incubator</td>
<td>Binder</td>
</tr>
<tr>
<td>Microscope</td>
<td>Wilovert</td>
</tr>
<tr>
<td>Plate Reader</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>Radioisotope Calibrator</td>
<td>Capintec</td>
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<tr>
<td>IBL 637 Blood Irradiator</td>
<td>CIS Biointernational</td>
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### 2.1.3 Software

<table>
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<td>Graphpad Prism5 (Statistics)</td>
<td>Graphpad Inc</td>
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<tr>
<td>ImageJ</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>PENELOPE Monte Carlo</td>
<td>Nuclear Energy Agency</td>
</tr>
</tbody>
</table>
2.2 Cell Culture

2.2.1 Cell Lines
A number of different cell lines were used during this work. All cell lines were cultured in a 37°C incubator containing 5% CO₂. Non-adherent human HL60 acute promyelocytic leukaemia cells were grown in RPMI-1640 medium, supplemented with 10% Fetal Calf Serum (FCS). Human lymphoblastic MOLT-4 cells were also grown in identical conditions. Cells were split and media was replaced three times per week.

PC-3 human prostate adenocarcinoma cells were grown in RPMI-1640 (10% FCS) at 37°C in 5% CO₂. Cells were split using trypsin-EDTA 2-3 times per week. BT-474 breast cancer cells were grown in RPMI-1640 supplemented with 20% FCS at 37°C in 5% CO₂. Cells were split once per week, using trypsin-EDTA, although media was changed every 2-3 days. All cells were regularly tested for mycoplasma and were all also type tested to confirm their identity.

2.2.2 BT-474 Spheroid Formation
BT-474 cells were grown as spheroids, using a method based on that described by Ivascu and Kubbies [66]. The monolayer of cells was detached using trypsin-EDTA and resuspended in fresh media. Cells were pipetted to ensure clumps of cells were separated into single cells. The absolute cell concentration was determined using a haemocytometer and the Trypan Blue assay. Cell numbers ranging from 1,000 to 20,000 per well were added to round bottomed 96 well plates, depending on the size of spheroid required. The 96 well plates had been precoated with 80μl 0.5% poly-hydroxyethylmethacrylate (Poly-HEMA) dissolved in 95% ethanol, and air dried at 37°C for 48 hours. The purpose of the Poly-HEMA was to prevent cells adhering to the well plates. Spheroid formation was encouraged by initially centrifuging the plates at 400 rpm for 8 minutes before leaving the plates for 24 hours at 37°C in 5% CO₂.
Spheroid diameters were measured by pipetting the formed spheroid onto a haemocytometer. Spheroids of 1,000 cells were found to have a diameter of 375µm.

### 2.3 External Beam Irradiation

Single Fraction EBRT was delivered using an IBL 637 blood irradiator (CIS Biointernational) containing a caesium-137 ($^{137}$Cs) source. The irradiator was made available by Cancer Research UK, who also maintained it with a regular program of Quality Control.

Samples were placed in a shielded chamber. When the machine was activated the chamber became exposed to the $^{137}$Cs source with an activity of the order of TBq resulting in a dose-rate of approximately 4Gy/min. Absorbed doses to samples were controlled by presetting an appropriate exposure time.

All cell cultures exposed to EBRT in this way were aliquoted in 10ml samples into 25cm$^2$ flasks and placed at the centre of the irradiation chamber in a position of known dose-rate.

In addition to making dosimetric calculations based upon the known dose-rate in the chamber, absorbed doses were verified using an instant read-out dosimeter.

### 2.4 Targeted Radionuclide Therapy Irradiation

Three alternative radiopharmaceuticals were used to provide low dose-rate irradiation to cell cultures. In the first instance, simulation of low dose-rate TRT was provided by $^{90}$Y- Ethylenediaminetetraacetic acid (EDTA). This provided a means of creating a uniformly radioactive media due to the lack of specific uptake by cells. This provided a mode of irradiation which could be modelled by Monte Carlo methods as described more fully in chapter 3. This radiopharmaceutical was used to investigate dose-response relationships in apoptotic cell lines (chapter 4), hypersensitive cell lines (chapter 5) and in BT-474 cells (chapter 6).

In the case of the BT-474 cell line used in chapter 6, a means of targeting the cells specifically was also required. The BT-474 cells are known to express the
HER-2 receptor [67] which is the target of the commercial immunotherapy Trastuzumab (Herceptin). Therefore Herceptin was labelled with $^{90}$Y.

For the purposes of bystander effect experiments with BT-474, a radiopharmaceutical was sought that could subsequently be extracted from cell culture media. Therefore $^{90}$Y was also labelled to the YAML568 antibody, thus providing a means of non-specific irradiation that could subsequently be separated from the media (unlike $^{90}$Y-EDTA).

### 2.4.1 $^{90}$Y-EDTA Labelling

$^{90}$Y was provided in the chemical form of $^{90}$YCl$_3$ (Perkin Elmer) in 102$\mu$l 0.05M HCl. $^{90}$Y-EDTA was produced as follows. 100$\mu$l 0.1M EDTA was added to 900$\mu$l 0.1M ammonium acetate. An equal volume of the buffered EDTA was then added to an equal volume of $^{90}$YCl, depending on the amount of radioactivity required.

### 2.4.2 $^{90}$Y-DOTA-Herceptin Labelling and Binding Assay

Expired clinical grade Herceptin, with a concentration of 25mg/ml, was obtained from the Chemotherapy unit of Barts and the London NHS Trust. The Herceptin was tested for aggregation or contamination using High Pressure Liquid Chromatography (HPLC). This showed a single peak at a molecular weight of 150kDa indicating no aggregation or contamination of the antibody.

In order to radiolabel Herceptin with $^{90}$Y, tetraazacyclododecanetetraacetic acid (DOTA) was used as a chelator, to link the Herceptin antibody to the yttrium metal ions. DOTA molecules were conjugated with the Herceptin as outlined by Cooper et al [68]. The conjugated antibody was aliquoted into 60x12ml aliquots, each containing 50$\mu$g of Herceptin, and stored at -20°C.

Conjugated DOTA-Herceptin aliquots were labelled with radioactivity as follows:- 1$\mu$l of 0.1M ammonium acetate was added to the volume of $^{90}$YCl$_3$ required (generally 10$\mu$l, equivalent to 20MBq) in an Eppendorf tube. The purpose of the ammonium acetate was to maintain a pH of 5.5 during the labelling reaction. 2 x 10$\mu$l of the DOTA-Herceptin was then added, such that the total volume within the Eppendorf was no more than 30$\mu$l. If the combined volume of the above was less than 30$\mu$l, the difference was made up using
sterile water for injection. The reaction mixture was incubated at 37°C for 4 hours.

Radiolabelling was verified using Thin Layer Chromatography (TLC) on a routine basis. Briefly, 0.1M ammonium acetate with 50mM EDTA solvent was added to a 50ml Falcon tube to a depth of 0.5-1cm. A 1µl sample of the radiolabelled Herceptin was dropped onto a strip of Whatman paper, such that when the strip was placed vertically in the Falcon tube, the site of the sample was just above the level of the solvent. After the solvent front had risen along the strip to its far end, the strip was removed and left to dry. The dried strip was placed on a piece of radiosensitive film (within a cassette) for approximately 1 minute. The film was then imaged on a phosphor imager and the resulting images analysed to identify the proportion of activity that had not moved along the silica strip with the solvent. This activity was assumed to be the radiolabelled antibody.

Labelling was initially tested using $^{111}$InCl$_3$ instead of $^{90}$YCl$_3$. In the case of $^{111}$In, labelling efficiencies (i.e. the proportion of radioactivity bound to Herceptin) of 88% were measured after 1 hour incubation (using TLC), rising to 93% after 4 hours. This compared to an HPLC measurement of labelling efficiency of 91% after 4 hours. When the procedure was repeated using $^{90}$Y, labelling efficiencies of >95% were achieved at 4 hours, as measured using TLC. Before carrying out dose-response experiments, a binding assay was carried out to ensure specific binding between $^{90}$Y-DOTA-Herceptin and the BT-474 cell line.

For the binding assay, cells were prepared by harvesting half a large flask of cells and centrifuging at 1000rpm. The cells were then washed with 0.5% albumin in Phosphate Buffer Solution (PBS) and resuspended in a volume of 4ml. A 1ml sample of this cell culture was added to an Eppendorf tube and then serially diluted into further tubes, such that 5 x 0.5ml aliquots of cell culture were produced with relative concentrations of 1: 2: 4: 8: 16. A further 0.5ml sample of the undiluted cell culture was added to a tube labelled as “non-specific binding”. A 50µl sample of 2mg/ml Herceptin was added to this sample in order to saturate all the HER2 binding sites. Meanwhile, the radiolabelled DOTA-Herceptin was diluted to a concentration of 50ng/ml. 250µl
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Aliquots of the antibody were added to all cell preparations as well as to an empty Eppendorf tube. The purpose of this tube was to provide a measure of the maximum potential radioactivity that could bind to the cells. All of the above preparations were carried out in duplicate.

The Eppendorf tubes were placed in 50ml Falcon tubes and left on rollers for 2 hours to allow any binding of the radiolabelled antibody to the cells. After 2 hours cell samples were spun down and the pellets washed with 0.5% albumin PBS. The pellets were then assayed for radioactivity in a gamma counter alongside the tubes representative of the total activity added to each cell sample.

![Graph A](image1)

![Graph B](image2)

Figure 2.1 Binding Assay results for A) In-111 DOTA-Herceptin and B) Y-90 DOTA Herceptin with BT-474 HER2 expressing breast cancer cell line
As figure 2.1 shows, there was clearly specific binding of both $^{111}$In-DOTA-Herceptin and $^{90}$Y-DOTA-Herceptin to the BT-474 cell line. In the case of $^{90}$Y-DOTA-Herceptin absolute measurements of the radioactivity of the cell pellets were made by using a Gamma Counter to compare the counts from the samples with those from a standard derived from the original $^{90}$YCl$_3$ of known concentration. These were combined with measurements of cell number made using a haemocytometer in order to produce figure 2.2. The results suggested that a monolayer of BT-474 cells in a 6 well plate, would not take up the amounts of radioactivity required for the dose-response experiments.

![Figure 2.2 Binding of Y90-DOTA-Herceptin to BT-474 cells in terms of absolute levels of radioactivity and cell number](image)

Therefore attempts were made to increase the specific activity of the $^{90}$Y-DOTA-Herceptin by decreasing the ratio of DOTA-Herceptin (by volume) relative to the $^{90}$YCl$_3$ during the labelling reaction. The original ratio used was 2:1 as described above. The effect of reducing this ratio was to rapidly decrease the labelling efficiency achieved (see Table 2.1). Therefore, it was decided to maintain the ratio of 2:1 for all dose-response experiments. In any case, a wide variation in cell binding was noted during the course of several dose-response experiments. The reason for this is potentially
explained by variations in specific activity / impurity content for different production runs of the $^{90}$YCl$_3$.

Table 2.1 Labelling Efficiency of $^{90}$Y-DOTA-Herceptin

<table>
<thead>
<tr>
<th>Ratio of DOTA-Herceptin to $^{90}$YCl$_3$ (by volume)</th>
<th>Labelling Efficiency after 1 hr</th>
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<tbody>
<tr>
<td>2:1</td>
<td>91.0%</td>
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<tr>
<td>1:2.5</td>
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<td>64.9%</td>
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<tr>
<td>1:25</td>
<td>0%</td>
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</table>

2.4.3 $^{90}$Y-DTPA-YAML568 Labelling and Binding Assay

For the purpose of bystander effect experiments, a radiolabelled antibody that would not bind to BT-474 cells, and could subsequently be extracted from the cell culture media in which irradiated cells were incubated, was required. YAML568 is a rat IG2A monoclonal antibody which specifically binds to the CD45 antigen. This antigen was not thought to be expressed by the BT-474 cells (YAML568 was developed to target malignant cells in patients with acute myeloid leukemia prior to undergoing stem cell transplantation [58].)

A stock of already conjugated DTPA-YAML568 (4mg/ml) was already available within the laboratory at the Barts Cancer Institute. The labelling was carried out in a similar way to the labelling of $^{90}$Y-DOTA-Herceptin. 1µl of 0.1M ammonium acetate was added to 20µl of $^{90}$YCl$_3$, (40MBq) in an Eppendorf tube. Two test labellings were carried out – in the first case 10µl DTPA-YAML568 was added to the radioactivity and in the second case 40µl DTPA-YAML568 was added. Radiolabelling was assessed as described in chapter 2.4.2 using TLC. After 4 hours, labelling efficiency was found to be 90% and 95% respectively for the labellings described above.

A binding assay was carried out (as described in chapter 2.4.2) in order to confirm that CD45 was not expressed by BT-474 cells, and that the radiolabelled antibody would not bind to the cells. This was indeed confirmed to be the case. (See figure 2.3.)
2.4.4 Media Transfer Experiments
In order to explore a bystander response in cell lines exposed to external beam irradiation, the following protocol was used. Donor cell cultures were exposed to $^{137}$Cs irradiation as previously described. Following irradiation, cells were further incubated for a period of 24 hours. After this time, suspension cell cultures were spun at 1000rpm for five minutes. The resulting supernatant was then removed and added to a recipient cell culture of the same cell type, such that 50% of the total cell media in the recipient cell culture was derived from the irradiated cell culture.

In the case of adherent cell cultures, the entire cell culture volume was aspirated from the recipient cell cultures and replaced with the cell culture taken from the donor cells. The donor cells were subsequently discarded. Following media transfer, all recipient cell cultures were incubated for a further 24 hours prior to clonogenic assay.

For treatments using TRT, (namely $^{90}$Y-DOTA-Herceptin or $^{90}$Y-DTPA-YAML568) it was necessary to extract the radioactivity from the cell culture media. A method based on that used for purifying antibodies was used, namely Protein A or Protein L affinity chromatography. Protein A and Protein L are immunoglobulin binding proteins which specifically bind to particular antibody
isotypes. Protein A was required to extract $^{90}$Y-DOTA-Herceptin whilst Protein L was required to extract $^{90}$Y-DTPA- YAML568.

Single use chromatography columns were loaded with 0.4ml Protein A/L beads in 70% ethanol (GE Healthcare). This column was then washed 5 times with 10% FCS RPMI media in order to remove the ethanol, before loading the column with the 2.5ml of cell culture volume used to irradiate plated BT-474 cells. This media was collected separately from the media used for washing the column, and repeatedly passed through the column in order to remove the radiolabelled antibody. All of the above was carried out in a laminar flow cabinet, so as to maintain sterility. Relative measurements of the radioactivity of the Protein A/L column and the filtered cell culture media were made using a radionuclide calibrator.

Complete extraction of the radioactivity was not possible in either case. For both Protein A / $^{90}$Y-DOTA-Herceptin and Protein L / $^{90}$Y-DTPA-YAML568, the amount of radioactivity removed from the media saturated at 85%. (See figure 2.4.)

![Graph showing extraction of $^{90}$Y-DTPA-YAML568 from RPMI media using Protein L Column](image-url)

Figure 2.4 Extraction of Y90-DTPA-YAML568 from RPMI media using Protein L Column
2.5 Internal Dosimetry Calculations

For all investigations of response to low dose-rate irradiation, it was necessary to establish a link between the radioactivity added to the cell cultures and the resulting absorbed dose to the cells. Unlike the case of the external beam treatments with $^{137}$Cs, it was not possible to establish in-vitro measuring techniques that could verify the absorbed dose at the cellular level. Therefore the dose-response investigations presented in chapters 4-6 make use of calculations of absorbed dose which took the following form.

$$D = S \times \tilde{A}$$

$$= S \times \frac{A_0}{\lambda} \left(1 - e^{-\lambda T}\right)$$

Equation 2.1

$\tilde{A}$ is the cumulative activity, i.e. the total number of atomic emissions over the period of time $T$ arising from an initial activity $A_0$ of a radionuclide with a half-life of $\ln(2)/\lambda$. $S$ is the factor which relates $\tilde{A}$ to the delivered Dose. The $S$-value accounts for the energy of the emissions as well as the particular geometry of the situation under consideration. $S$-values are available for establishing the absorbed dose to various organs of the body from radioactivity within those organs, as well as for establishing the absorbed dose to homogenously filled spheres of radioactivity, or even to individual cells. However, appropriate $S$-values were not available for the type of in-vitro experiments described in this thesis.

Therefore the use of a Monte Carlo code was investigated for calculating the absorbed dose to the cells treated with the radiopharmaceuticals described in the preceding section.

Table 2.2 summarises key properties of some of the Monte Carlo codes more frequently applied to Nuclear Medicine. The class refers to the degree to which every possible event is simulated – in the context of microdosimetry, class II codes treat knock-on electrons as individual events, whereas in class I codes these are grouped.
Materials and Methods

Table 2.2 Key Properties of Several Monte Carlo Codes relevant to Nuclear Medicine

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<th>Code</th>
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<th>Energy Range</th>
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<td>1KEV to 1TEV</td>
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<td>GEANT</td>
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<td>II</td>
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<td>PENELOPE 2006</td>
<td>$\beta, \gamma$</td>
<td>100EVS to 1GEV</td>
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</tr>
<tr>
<td>MCNP</td>
<td>$N, \beta, \gamma$</td>
<td>&gt; 1KEV</td>
<td>I</td>
</tr>
</tbody>
</table>

It was decided to make use of PENELOPE (Penetration and Energy Loss of Photons and Electrons) for all dosimetric calculations. The reasons for this principally related to the lower energy range simulated by the code, as well as the fact that the system proved to be relatively straight forward to set up on a single desktop PC (3GHz Pentium 4 processor, 1GB RAM). For all simulations of beta emitting radionuclides, energy spectra were taken from the data used in the publication of ICRP report 38 and made available by the Oak Ridge Laboratory [69].

A full appraisal of the application of PENELOPE to measuring the distribution of absorbed dose and establishing relevant S-values for use with equation 2.1 is presented in chapter 3. However, table 2.3 provides an illustration of the established relationship between radioactivity and absorbed dose.

Table 2.3 Absorbed Dose (Gy) to cells in 6 well plate incubated in 2.5ml RPMI containing $^{90}$Y-EDTA

<table>
<thead>
<tr>
<th>Concentration / MBq/ml</th>
<th>Time after treatment / hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
</tr>
<tr>
<td>0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>0.4</td>
<td>2.4</td>
</tr>
<tr>
<td>1.1</td>
<td>6.6</td>
</tr>
<tr>
<td>2.2</td>
<td>13.2</td>
</tr>
</tbody>
</table>
2.6 Measuring Response to Irradiation

A number of different techniques were used to assess the response of cells to both EBRT and TRT. For the purposes of cell survival studies, the clonogenic assay was used to measure cell survival. Assays of adenosine triphosphate (ATP) were used to provide a measure of temporal changes in cell viability following treatment.

Functional responses to irradiation were also investigated. A simple luminescence based assay was used to measure apoptosis via the detection of caspase effector proteins that form part of the apoptotic pathway. Specific molecular pathways were investigated through the use of Western Blot techniques.

2.6.1 Clonogenic Survival Assay

The clonogenic assay was used as an objective measure of cell survival. The assay is based upon observing the ability of single cells to reproduce and replicate to the point that a small colony of viable cells is formed. Following irradiation, both treated aliquots of cells and a negative untreated control aliquot of cells (in triplicate) were serially diluted into 6 well plates, such that cells were seeded at $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$ concentrations. After a period of time (depending on the cell line in question) these well plates were assessed for the formation of viable colonies. Colonies were counted relative to the negative control sample. If the formation of colonies was not observed for treated aliquots at the same concentration of cell seeding as that seen for the negative controls, then the relative difference in seeding dilution was taken into account.

\[
\text{Surviving Fraction} = \frac{n^0_{-colonies_{\text{treated cells}}}}{n^0_{-colonies_{\text{negative control cells}}}} \times \frac{\text{seed dilution}_{\text{negative control cells}}}{\text{seed dilution}_{\text{treated cells}}}
\]

Equation 2.2

Two methods for carrying out this assay were used, depending on the nature of the cells. In the case of adherent cell lines (e.g. BT-474, PC-3), colonies were assessed as follows.
Well plates were placed on ice and the contents washed twice with Phosphate Buffer Solution (PBS). Cells were then fixed by adding enough methanol (kept at -20°C) to cover the well plate surfaces and left for 10 minutes. After 10 minutes, the methanol was aspirated and replaced with a 0.5% Crystal Violet solution (in 25% methanol) and left for a further 10 minutes. The well plates were then rinsed carefully until no colour ran off in solution and left to dry. Images of the stained colonies in the well plates were scanned and subsequently counted using the ImageJ software.

In order to support the development of suspension cell colonies (e.g. HL60 and MOLT-4) a viscous medium consisting of 50% methylcellulose (3%) Iscove’s cell culture media (R&D Systems); 30% FCS; and 20% conditioned HL60 media, was used to incubate cells. After 2-3 weeks colonies were sufficiently well formed to allow visual counting.

![Example of Crystal Violet Stained PC-3 Prostate Adenocarcinoma Clonogenic Colonies grown from A) negative control cells and B) cells treated with 2Gy EBRT. In each case cells were seeded at 10^4 concentration relative to the treated cells](image)

### 2.6.2 ATP Viability Assay

Although clonogenic survival assays were used as an objective end point to measure cell death in response to irradiation, an alternative assay was used to observe the transient response to irradiation. The CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation) is a method of determining the
Materials and Methods

number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells.

The assay is sensitive enough to be carried out using 100μl samples of a cell culture mixed with 100 μl aliquots of the single reagent in 96-well plates. The effect of the reagent is to cause cell lysis, and the production of a luminescent signal proportional to the amount of ATP present, which is assumed to be directly proportional to the number of viable cells present in the sample. The assay uses a proprietary luciferase. The resultant light output signal has a half-life of approximately 5 hours, although for time course experiments measurements were made at a standard time of 20 minutes after mixing.

The assay was applied to irradiated cell cultures in order to observe changes in viability as a function of time, post treatment. Samples were tested prior to treatment initiation, 1 hour post treatment initiation and then every 24 hours. Samples were taken in duplicate.

2.6.3 Caspase-3/7 Apoptosis Assay

A similar technique to that described above was used in order to detect and measure apoptotic activity. Caspase-3 and caspase-7 are proteins that play an important effector role in apoptosis. Activation of caspase-3 in particular, occurs at the convergence of the intrinsic and extrinsic pathways [70]. The Caspase-Glo® 3/7 Assay (Promega) is an assay that detects these proteins using the luciferase reaction. A caspase-3/7 substrate in the reagent is cleaved following cell lysis, allowing a luciferase reaction to occur and thus provide a light signal that is proportional to the level of caspase-3/7. As with the ATP viability assay, duplicate 100 μl cell culture samples are mixed with equal volumes of the reagent in 96-well plates. The resulting light signal was observed to increase for a period of approximately 1 hour following the addition of the reagent, before falling off with a relatively long half-life. Hence all measures of luminescence were made 1 hour after mixing the cell culture and the Caspase-Glo® 3/7 reagent.
2.6.4 Western Blots
Western blotting is a technique which is used to identify specific proteins within a particular cell culture. Proteins are separated by size using electrophoresis and then transferred to a membrane. Proteins of interest are tagged using an antibody specific to that protein, which may then be detected by chemiluminescence methods.

Following incubation with \(^{90}\)Y-EDTA, the 6 well plates containing treated cells were placed on ice. The media was removed and the cells washed with ice cold PBS. After washing, the cells were detached from the well plates using a cell scraper and transferred to Eppendorf tubes. These were spun at 8,000 rpm for 10 minutes (4°C). The PBS was aspirated and replaced with 200\(\mu\)l cell lysis buffer containing 1% protease inhibitor. The tubes were then spun again at 12,000 rpm for 10 minutes (4°C) and the lysates transferred to further Eppendorfs. A luminescence based protein assay (Biorad) was used to assess the protein concentration of the lysates.

A stacking gel was created on top of a bottom gel, between glass plates. Polyacrylamide gels were created using the following formations. The bottom gel was created using 8ml protogel, 7.5ml resolving gel, 14.2ml distilled water, 300\(\mu\)l 10% ammonium persulphate and 30\(\mu\)l TEMED. The stacking gel was made up with 2.1ml protogel, 4ml stacking protogel, 9.8ml distilled water, 80\(\mu\)l 10% ammonium persulphate, 80\(\mu\)l Bromophenol blue and 16\(\mu\)l TEMED. Wells were created by pouring the stacking gel around a plastic comb, before leaving the gel to set.

Cell lysates were prepared for electrophoresis by adding 5\(\mu\)l loading buffer and heating at 95°C for 3 minutes in order to denature the protein. A volume of lysate equivalent to 20\(\mu\)g protein was loaded into each well whilst one well was set aside for a molecular weight marker. The gels held between glass plates were loaded into an electrophoresis tank containing a Sodium Dodecyl Suplhate (SDS) running buffer (Tris-Glycine) and run at 150V until the molecular weight markers were seen to have separated down the length of the gel (~1hr).

To transfer the proteins, a transfer buffer was made up from 200ml TRIS (no SDS) Glycine, 200ml methanol and 1600ml distilled water. The blotting
membrane was activated by placing in methanol for 30 seconds, and then placed in a tray containing a covering of transfer buffer. Two sheets of Whatman paper and sponge, cut to the size of the gels were also placed in the buffer, as were the gels themselves. The gel was then assembled next to the membrane, held between the sheets of Whatman paper, which were themselves held between the sponge. A 200mA current was applied to the assembly for 1hr in order to transfer the proteins from the gel to the membrane. Following transfer of protein, the membrane was incubated in 10ml 1-2% BSA in TBST for 40 minutes, in order to block non-specific uptake of the primary antibody. This was followed by overnight incubation at 4°C of the membrane in a TBST solution containing 0.3% BSA as well as the primary antibody of interest. The following day, the membrane was washed with TBST, before incubation with a secondary antibody, appropriate to the nature of the primary antibody. After further washes, ECL reagent (GE Healthcare) was applied to the membrane, before placing the membrane in contact with photographic film within a cassette. Films were kept in contact for standard periods of time in order to allow comparison between different membranes. Films were developed using an AGFA film developer.
3 Microdosimetry Using PENELOPE

The aim of this chapter is to appraise and describe the use of the PENELOPE Monte Carlo code as a means of providing accurate dosimetry in the context of the experiments described in later chapters.

Following a description of the implementation of PENELOPE used for these experiments (chapter 3.1), validation experiments are presented (chapter 3.2), both at the macroscopic and microscopic scale, in order to verify the techniques used to produce the findings presented later in this chapter.

Following validation, the PENELOPE code was used to model the absorbed dose to cells cultured in multi-well plates, both for the situation in which the cells are irradiated by a bound radiopharmaceutical and for the case of cells cultured in a radioactive media which does not result in specific uptake.

Results are primarily presented for the beta emitting isotopes $^{90}Y$ and $^{131}I$, taken to be representative of high and low energy beta emitters respectively. In chapter 3.3.2, relationships are defined between the absorbed dose to cells and both the amount and volume of radioactivity added to these cell cultures. These are presented as S-Factors in keeping with the MIRD convention.

In chapter 3.3.3, the relative influence of radioactivity in neighbouring wells is considered. Furthermore, the importance of modelling interactions with the Perspex labware itself is presented in chapter 3.3.4. Chapter 3.3.5 shows the results of an investigation on the effect that a non-confluent distribution of cells may have on the accuracy of the modelling of dosimetry.

In chapter 3.4 results are also presented for the modelling of radioactivity bound to the surface of tumour spheroids in order to support such investigations.
3.1 PENELROPE Workflow

3.1.1 Program Structure
The PENELROPE Monte Carlo program consists of a set of Fortran subroutines designed to simulate different aspects of potential photon / electron interactions [71]. Different subroutines address the simulation of different physical interactions including both elastic and inelastic scattering (different routines cater for photons and electrons) as well as bremsstrahlung radiation. Random sampling of the probability distributions is covered by further subroutines within this source code. In addition to this source code, a number of database files are supplied which allow for the generation of files detailing the physical properties of the geometry under consideration. In principle data can be generated for any arbitrary material although database files describing 280 different materials are supplied with the distribution.

The PENELROPE package also contains three driving programs which can be used to direct these subroutines and track the particle simulations in a meaningful way. The models used in the following chapter use the PENCYL program. This program is based around the definition of cylindrical geometries and allows both point source and distributed source arrangements. (The other programs PENSLAB and PENMAIN are restricted to point source arrangements only and are therefore not suitable for TRT models.) In order to run a simulation two input files must be set up by the user. Namely,

- Material Definition File
- Model Input File

Together, these two files define the specific model to be simulated and evaluated.

3.1.2 Material Definition File
An auxiliary program “material.exe” is included with the PENELROPE distribution. When run by the user, this program draws upon a database of
atomic interaction data to define a file containing all relevant information (such as cross-sections for particular radiation interactions) for each material specified. The resulting datafiles are then concatenated to produce a single file containing all necessary data for the simulation.

3.1.3 Model Input File
A model input file must be defined by the user. The principal components of this file are as follows:

- Model geometry
- Nature of source radiation (gamma / electron)
- Energy spectrum of source radiation
- Distribution of source radiation
- Specification of the material definition file to be used.
- Simulation parameters (e.g. cut-off energies)
- Specification of the geometric elements of interest.

Firstly, the geometry is defined by a set of cylinders or annuli which are assigned an axial thickness, inner and outer radius and a material. No overlap of these geometric elements is permitted.

The source radiation is defined by a number of parameters. First the nature of the radiation (photons, electrons or positrons) is specified. Therefore in order to simulate radionuclides that emit both electrons and photons it is necessary to run separate simulations.

The energy distribution of the photons/electrons is defined either as being monoenergetic or as a spectrum described in terms of energy bins and relative probability of emission for a given bin. The source position may be defined as a point with a given position. Alternatively any number of the defined geometrical elements may be assigned as being “radioactive”. If desired, the relative activity of these elements to each other may also be defined. Otherwise, the probability of emission is assumed to be equal between all assigned bodies.

The third part of the input file specifies the number of different materials involved in the simulation and points towards the specifically generated material file that contains all the necessary information for the simulation. Furthermore, a cut-off energy below which particles are no longer explicitly
tracked must be specified for each material. The remainder of the input file determines details of the way in which the output data are formatted for interpretation and analysis, and is further described in the next section.

### 3.1.4 Dosimetry Output

The purpose of running the PENELOPE Monte Carlo simulations was to derive S-factors that could be applied to specific in-vitro situations. Information regarding the deposition of energy within the geometric model is specified in a number of ways. Altogether, the PENCYL program produces a number of data files which describe the nature of the charge and energy distribution throughout the simulation in different ways. However, for the purposes of the model evaluation, the following output files were used most extensively and are described in more detail below.

- Pencyl output summary file (pencylres.dat)
- 3D absorbed dose distribution data (pc-dosc1.dat)
- Depth absorbed dose distribution (pc-ddose.dat)

The pencylres.dat file provides an overview of the energy deposition across the whole model. For each geometric cylinder or annulus defined in the model input file, the energy absorbed per primary particle is reported in eV. Furthermore a breakdown is provided of the number of primary particles simulated and the fate of those particles in terms of whether they are absorbed, transmitted or backscattered into the geometric element that is their source. Further data are provided on the nature and number of the simulated collisions of secondary particles. However, depending on the size of the geometric elements this may not necessarily provide information on a microscopic scale. The pc-dosc1.dat output files provided absorbed dose distributions (in eV/g) for specified bodies as a function of both the axial position (z) and the radius from the geometric centre (r). This data was provided in both a graphical form, as well as a tabulated form to allow further analysis of the data. Alternatively, the ddose.dat
files provided an axial absorbed dose profile averaged over the radial plane of the model throughout all geometric bodies.
3.2 Validation of PENELOPE Model

Radiation exposures made using the $^{137}$Cs blood irradiator were measured in situ in order to confirm that the expected absorbed dose was delivered. In the case of experiments simulating TRT, the microscopic scale of the geometries involved meant it was not possible to make in situ measurements in order to verify the prediction of absorbed dose to radio-labelled cells, or even to cells bathed in a radioactive media. Therefore two validation exercises were carried out whereby PENELOPE simulations were tested against reference datasets. The first validation exercise involved the testing of macroscopic models against reference datasets, whilst the second validation was carried out at the microscopic scale.

3.2.1 MIRD Sphere Self-Dose

Calculations have been made and published detailing the radiation dose absorbed by different sized homogenous spherical distributions of a number of radionuclides. These are specified in terms of S-Factors [72]. Therefore in order to verify the implementation of PENELOPE, a number of simulations were run. Each simulation consisted of a cylinder of water uniformly filled with radioactivity - either $^{90}$Y or $^{131}$I – surrounded by a volume of air. In order to match the spherical geometry as closely as possible, the diameter and height of the cylinders were kept equal. The mass of these cylinders were 0.02g; 0.1g; 0.8g; 20g; 100g; 400g; 800g; and 3kg. Simulations were performed using 10 million primary electrons. Particles were tracked down to a minimum energy of 1keV.

The dosimetry information written to the pencylres.dat files was expressed as the energy deposited per primary particle. A conversion to an S-Factor (mGy/MBq-s) was made as outlined in Appendix 1.

As shown in figures 3.1 these derived S-Factors compare favourably with the published MIRD data on self-dose to small uniform spheres and no significant difference was found between the MIRD datasets and the PENELOPE simulations for either $^{90}$Y or $^{131}$I ($r^2=0.99$).
Figure 3.1 Comparison of PENELOPE Dosimetry vs published MIRD Dosimetry of Uniformly Filled Spheres of Radioactivity for A) Y-90, B) I-131. Uncertainties are too small to be visualised.

The results of this validation indicate both a correct setup of the PENELOPE input files and subsequent interpretation of the PENELOPE data output. Initially, a closer correlation between the MIRD reference values and the
PENELOPE cylindrical models was observed for the $^{90}\text{Y}$ data compared to the $^{131}\text{I}$ data. However, it should be noted that the simulations of $^{131}\text{I}$ did not initially include the modelling of absorbed dose deposition from the photon emissions of this radionuclide. The significance of this omission becomes greater at the larger volumes considered (see figure 3.2). Inclusion of the photon component resolves the differences observed. The photon contribution becomes increasingly less significant at smaller volumes, a situation more relevant to the scale of in-vitro experiments. However, it is interesting to note that energy deposition from photon emissions is of the order of 10% for a volume of 20ml and could potentially need to be taken into account when deriving S-Factors for in-vitro experiments at larger scales.

![Figure 3.2 Relative Energy Deposition in Water Sphere Uniformly Filled with I-131](image)

The non-linear nature of the S-Factor reciprocal vs mass relationship for $^{90}\text{Y}$ is explained when consideration is given to the average energy absorbed by the water volume, per primary particle emitted. If all the beta particles emitted were absorbed within the source element of the model then this figure would simply be equivalent to the average energy of the beta spectrum simulated.
However, it can be conceived that emissions occurring near the edge of the volume would scatter or progress out of the source element and would therefore not be absorbed by the source itself. As the energy (and therefore the range) of the emissions increased, this would become a more significant effect.

As illustrated in figure 3.3, the higher energy $^{90}$Y beta emissions have a higher probability of escaping the source element than those of $^{131}$I as the volume considered is reduced.

![Figure 3.3 Energy Deposition as a function of Uniform Sphere Size](image-url)
3.2.2 Point Source Kernel

In order to validate the performance and correct interpretation of PENELOPE simulations at the microscopic scale, simulations were made of a point source of $^{90}$Y surrounded by water. A geometric model of a point source of $^{90}$Y surrounded 5cm of water in any direction was created. The absorbed dose deposited along the radial axis of the model was scored at intervals of 9μm between 0μm and 198μm and at intervals of 220μm from 198μm to 1cm (figure 3.4). (In order to score the energy deposition at these intervals two concentric cylinders were modelled, $r=0\rightarrow198\mu m$ and $r=198\rightarrow10,000\mu m$ respectively, surrounded by a third with radius 5cm). Data was obtained from the 3D absorbed dose distribution tabulation described in section 3.1.4.

As shown, the absorbed dose varies rapidly as a function of distance over several orders of magnitude.

Therefore, the normal convention when presenting dose point kernels is to make use of a dimensionless scaled dose kernel [73].
\[ j\left( \frac{r}{X_{90}}, E \right) = \frac{4\pi r^2 D(r, E) X_{90}}{E} \]

Equation 3.1 Scaled dose kernel

\( X_{90} \) is the distance at which 90% of the energy emitted has been absorbed by the surrounding material (5.4mm); \( E \) is the average energy of the beta emission spectrum (0.934MeV). The purpose of the scaled dose kernel is to facilitate comparison between other isotopes as well as monoenergetic electron sources. Figure 3.5 shows the scaled dose kernel obtained from the model described overlaid on reference data previously published for the same dose kernel [73].

Figure 3.5 Scaled Point Dose Kernel for Y-90 Derived Using PENELOPE

The point dose kernel derived from the PENELOPE data shows a close association with the EGSnrc data previously published. Minor deviations occur at the transition between the inner cylindrical body and the outer, as well as at the singularity where \( r = 0 \). Further comparisons with published dose point kernels are made in chapter 3.5.
The radial absorbed dose profile was also compared with an axial absorbed dose profile in order to check the isotropic performance of the PENELOPE code. This comparison is shown in figure 3.6 and demonstrates that absorbed dose profiles can be measured along either the z-axis or the radial axis of a given model, although there is less uncertainty associated with the radial profiles.

The purpose of the two validation experiments was primarily to give confidence in subsequent dosimetry models developed for the purpose of the dose-response experiments described in the following chapters, and specifically the way in which output from PENELOPE has been interpreted.

The validation of absorbed dose to uniform volumes of activity as described in 3.2.1 primarily provides confidence in the correct set up of the model input files, particularly with respect to the definition of the energy spectra, the description of the radioactivity deposition and the material specification.

The simulation of the point source provides further confidence in the simulation of the underlying physics as well as the way in which the output data are analysed and processed.
3.3 Dosimetry of Multi-Well Plates

For the purposes of the experiments described in the remainder of this thesis, S-Factors were required predominantly for 6 well plates. However, the dosimetry of standard 12, 24, 48 and 96 well plates is also considered. The aim of this section is to present the results of this work and moreover to investigate whether a universal set of S-Factors could be derived for in-vitro investigations of TRT that would avoid the need for future Monte Carlo modelling of each individual situation that could arise.

3.3.1 Modelling of Cell Culture Well Plates

As well as using $^{90}$Y-EDTA as a means of providing low dose-rate irradiation to cell cultures, it was also anticipated that experiments would be carried out using a radio-labelled antibody or peptide as a more realistic model of targeted radionuclide therapy. Therefore, two distinct situations were considered with respect to in-vitro cell culture experiments: the first was that of a monolayer of cells incubated with homogenously radioactive cell culture media; the second was that of a monolayer of cells incorporating a bound radiopharmaceutical incubated in non-radioactive cell culture media (See figure 3.7).

The materials.exe program was used to define PENELOPE material files for polystyrene (to model the cell culture flasks); air; water (for cells and cell culture media); and iron (to model the steel shelf of the incubator). These were then concatenated into a single file for use with these simulations. Both the situations outlined above were simulated for 6, 12, 24, 48 and 96 well plates. The dimensions used to describe the geometry are shown in table 3.1. In the case of a monolayer of cells incorporating a radiopharmaceutical, a finite dimension must be assigned to the height of the radioactive element of the model. Cells can vary in size from as small as 5$\mu$m diameter up to 100$\mu$m diameter. However, the majority of cells are of the order of 10$\mu$m diameter so this was used as the height of the radioactive layer. The thickness of the steel
shelf upon which the well plates were placed whilst in incubation was set to 2mm.

Figure 3.7 Schematic Diagram of Cylindrical Geometries Used to Simulate Dose Deposition to Cell Cultures in Multiwell Plates for the Situation of A) Uniformly Radioactive Cell Culture Media and B) A Monolayer of Cells With Radiopharmaceutical Uptake

![Diagram of cylindrical geometries](image)

Table 3.1 Specification of Plastic Cell Culture Labware

<table>
<thead>
<tr>
<th>Number of Wells</th>
<th>Well Diameter / MM</th>
<th>Polystyrene Thickness / MM</th>
<th>Maximum Volume / ML</th>
<th>Volume Modeled / ML</th>
<th>Height of Water Component / CM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>35.4</td>
<td></td>
<td>16.8</td>
<td>2.5</td>
<td>0.25</td>
</tr>
<tr>
<td>12</td>
<td>22.7</td>
<td></td>
<td>6.9</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>24</td>
<td>16.3</td>
<td>1.3</td>
<td>1.90</td>
<td>0.5</td>
<td>0.26</td>
</tr>
<tr>
<td>48</td>
<td>11.6</td>
<td></td>
<td>0.95</td>
<td>0.3</td>
<td>0.29</td>
</tr>
<tr>
<td>96</td>
<td>6.86</td>
<td></td>
<td>0.36</td>
<td>0.1</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Figure 3.8 shows the average energy absorbed per primary electron simulated, for the above models in the cases of both ⁹⁰Y and ¹³¹I. As with the validation experiments described in chapter 3.2.1 this should be no more than the average energy of the respective beta energy spectra.

When any dimension of the volume considered is less than the range of the beta particles, this results in a significant number of particles escaping the target volume and hence a fall in the absorbed energy. This is most clearly observed with ⁹⁰Y. When the activity was restricted to a 10μm layer then this effect is even more pronounced.
Figure 3.8 Energy Deposition in Multiwell Plates for A) $^{90}Y$ and B) $^{131}I$
3.3.2 Absorbed Dose Distribution to Cells

Due to the finite range of both beta and secondary electrons, cells at the edges of the wells receive a lower absorbed dose than those at the centre (see figure 3.9). Therefore consideration must be given to the distribution of absorbed dose over the area of the well plates.

Figure 3.9 Dose Distribution Across 6 Well Plate From Uniform Distribution of a) $^{90}$Y and b) $^{131}$I
The absorbed dose distribution along the surface of the well plate can be analysed to provide both a conventional histogram of absorbed dose over the area of the plate as well as a dose volume histogram showing the dose absorbed over a given volume. (See figure 3.10-3.13) These show how a radionuclide with lower energy emissions such as \(^{131}\text{I}\) is much more likely to achieve a consistent absorbed dose over the whole cell population than a higher energy beta emitter such as \(^{90}\text{Y}\).
Figure 3.10 Cumulative Dose Volume Histogram Resulting From Uniform Distribution of Radioactivity in Multiwell Plates
Figure 3.11 Differential Histogram of Absorbed Dose Resulting From Uniform Distribution of Radioactivity in Multiwell Plates
Figure 3.12 Cumulative Dose Volume Histogram Resulting From Bound Monolayer Distribution of Radioactivity in Multiwell Plates
Figure 3.13 Differential Histogram of Absorbed Dose Resulting From Bound Monolayer Distribution of Radioactivity in Multiwell Plates
The 3D absorbed dose distribution outputs were analysed to provide dose volume histograms of absorbed dose in the case of radioactive media and cell bound radioactivity (see figure 3.10 and 3.12). These were then modified to provide a histogram of absorbed dose over the volume considered to be occupied by a layer of cells. (See figures 3.11 and 3.13.)

As the size of the well plate considered is reduced and energy losses at the edges of the wells become more important, there is increasingly a spread in the distribution of absorbed dose over the area of that well, especially in the case of $^{90}$Y.

The histograms of absorbed dose were fitted with a Gaussian model, in order to provide a single value (along with an appropriate uncertainty) for the absorbed dose per primary particle emitted. (The histograms are not always symmetrical – in such cases the Gaussian model provides a more appropriate description of the absorbed dose than the mean.) These values were converted to S-Factors as outlined in Appendix 1 and are presented in tables 3.2 and 3.3.

<table>
<thead>
<tr>
<th>ISOTOPE</th>
<th>6 WELL (2.5ML)</th>
<th>12 WELL (1ML)</th>
<th>24 WELL (0.5ML)</th>
<th>48 WELL (300µL)</th>
<th>96 WELL (100µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{90}$Y</td>
<td>0.026±0.0004</td>
<td>0.061±0.001</td>
<td>0.12±0.01</td>
<td>0.113±0.02</td>
<td>0.48±0.09</td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>0.007±0.0001</td>
<td>0.0165±0.0005</td>
<td>0.033±0.001</td>
<td>0.0665±0.001</td>
<td>0.185±0.005</td>
</tr>
</tbody>
</table>

Table 3.2 S-Factors for Dose to Cells in Uniformly Radioactive Cell Culture (mGy/MBq-s)

<table>
<thead>
<tr>
<th>ISOTOPE</th>
<th>6 WELL (2.5ML)</th>
<th>12 WELL (1ML)</th>
<th>24 WELL (0.5ML)</th>
<th>48 WELL (300µL)</th>
<th>96 WELL (100µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{90}$Y</td>
<td>0.103±0.004</td>
<td>0.238±0.016</td>
<td>0.472±0.03</td>
<td>0.887±0.06</td>
<td>2.46±0.18</td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>0.117±0.007</td>
<td>0.272±0.016</td>
<td>0.534±0.03</td>
<td>1.04±0.08</td>
<td>2.97±0.25</td>
</tr>
</tbody>
</table>

Table 3.3 S-Factors for Dose to Monolayer of Cells Resulting from a Bound Radiopharmaceutical (mGy/MBq-s)

The tables above enabling the calculation of absorbed dose have been derived in relation to specific volumes of cell cultures. Nonetheless, in the case of a monolayer of bound radiopharmaceutical, the absorbed dose delivered will be largely independent of the volume of cell culture media. (However, the thickness of the layer representing the monolayer of cells can have a
significant impact on the magnitude of the S-Factors. When the thickness of
the layer was set to be 50µm rather than 10µm, then the calculation of
absorbed dose was decreased by 25% for $^{90}$Y and 50% for $^{131}$I.

In the case of a uniformly radioactive cell culture, the volume of cell culture
media added will have a significant effect on the absorbed dose. If the total
energy (per primary electron) absorbed within the cell culture media remained
constant then S-Factors for any volume could be calculated relative to the
reference data presented in table 3.2 using equation 1.1. As figure 3.14
illustrates though, this is not the case.

![Graph showing energy absorbed vs. cell culture media volume](image)

**Figure 3.14** Average energy absorbed as a function of cell culture media volume in 6 well plate

As the height of the active volume is reduced below the range of the beta
particles, the energy absorbed by the cell culture media falls sharply. This
relationship was analysed and empirically found to be described by the
following equation.

$$E = \frac{E_0 x}{(k + x)}$$

**Equation 3.2**
$E_0$ is close to the average energy of the emission spectrum; the difference is due to the fact some energy will always be absorbed by the well plate bottom and sides. The parameter $k$ relates to both the range of the beta particles and to the geometry under consideration, whilst $x$ is the height of the cell culture media.

However, the above curve describes the energy absorbed over the whole volume and relates to the average absorbed dose over the whole volume. If the absorbed dose across the bottom of the well (derived from the dose histograms) is multiplied by the volume of the well, then similar curves are derived, which can be fitted with the type of function described in equation 3.2. Thus, the absorbed dose per primary particle to any volume of radioactivity can be defined by the following expression.

$$D = \frac{1}{V} \times \frac{E_0 \left( \frac{V}{\pi r^2} \right)}{\left( k + \left[ \frac{V}{\pi r^2} \right] \right)}$$

Equation 3.3

Values of $E_0$, and $k$ specific to combinations of well plates and isotopes are presented in the table 3.4.
### Table 3.4 Parameters for determining absorbed dose to cells incubated in an arbitrary volume of radioactivity. (Dose given as eV/g per unit cumulative activity.)

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Well Plate</th>
<th>$E_0$</th>
<th>$k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{90}Y$</td>
<td>96</td>
<td>$4.3 \times 10^5$</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>$5.4 \times 10^5$</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>$5.8 \times 10^5$</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>$5.0 \times 10^5$</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>$5.4 \times 10^5$</td>
<td>0.12</td>
</tr>
<tr>
<td>$^{131}I$</td>
<td>96</td>
<td>$1.4 \times 10^5$</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>$1.4 \times 10^5$</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>$1.4 \times 10^5$</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>$1.4 \times 10^5$</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>$1.1 \times 10^5$</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Figure 3.15 Accuracy of Empirical Dose Estimate**

The figure above shows the accuracy of these empirical calculations of absorbed dose for any given volume of activity, in comparison with the results of the PENELOPE simulations of defined cell culture volumes.
3.3.3 Investigation of the absorbed dose to adjacent wells

One of the limitations of the pencyl program is the fact that only cylindrical geometries can be modelled. Therefore all of the models consider only a single well. An important question to answer from a practical point of view is whether activity within one well can give rise to an unaccounted radiation dose in a neighbouring well. i.e. can single wells in a multi-well plate be considered as being independent of their neighbours?

Although the cylindrical constraints of pencyl prevent a direct simulation of the situation, it was possible to analyse the models of the single wells to explore the potential absorbed dose from one radioactive well to its neighbour.

Figure 3.16 Absorption of Energy Emitted by $^{131}$I in a Single Well
If the energies per primary particle absorbed by all components of the model are considered then it possible to estimate how many electrons have “escaped” the simulation of a single well. In the case of $^{131}$I these losses are less than 1% of the average energy of the beta spectrum (figure 3.16). Therefore it can be concluded that the polystyrene of the labware is sufficient to absorb the beta emissions of $^{131}$I and that cells in neighbouring wells will not receive any significant radiation dose for which dosimetry information is unavailable.

However, in the case of $^{90}$Y it can be seen that approximately 10% of the energy emitted is unaccounted for (figure 3.17). Although this energy would be emitted isotropically and that one would therefore assume that the solid angle subtended by a layer of cells in an adjacent well would render the absorbed dose to these cells insignificant, a further simulation was run to confirm this was the case. The existing model was extended as shown in figure 3.18.
In order to model the “worst-case” scenario, the model was based upon the dimensions of the 96 well plate, and neighbouring wells were considered to be in immediate proximity to the active well with no air gap.

![Geometric Model Used to Investigate Dose to Adjacent Wells](image)

The results of the simulation demonstrated that the absorbed dose per primary particle to the radioactive water was $3.81 \times 10^6 \text{eV/g}$ compared with $2.36 \times 10^4 \text{eV/g}$ delivered to the surrounding water. i.e. the absorbed dose from one well to its neighbour can be considered to be <1% of the absorbed dose absorbed by that well.

### 3.3.4 Influence of Polystyrene

Attempts have been made to calculate dosimetry factors for in-vitro work based upon published data considering the spherical models described earlier. Such approaches do not take interactions within the polystyrene labware itself into account. Moreover, the thickness of the polystyrene was modelled as being 1.3mm, a value which was specific to the equipment used in this laboratory but may not be true across all manufacturers.

The aim of the following section was to investigate to what extent the surroundings of a radioactive source representing an in-vitro experimental investigation need to be considered.
In the first instance, the simulation of radioactivity in a 6 well plate was repeated for the case of homogenously radioactive water. However, the polystyrene and iron components of the model were designated as air. This resulted in a reduction in absorbed energy within the water of 3.3% and 0.6% for $^{90}$Y and $^{131}$I respectively. However, this represents the difference in the average energy absorbed throughout the whole volume of water. Consideration must be given to the fact that although cell culture media may be produced that is uniformly radioactive, the cells themselves will lie on the bottom surface of the well plate. i.e. the area in which backscatter is likely to have the most significant impact.

If the axial absorbed dose profile is considered for the case of the simulation consisting solely of water and air compartments then it is observed to be symmetrical around the centre of the water component of the model (see figure 3.19). In this situation there is no backscatter of radiation into the water. In the situation for which the plastic of the labware is included, then it can be seen that the profile becomes asymmetric, presumably due to the addition of backscatter from the polystyrene into the water. In fact, at the bottom of the well plate there is a 25% increase in the dose absorbed when the polystyrene labware is accurately modelled in the case of $^{90}$Y and 13% in the case of $^{131}$I.

The replacement of polystyrene and iron components by air was also applied to the model described in figure 3.7b, used to simulate a monolayer of radiolabelled cells. In this case reductions of 23.1% and 11.1% in absorbed energy were observed for $^{90}$Y and $^{131}$I respectively.
Figure 3.19 Axial Profile of Absorbed Dose Delivered to Uniformly Radioactive Water in 6 Well Plate from A) 90Y and B) 131I
Therefore it would appear that modelling the backscatter of electrons at the water polystyrene interface can make a considerable difference to the dosimetry of cell cultures, particularly for higher energy beta emitters. This then leads to a further question as to whether the thickness of the polystyrene is of any significance regarding the magnitude of the backscatter. In order to explore this further, a series of simulations were run in which the thickness of the polystyrene layer was successively reduced from 1.3mm to 10µm (see figure 3.20). Initially it was anticipated that this would lead to a reduction in the absorbed energy, towards the result of the models using only water and air. However, for both $^{90}$Y and $^{131}$I this was not the case. From this, one can infer that there is also a large degree of backscattered radiation from the iron shelf of the incubator that becomes more significant as the attenuating property of the polystyrene is reduced. Indeed, when the simulations were repeated for the case of a 1.0µm thick iron shelf the expected trend was observed. The magnitude of the energy absorbed appears to be more sensitive to changes in the thickness of the polystyrene in the case of $^{90}$Y. The results suggest that $^{131}$I beta emissions will backscatter from depths of up to 0.1mm in polystyrene plastic. The higher energy $^{90}$Y emissions will backscatter from depths of up to 0.5mm. i.e. as long as the labware in which cells are cultured in thicker than 0.5mm then the S-Factors presented in 3.3.2 may be considered as universally applicable to all manufacturers.
Figure 3.20 Dose Absorbed by Radioactive Monolayer as a Function of Plastic And Iron Thickness for A) $^{90}$Y and B) $^{131}$I
3.3.5 Non-Confluent Cell Distributions

Two distinct situations have already been modelled. Firstly, a situation in which cells are exposed to a radioactive cell culture media whilst in the second case the radioactivity is not free in the cell culture media, but is instead attached to the monolayer of cells via an appropriate radiopharmaceutical. However, it is worth considering that not all cell lines will grow as a continuous sheet of cells or reach full confluence. In the case of cells exposed to a radioactive media, the absorbed dose is not a function of the cell distribution. However, in the case of cells which incorporate a radiopharmaceutical, it can be seen that the distribution of the cells over the well plate surface needs to be considered.

For example if a short range (< 10μm) emitter is incorporated into a 50% confluent cell culture, then the model described in chapter 3.3.1 will underestimate the absorbed dose by a factor of 2. (The energy emitted will be considered over the whole surface area of the well plate. In reality the energy is restricted to cells covering half that surface area meaning that the radiation intensity will be twice as high.)

For medium range beta emitters a proportion of the energy emitted will not be absorbed by cells but will instead be deposited between cell clusters due to the crossfire effect. This proportion is likely to be a function of the distance between cell clusters as well as the energy of the beta emissions.

Specifically, in the case of the BT-474 breast carcinoma cell line, the cells do not grow to 100% confluence, but instead grow in adherent patches as illustrated in figure 3.21 to a maximum of ~50% confluence.

To investigate the dosimetry of such a non-confluent cell distribution, the model previously used to simulate a radioactive monolayer in a 6 well plate was modified as follows. The radioactive water layer was modified such that the layer was defined by a number of concentric cylinders which were alternately designated as being radioactive or non-radioactive. The width of the rings was varied to simulate varying sized cell clusters and varying distances between them. Simulations were carried out of $^{131}$I emissions (beta spectrum only) as well as for $^{90}$Y.
As anticipated, for the case of $^{131}$I when 50% of the well surface area is covered by cell clusters separated by a large distance relative to the range of the beta particles, the absorbed dose to the cells per emission is nearly twice as high as when the cells are evenly distributed over the entire surface area. However, as can be seen in figure 3.22, the finite range of the beta particles results in energy losses to the volume between the cell clusters, resulting in a reduction of the absorbed dose at the edges of these clusters. As the size of the clusters and the distance between them is reduced, an increasingly high proportion of cells will find themselves near such edges and as a consequence the absorbed dose per emission decreases. However, even for cell clusters as small (and close) as 100$\mu$m, the absorbed dose is still up to 50% higher than is the case for a confluent distribution.

For $^{90}$Y emissions which have a higher energy than those of $^{131}$I, the same initial result is seen whereby for 1.7mm cell clusters, the maximum dose-rate is twice that calculated in the original simulation of a confluent layer. There is however, a much faster tendency for the dose distribution to approach that associated with an even distribution of radioactivity as the separation of cell clusters is reduced.
Figure 3.22 Radial Profile of Absorbed Dose to Concentric Arrangement of Alternatively Radioactive and Non-Radioactive Cells in 6 well plate for A) I-131 and B) Y-90

(In the case of the BT-474 cells treated with $^{90}$Y-Herceptin an alternative S-Factor was used based upon an assumption of 50% confluence and a cluster size of 100-200$\mu$m.)
3.4 Dosimetry of Multicellular Spheroids

Multicellular spheroids are a commonly used in-vitro model of micrometastases in cancer studies [74, 75]. Although chapter 3.2.1 discussed the dosimetry of uniformly radioactive spheres, the in-vitro application of radiopharmaceuticals to such spheroids will not necessarily result in such a distribution of radioactivity. Instead the radiopharmaceutical is more likely to bind to the surface of the spheroid resulting in a heterogeneous absorbed dose distribution over the spheroid.

In order to assess the likely absorbed dose distribution across such spheroids, geometric models were created for use with PENCYL as follows. Spherical geometries were created using a stack of cylinders of diameters such that they formed an approximately spherical volume as shown in figure 3.23.

![Figure 3.23 Stack of cylinders arranged to approximate a spherical volume](image)

In terms of defining the material composition, all cylinders were designated as water as was the media around the spheroid. Radioactivity was assigned to the outer 5% of each cylinder. The figure of 5% was chosen to represent some diffusion of radioactivity from the outer edge of the spheroids as has been observed in some experimental models [76].
A detailed assessment of absorbed dose over the central cylinder with a diameter equivalent to the diameter of the spheroid was made in order to obtain radial profiles of the absorbed dose over the whole volume. Studies were made of the dose distribution resulting from $^{90}Y$, $^{131}I$ and phosphor-32 ($^{32}P$) in spheroids of 5mm, 1mm, 0.5mm and 0.1mm radius. (A radius of 5mm is clearly unlikely for an in-vitro cell spheroid – however such a spheroid was considered as it represented a distance approaching the maximum range of the $^{90}Y$ beta particles.)

As the energy of the beta emissions decreases ($^{90}Y E_{\text{max}} = 2.28\text{MeV}; ^{32}P E_{\text{max}} = 1.71\text{MeV}; ^{131}I E_{\text{max}} = 0.606\text{MeV}$) the decreased range of the electrons means that dose deposition becomes increasingly confined to the outer zone of the spheroid. (See figure 3.24.) In all cases the absorbed dose deposited per primary emission increases dramatically as the radius of the spheroid decreases, since the emissions are effectively limited to an increasingly confined volume.

The total number of emissions of course depends upon the amount of radioactivity taken up by the cells making up the spheroid. In the case of a non-internalised antibody or peptide it seems reasonable to assume that this will be proportional to the surface area of the spheroid.

Therefore figure 3.25 shows dose volume histograms for the case where the absorbed dose distribution throughout the 1mm, 0.5mm and 0.1mm radius spheroids has been normalised to the case of the 5mm radius spheroid.

In the case of an $^{131}I$ labelled spheroid, the absorbed dose absorbed over the volume of the entire spheroid increases as the size of the spheroid considered decreases. Initially this is true of both a $^{32}P$ and a $^{90}Y$ labelled spheroid. However, in the case of these two isotopes the results suggest that for spheroids of 1mm or less radius, the dose distribution will be the same regardless of the size of the spheroid.
Figure 3.24 Radial dose profile to spheroids resulting from radionuclides localised over surface of spheroids
Figure 3.25 Dose Volume Histograms for Various Sized Spheroids
3.5 Discussion

The issue of dosimetry is crucial to establishing reproducible and meaningful dose-response relationships, whether in the context of clinical treatment planning studies or in in-vitro studies such as those presented in this thesis.

At the clinical level, there have been numerous publications detailing methods to convert both planar [77] and/or tomographic image data into assessments of absorbed dose. To date there have been several implementations of the conversion of SPECT activity distributions to absorbed dose distributions using both kernel techniques [78, 79] or direct Monte Carlo simulation [38, 80, 81].

As discussed previously, kernel techniques involve the convolution of the absorbed dose distribution around a point source with the spatial distribution of radioactivity. Originally dose point-kernels for monoenergetic electrons were derived analytically by Spencer [82]. These were then subsequently averaged over the spectrum of various commonly used radionuclides to produce dose point-kernels appropriate for applications in nuclear medicine [83]. The first application of Monte Carlo to monoenergetic dose point-kernels was published by Berger et al in 1971 using ETRAN [84]. Various refinements have subsequently been published as new codes are released or old codes updated, including dose point-kernels for beta emitting radionuclides [85-87]. To date these kernels have generally been produced by either the Electron Gamma Shower (EGS) or by the Monte Carlo N-Particle (MCNP) codes. A comparison of dose point-kernels produced using either EGSnrc, MCNP or GEANT4 published by Pacilio et al concluded there was relatively little difference between them for higher energy beta emitters such as $^{90}$Y or $^{188}$Re. For $^{131}$I discrepancies for low energy electrons led to differences of ~10% at short source to target distances [88]. Uusijärvi et al carried out a similar exercise comparing the PENELOPE code to GEANT4, MCNP and ETRAN [89]. In their evaluation of monoenergetic point dose-kernels they observed average differences of <10% for energies ranging from 10keV to 1MeV. The $^{90}$Y point dose-kernel presented in figure 3.5 is overlaid in figure 3.26 onto data presented by Mainegra-Hing et al [73] showing the point dose-kernel they derived using EGSnrc as well as both the analytical kernel produced by Prestwich et al and the first publication of a Monte Carlo derived kernel by Berger.
As can be seen the data produced as a validation of the implementation of PENELOPE used in this chapter is most closely matched to the more recent EGSnrc / EGS4 derived kernels.

At the microscopic level, the dosimetry of single cells has been more extensively studied compared to the situation regarding multicellular arrangements. In general the MIRD approach has been taken to dosimetry of single cells, but rather than defining source and target organs, cellular compartments such as the nucleus and cytoplasm are used. As with dose point-kernels analytic approaches were originally used to define relationships between the absorbed dose to a target compartment of a cell and the cumulative activity in source compartments [31]. These have subsequently been compared to calculations based upon Monte Carlo codes [90-92]. The primary application of such single cell models has been towards dosimetry calculations involving Auger emitting radionuclides, for which the range of the particles is of the order of the cell dimensions [93, 94]. Although the case of a single cell and beta emitting radionuclides has also been considered, models of absorbed dose distribution in multicellular situations accounting for cross fire have also been described.
The major application of these models has been to explore the consequences of radiopharmaceutical heterogeneity at the microscopic scale [95, 96]. For example, Hindie et al have considered the microstructure of thyroid follicles and demonstrated differences of ~10% between the average absorbed dose to the thyroid and the absorbed dose delivered to the thyroid cells themselves [97]. Zhu et al considered the microdosimetry of tumour cells targeted by radiopharmaceuticals localised to endothelial cells within tumour vasculature in order to objectively optimise the potential choice of radionuclide [98]. This type of approach is analogous to that presented in chapter 3.3.5 in which the effect of a non-confluent cell distribution on the absorbed dose to those cells was shown to be up to 100%. In common with the literature, it is demonstrated that this is an effect that is even more pronounced for lower energy beta emissions. Nonetheless, even for cell clusters regularly spaced at 0.44mm (~5% of the $^{90}$Y maximum beta particle range) this leads to a difference of ~50% between the absorbed dose calculated on the assumption of a continuous distribution of radioactivity and that when the heterogeneity is accounted for.

The distribution of cells within a tissue culture flask is of course a somewhat simpler biological situation than those discussed above. Nonetheless, for many in-vitro experiments involving radionuclides, absorbed dose may be expressed in surrogate units such as radioactivity concentration, making comparison with EBRT treatments difficult. The only publication to date of S-Factors for use in multi-well plate experiments was published by Freudenberg et al [99]. They used a convolution technique to calculate absorbed dose within the wells, based upon the dose kernel of Prestwich. It is important to note that such an approach will not account for interactions within the well plate itself and that as presented in chapter 3.3.4, for high energy beta emitters, neglecting to consider these interactions may lead to errors of ~25% in the calculation of absorbed dose. Indeed, comparison of their results with those presented in this thesis, reveal differences of -17% and -20% in the dosimetry of 6 well plates containing $^{90}$Y and $^{131}$I respectively.

The dosimetry of multicellular spheroids was originally discussed in the context of theoretical discussions of the most appropriate radionuclides to be used in the treatment of micro-metastases [53, 100]. The analysis of these situations tended to assume a homogenous distribution of the radionuclide throughout
the spheroid. The general findings of these investigations were that lower energy beta emitters were more efficient in terms of self-dose to spheroids of a size smaller than the range of the higher energy beta emitters. A comparison of the dose volume histograms presented in chapter 3.4 shows that for the smallest 100μm radius spheroid $^{131}$I is indeed the most effective radionuclide compared to $^{32}$P and $^{90}$Y. However, by confining the activity to the spheroid surface, the use of $^{131}$I becomes increasingly ineffective as the size of the spheroid is increased. Strikingly, this does not appear to be the case for either $^{32}$P or $^{90}$Y – the results presented suggest that for spheroids of diameter <2mm, the size of the spheroid would not affect treatment outcome (assuming an uptake proportional to the spheroid surface area). Although this appears somewhat counterintuitive, it is possible to compare the PENELOPE dosimetry with alternative derivations. Bardies et al used analytical expressions to explore absorbed dose profiles through spheroids containing either homogenous or surface bound activity for 22 different radionuclides [41]. When compared with the case of $^{90}$Y, there is no difference in the absorbed dose distribution at the centre of the spheroid for either 100μm or 1mm radius spheres. Bardies et al found the absorbed dose at the surface to be 2.5 times greater in both instances, whilst the PENELOPE simulation predicts an absorbed dose 3.2 times higher. (The reasons for this are not clear although it can be seen from figure 3.26 that the PENELOPE derived scaled dose point-kernel is greater than that derived analytically by Prestwich al lower values of r.) Moreover Bardies et al published their data in terms of S-Factors (cGy / Bq-s). The difference between the respective S-Factors for 100μm and 1mm is 100. i.e. the respective difference in surface area. Thus, this would appear to confirm the observation made above regarding the apparent unimportance of the spheroid radius relative to the efficacy of treatment with high energy beta emitters such as $^{90}$Y.

Although the focus of the work carried out in this chapter has been the use of Monte Carlo techniques to establish a link between the amount of radioactivity used in-vitro and the radiation absorbed by cell cultures, it is worth pointing out that alternative techniques have been explored. Gear et al have explored the potential of the type of polymer gels normally used to verify intensity modulated
radiotherapy [101]. They concluded that such a gel mixed with a radioactive source and subsequently imaged using MRI, provided a potential means of obtaining absorbed dose distributions in any arbitrary geometry. Others have used radiochromic film to measure the dose distribution within a liquid source of $^{153}$Sm [102].

There are of course limitations to the approach taken to dosimetry in this chapter. Although the application of a MIRD S-factor model to a simulation of homogenously distributed activity works well, this approach is increasingly limited when applied to lower energy emissions originating from radiopharmaceuticals bound to a heterogeneous distribution of cells. However, for the purposes of the experiments presented in the subsequent chapters, the Monte Carlo derived S-Factors relating radioactivity in 6 well plates to absorbed dose in cell cultures, represent a validated and accurate method of assessing the delivery of radiation dose.
4 Low Dose-Rate Irradiation Applied to Hematopoietic Cells

4.1 Introduction
The interpretation of the LQ model is that fatal damage to a cell is caused by either multiple single strand breaks or double strand breaks in the DNA helix caused directly by ionising radiation. However, in light of alternative biological phenomena such as hypersensitivity and the bystander effect as described in chapter 1.3, as well as the complexity of the response to radiation at the level of molecular biology, it should not necessarily be taken for granted that the LQ model developed for optimising fractionation regimes in EBRT can be directly translated to applications in TRT. In particular, it has been proposed that apoptosis may be a mode of cell death more likely to be associated with low dose-rate irradiation and that therefore models of cell survival developed to model responses to acute irradiation of DNA, may not be appropriate.

Hence, the aim of the following chapter was to describe the response of different hematopoietic cell lines (already known to enter into apoptosis in response to ionising radiation) exposed to EBRT using the LQ model. The model parameters were then used in conjunction with versions of the LQ model developed for exponentially decreasing irradiation, to make predictions of the response of these cell lines to an in-vitro simulation of TRT. The results of these predictions of clonogenic survival are compared to measured data.

In parallel with this work the relative importance of apoptosis in TRT vs EBRT was also explored. Following irradiation with either EBRT or TRT, temporal changes in ATP levels as well as caspase 3/7 levels were measured, in order to investigate the hypothesis that apoptotic response is more likely following prolonged low dose-rate irradiation.

4.1.1 Materials and Methods
The HL60 and MOLT-4 leukaemic cell lines were grown in suspension as described in chapter 2.2. After splitting the cells, 6x10ml aliquots of each cell culture were dispensed into 25cm² flasks and exposed to EBRT as described in chapter 2.3. Each aliquot was treated with 0Gy, 0.5Gy, 1Gy, 2Gy, 5Gy and
10Gy respectively using the $^{137}$Cs irradiator. Assessments of absorbed dose were made in-situ. After 48 hours incubation, each sample was set up for a clonogenic assay as outlined in chapter 2.6.1. These experiments were carried out in duplicate.

For the purposes of simulating TRT in-vitro, radioactivity concentrations of $^{90}$Y-EDTA ranging from 110kBq/ml – 4MBq/ml were used to deliver absorbed doses up to 23Gy to 2.5ml aliquots of HL60 and MOLT-4 cells cultured in 6 well plates. The S-Factors presented in table 3.2 were used in these calculations. These aliquots were subsequently incubated and assayed for survival in an identical manner to the EBRT treated cells.

The clonogenic cell survival curves for HL60 and MOLT-4 cells treated with both EBRT and TRT are presented in chapter 4.2.1. Chapter 4.2.2 describes the immediate response of the cell lines following treatment in terms of changes in viability and levels of caspase-3/7. An extension of the LQ model derived by Dale is explored in chapter 4.2.3 and compared to the experimental data. The results are discussed in the context of current literature in chapter 4.2.4.
4.2 Response to Radiation of Hematopoietic Cell Lines

4.2.1 Clonogenic Survival Results
The initial aim was to characterise the response of cells to a single non-fractionated dose of irradiation. The clonogenic survival of both MOLT-4 and HL60 cells in response to a single non-fractionated dose of external radiation is shown in figure 4.1. Both cell lines are observed to be relatively sensitive to ionising radiation delivered in this manner, although MOLT-4 cells are more radiosensitive.

A least squares fit was used to apply a Linear Quadratic model to both the HL60 and MOLT-4 survival data. Each survival curve displayed an initial shoulder that then fell off with increasing dose. MOLT-4 cells were seen to be more radiosensitive than the HL60 cells. The LQ parameters were $\alpha = 0.25$, $\beta = 0.076$, $\alpha/\beta = 3.2$; $\alpha = 0.8$, $\beta = 0.21$, $\alpha/\beta = 4$; for the HL60 and MOLT-4 cells respectively. These parameters were used as the basis for the subsequent application of the radiobiological modelling of cell survival in response to TRT, presented in chapter 4.3.

When the clonogenic survival of both HL60 and MOLT-4 cells, exposed to $^{90}$Y-EDTA, is compared to their respective responses to the delivery of an acute dose of irradiation, marked differences are observed (See figure 4.2). Protracted low dose-rate irradiation was seen to be significantly less effective than a single acute dose of gamma radiation. At 10Gy the simulation of TRT was less effective than EBRT by at least three orders of magnitude for treatment of both HL60 and MOLT-4 cell lines.

In contrast to the EBRT survival curves, there was no “shoulder” to the observed curves which are much closer to a linear behaviour. In the case of the HL60 cells, there is little difference in the dose-response relationship whether treatment is delivered over 12 hrs or at a lower dose-rate over 48 hours.

The response of the MOLT-4 cells, on the other hand appears to be more sensitive to differences in treatment time. By treating over 12 hours instead of 48 hours (i.e. a x4 increase in dose-rate for corresponding dose-points) the
Figure 4.1 Clonogenic Survival in Response to Gamma Irradiation of A) HL60 and B) MOLT-4 cell lines
Low Dose-Rate Irradiation Applied to Hematopoietic Cells

Figure 4.2 Clonogenic Survival in Response to Irradiation with $^{90}$Y-EDTA of A) HL60 and B) MOLT-4 cell lines. (Errors too small to be visualised)
efficacy of treatment was brought closer to that of an acute single delivery of radiation.

4.2.2 Immediate Changes in Cell Viability and Caspase-3/7 Levels
As well as measuring response to treatment in terms of clonogenic survival, post irradiation changes in levels of ATP and caspase 3/7 activation were measured using the techniques described in chapter 2.6.2 and 2.6.3. The aim of these experiments was to compare the immediate biological response to TRT in comparison to EBRT – in particular to determine the relative role of apoptosis in each treatment modality.

Figure 4.3 compares the response of HL60 cells to either EBRT or TRT. In the case of the EBRT treatment there was an immediate loss of viability following a 10Gy absorbed dose of gamma irradiation. At the lower 5Gy absorbed dose there was neither a decrease nor increase in viability immediately post irradiation. Instead a decrease of ATP was not seen until 48 hours following treatment. A similar delayed reaction was observed after initiation of treatment with ⁹⁰⁰⁹⁰Y-EDTA for the TRT treated cells at all doses of ⁹⁰⁰⁹⁰Y-EDTA, although the relative loss of cell viability after 96 hours was seen to be dose-dependent.

Post treatment, no immediate change in caspase-3/7 levels are observed with the exception of the 10Gy EBRT HL60 cells. A delay of 24 hours was seen before cells exposed to lower doses of EBRT exhibited an increase in caspase-3/7 levels. In the case of TRT treated HL60 cells this delay was increased to 48 hours. The rise in caspase 3/7 levels would appear to precede the subsequent drops in cell viability. Hence it seems reasonable to conclude that apoptosis is a significant mode of cell death for HL60 cells treated with either TRT or EBRT.

However, for both treatment modalities, the magnitude of the change in caspase-3/7 levels does not appear to be directly proportional to the absorbed dose. As well as presenting caspase-3/7 levels as a function of time, it is also possible to present the cumulative apoptotic response by integrating the caspase-3/7 levels over time (see figure 4.3). Again it was seen that although there is an initial increase in the apoptotic response as the absorbed dose
increases, it was then observed to asymptote. This was the case for both EBRT and TRT.

Figure 4.3 Change in levels of ATP and Caspase 3/7 post treatment of HL60 cells by EBRT and TRT.
The equivalent set of results for the MOLT-4 cells are shown in figure 4.4. It can be seen that EBRT elicits an immediate change in the number of viable cells regardless of the absorbed dose. The decrease is dose-dependent, although after 80 hours the beginning of a recovery regrowth phase is observed in the 2Gy sample. For TRT the higher concentrations of $^{90}$Y-EDTA resulted in an immediate loss of viable cells. As the radioactivity concentration was lowered a slower response was seen. For the lower concentrations of radioactivity, the rate of cell proliferation is initially matched to that of the negative control cells. As the accumulation of absorbed dose causes the rate of cell death to outweigh proliferation, this is reflected as a loss of viability. In comparison to the HL60 cell responses, the increased radiosensitivity of the MOLT-4 cells was apparent. Despite their greater radiosensitivity and the larger loss of cell viability, the caspase-3/7 level responses of the MOLT-4 cells are not significantly higher than those displayed by HL60 cells.

In the case of the external beam treatment, at 24 hours post irradiation the caspase 3/7 levels demonstrated clear dose dependence, although the increase in caspase 3/7 activation per unit absorbed dose decreases as the absorbed dose rises towards 10Gy. The same is true of the cells treated with $^{90}$Y-EDTA. Beyond 24 hours the level of caspase-3/7 in cells exposed to lower radiation doses continued to rise, whereas for MOLT-4 cells exposed to higher doses, caspase-3/7 levels either remained stable or began to decrease. The net effect was that, particularly for MOLT-4 cells exposed to TRT, the cumulative total of caspase-3/7 activation again asymptotes beyond a certain dose.

Overall, these results would suggest that there is a limit on the role played by apoptosis and that at higher doses in particular, an alternative mode of cell death must be responsible for the significant decreases in clonogenic survival observed.

Although for both HL60 and MOLT-4, there are temporal differences in the response to irradiation between EBRT and TRT, the magnitude of caspase 3/7 activation relative to a negative control cell population is not significantly different between modalities. Hence it is not possible to conclude that for cells
with the potential to undergo apoptosis in response to radiation, a low dose-rate treatment modality is of particular advantage.

Figure 4.4 Change in levels of ATP and Caspase 3/7 post treatment of MOLT-4 cells by EBRT and TRT.
4.2.3 Bystander Effect Media Transfer
Media from irradiated HL60 and MOLT-4 cell cultures was transferred to recipient cell cultures which were then incubated for a further 48 hours. However clonogenic assays of these recipient cells showed no difference in cell survival to negative control samples. i.e. no bystander effect was demonstrated for these cell lines.

4.2.4 Applying the Linear Quadratic Model to TRT
In chapter 1.3.8 the concept of the Linear Quadratic model was used to explain fractionation effects. In order to compare the relative effect of different fractionation schemes the concept of the Biologically Effective Dose (BED) was introduced [102]. By definition this is given by,

$$BED = -\frac{\ln(S)}{\alpha}$$

Equation 4.1

The BED can also be expressed conceptually as,

$$BED = Total\ Dose \times Relative\ Effectiveness$$

Equation 4.2

For the case of fractionated EBRT delivery, it can be derived from equations 4.1 and 1.7 that,

$$BED = Nd \times \left(1 + \frac{d}{\alpha/\beta}\right)$$

Equation 4.3

Hence the relative effectiveness is given by \((1+d(\alpha/\beta))\). In this case it is simple to observe that increasing the dose per fraction will increase the relative effectiveness of a fractionation schedule.

Expressions for BED have also been developed by Dale for both the case of a continuously irradiating low dose-rate (CLDR) delivery as well as for a
monoexponentially decaying radiation (MDR) delivery [104, 105]. As well as calculating doses based upon the combination of (initial) dose-rate and treatment times, these models also introduce parameters that account for the repair of tissue over the course of radiation delivery and the time over which treatment is delivered.

**Continuous Low Dose-Rate Irradiation**,

\[
BED_{CLDR} = RT \times \left( 1 + \frac{2R}{\mu(\alpha/\beta)} \right)
\]

Equation 4.4

R=Dose-rate; T = Treatment Time;

\[\mu = \text{Sub-lethal repair constant} = \frac{\ln(2)}{t_r}\]

where \(t_r\) is an expression of the repair time.

For a **monoexponential dose-rate**, 

\[
BED_{MDR} = \frac{R_0}{\lambda} \times \left( 1 + \frac{R_0}{(\mu + \lambda)\left(\alpha/\beta\right)} \right)
\]

Equation 4.5

\(R_0 = \text{Initial Dose-rate}\) \(\lambda = \text{Decay constant}\) \(\mu = \text{Sub-lethal repair constant}\)

For the MDR model described above it is assumed that the radiation source is allowed to decay until infinity. For occasions when the treatment delivery is limited to a finite period of time, \(T\), then \(RE\) is given thus [103].
Low Dose-Rate Irradiation Applied to Hematopoietic Cells

\[ RE = 1 + \frac{2R_\lambda}{(\mu - \lambda)} \frac{A - B}{C} \]

where

\[ A = 1 - e^{-2\lambda T} \]
\[ B = 1 - e^{-(\mu + \lambda)T} \]
\[ C = 1 - e^{-\lambda T} \]

Equation 4.6

Thus, equations 4.1, 4.2 and 4.5 may be used to predict the response to TRT using the \( \alpha \) and \( \beta \) parameters derived from the results of EBRT experiments. The only unknown parameter is the sub-lethal repair constant, \( \mu \). The significance of the repair constant, on the predicted survival of both MOLT-4 and HL60 cells is shown in figures 4.5 and 4.6 respectively. For both cell lines, varying the repair constant causes the predicted survival to vary, between the linear quadratic survival observed in response to a single fraction of EBRT and a linear survival curve (normally described as a continuous repair model). As the ratio of repair time to treatment time is decreased, the predicted survival tends increasingly towards the latter.

For the MOLT-4 cells a repair time of 1 hour looks to be the most appropriate, showing good agreement with both sets of measured data, whilst for HL60 a repair time of 30 minutes may be more appropriate.

However, for the treatments used in clinical practice, the treatment time is typically to infinity and thus the precise magnitude of a repair factor becomes less important, so long as it can be assumed to be small relative to the treatment time. Both the experimental data observed, and the LQ formalism of Dale applied to the data, suggest that if the dose-rate is decreased and equivalent doses are delivered over longer periods of time, then the continuous repair model may be a reasonable approximation of the cell survival that could be anticipated.

One of the limitations of the experimental data presented is the restriction of the treatment time to 48 hours. However, as the analysis of caspase 3/7 activation showed, the cell cultures left unattended would pass into apoptosis spontaneously (due to a lack of media change and/or splitting of cell cultures).
As a result, it would have been problematic to have a negative control against which to compare treated aliquots of cells.

*Further limitations – could have used the same cell line with and without functional p53*
Figure 4.5 Modelling the Response of MOLT-4 cells treated with $^{90}$Y-EDTA over A) 12 hours and B) 48 hours.
Figure 4.6 Modelling the Response of HL60 cells treated with 90Y-EDTA over A) 12 hours and B) 48 hours
When discussing the application of the LQ model to prolonged treatments with ionising radiation, the BED has been further modified by the introduction of a repopulation factor, designed to account for the proliferation of cells during the course of treatment. Thus,

\[ \text{BED} = \text{Total Dose} \times \text{Relative Effectiveness} - \text{Repopulation Factor} \]

Equation 4.7

\[ RF = \frac{0.693T}{\alpha T_{av}} \]

Equation 4.8

\( T_{av} \) is the average doubling time of the cell population.

However, this repopulation factor has not been taken into account for the analysis of the experimental results presented. The clonogenic survival data shown is measured relative to a negative control in each case, thus accounting for inherent cell proliferation over the respective treatment times.
4.3 Discussion
There is a clear difference in the effectiveness of TRT in comparison to single fraction EBRT (See figure 4.2). The range of doses covered matches those measured clinically in trials of $^{90}$Y-Zevalin [183]. At low doses the difference is relatively small, but at higher absorbed doses TRT treated cells cease to display the “shoulder” associated with the quadratic term of the LQ model and are closer to a linear change in cell survival. However, despite this difference, it has been shown that modified versions of the LQ model can be used to predict the clonogenic survival of both HL60 and MOLT-4 cells exposed to TRT, using parameters derived from EBRT survival data.

A number of direct comparisons of radiosensitivity to TRT in comparison with EBRT have been made. Certain early investigations of the efficacy of radioimmunotherapy (RIT) in preclinical animal models suggested that RIT was more effective than EBRT. However, a review of these studies by Knox [106] demonstrated a wide variation in the findings of such studies, such that RIT was found to be “more effective, less effective, or as effective as equivalent doses of external beam XRT.” This somewhat broad finding even extended to investigations of xenografts of the same colorectal cell line (as carried out by different authors).

With regards to the cell lines studied in this chapter, a linear survival curve for both HL60 and MOLT-4 exposed to low dose-rate gamma irradiation was previously reported by Vavrova et al [107]. In the case of the HL60 cells, a similar difference in radiosensitivity was observed between EBRT and low dose-rate therapy (1.8mGy/min). However, they found no difference in sensitivity between EBRT and low dose-rate therapy for MOLT-4. The reason for the discrepancy between these results and those presented in this chapter is not clear, although differences in methodology regarding the irradiation of cells are noted. (Vavrova et al carried out low dose-rate irradiations under non-incubated conditions, presumably for up to 24 hours, using an attenuated Cobalt-60 beam. Thermoluminescent devices (TLDs) were used for dosimetry.)

A similar study of CA20948 rat pancreatic tumour cells, either incubated with $^{131}$I or exposed to single fraction EBRT, also demonstrated the greater efficacy of EBRT. The survival of cells exposed to $^{131}$I appeared to follow that of the
shoulder of the curve observed in response to EBRT [108]. Nonetheless, Zhuang et al have suggested that in the case of CL187 colonic cancer cell lines, the use of I-125 seeds to deliver low dose-rate irradiation is more effective than external beam treatment delivering equivalent doses [109].

According to the LQ model, the \( \alpha/\beta \) ratio is a quantitative indication of the sensitivity of cells to a change in fractionation or dose-rate. The lower this value, the greater the difference between the response to acute irradiation and low dose-rate treatment. Values for HL60 and MOLT-4 were found to be 3.3Gy and 3.8Gy respectively. Therefore, it could be expected that the sensitivity of each cell line to TRT relative to EBRT would be similar. This hypothesis is based upon the mechanistic concepts behind the LQ model and does not account for differing molecular biological pathways in response to irradiation.

One of the principal reasons for comparing the response of HL60 and MOLT-4 cells was their known differences regarding the biological response to irradiation. In particular HL60 cells are p53 deficient, whilst MOLT-4 are p53 wild.

When comparing the reaction of HL60 cells and MOLT-4 cells to gamma irradiation, one immediately apparent difference in the subsequent viability of the cells is the delayed response of the HL60 line. (See figures 4.3 and 4.4) This delay in both initiation of apoptosis and subsequent loss of viability is most likely to be related to a well known G2/M cell cycle delay that is induced by exposure to radiation [110, 111]. Furthermore the same groups have demonstrated that G2/M cell cycle arrest also occurred when low dose-rate exposures were made using exponentially decaying radionuclides. This is in agreement with the findings presented in chapter 4.2.2. The delayed caspase-3/7 activation and subsequent loss of HL60 cell viability is a phenomenon common to both the EBRT treatment model as well as the \(^{90}\text{Y}-\text{EDTA} \) TRT model.

Apoptosis has been put forward as the principal mode of cell death in TRT [27, 57]. However, the data presented in figures 4.3 and 4.4 do not necessarily support this hypothesis. An increased level of caspase 3/7 activation following irradiation is seen in both HL60 and MOLT-4 cells for both different methods of irradiation. In the case of gamma irradiated cells there appears to be an
association between the level of caspase 3/7 observed and the absorbed dose up to 2Gy and 5Gy for MOLT-4 and HL60 cells respectively. Beyond these absorbed doses the total level of caspase-3/7 detected appears to reach a limit, although the absorbed dose does influence the speed with which the changes occurred post irradiation. Thus, these results confirm what is already known - apoptosis is not the only mode of cell death in high dose-rate irradiated HL60 or MOLT-4 cell lines [112, 113]. In particular necrotic cell death is increasingly prevalent at high doses of ionising radiation.

In the case of TRT, Friesen et al used Flow Cytometry to analyse the percentage of apoptotic cells post irradiation of HL60 cells as well as CEM cells [58]. Although, they also observed temporal differences in changes in activation of apoptosis between beta and gamma irradiated cell lines, the authors “found higher apoptosis rates and earlier activation of apoptosis pathways after gamma-irradiation”. One disadvantage of the Flow Cytometry technique applied is that it analysed the population of viable cells and thus does not account for necrotic cell death. The advantage of the caspase 3/7 luminescence technique used in this chapter is that it allowed the investigation of relative levels of apoptosis across all absorbed dose points. Although the levels of caspase-3/7 observed initially were dependent on the radioactivity concentration, they did not rise to levels above those observed for gamma irradiated cells. Moreover, the relatively small differences in the levels of caspase-3/7 observed at increasing doses of low dose-rate irradiation do not account for the order of magnitude differences in clonogenic survival seen at successive absorbed dose points. Thus, it may be that as with EBRT there is a transition from apoptotic cell death to necrotic cell death as the dose-rate is increased.

As stated previously, a significant difference between HL60 and MOLT-4 cells is the mutation of the p53 gene in HL60. The presence of p53 has been linked to decreased radioresistance and is reflected in the greater radiosensitivity shown by the MOLT-4 cells. Although both cell lines displayed an apoptotic response to irradiation, as demonstrated both in this chapter as well as by others [114], the presence or absence of p53 does not appear to affect the sensitivity of either cell line to TRT relative to EBRT. i.e. regardless of the
differing biological pathways in the cell lines the LQ model is consistent in being able to predict the response to TRT. Nonetheless, improvements to experimental design regarding the effect of p53 status could be made. For example, it is possible to transfet cells such as HL60 so as to restore the p53 gene. A side by side comparison of p53 null HL60 vs transfected p53 wild-type HL60 would allow stronger conclusions to be drawn regarding the relevance of p53 status to the LQ model.
5 Application of the LQ model to a Hypersensitive Cell Model

5.1 Introduction and hypothesis
Chapter 1.3.6 introduced the concept of hypersensitivity and the possibility of low dose-rate effects eliciting greater cell damage than might be expected. The aim of this chapter was to characterise the response to low dose-rate irradiation of a cell line already reported to have a hypersensitive response to acute radiation absorbed doses.

The hypothesis tested was that the response of such a cell line to low dose-rate irradiation would not be adequately modelled by the extensions of the Linear Quadratic Model discussed in the previous chapter, and that furthermore the cells would display an inverse dose-rate effect. A further aim, in the event of an inverse dose-rate effect being observed, was to identify any threshold dose-rate above or below which the sensitivity to radiation underwent significant change. Furthermore, a biological explanation for any low dose-rate effects was explored at the level of molecular biology.

The selected cell line was PC-3, a cell line derived from human prostate adenocarcinoma. In chapter 5.2, the response of the cell line to acute irradiation and the characterisation of the survival curve are presented. Chapter 5.3 describes the results of exposing PC-3 cells to low dose-rate irradiation with $^{90}$Y-EDTA over a range of treatment times and dose-rates. Chapter 5.4 contains an application of the Linear-Quadratic model to the data presented in chapter 5.3, and introduces a suggested modification of the LQ model that could be applied to hypersensitive cells.

The biological basis for the inverse dose-rate effect is explored in chapter 5.5. In particular the molecular pathways suggested as being the cause of hypersensitivity to acute irradiation are investigated following treatment with low dose-rate irradiation. All results are discussed in the context of existing literature in chapter 5.6.
5.2 Response of PC-3 to Acute Irradiation

Adherent PC-3 cells were grown as described in chapter 2.1. For delivery of acute irradiation, the $^{137}$Cs Irradiator was used (chapter 2.2). Cell survival was assessed with a clonogenic assay using Crystal Violet staining (chapter 2.3.)

![Surviving Fraction vs Dose Graph](image.png)

Figure 5.1 Response of prostate adenocarcinoma PC-3 cells to acute gamma radiation

The cell survival curve for PC-3 in response to acute irradiation is shown in figure 5.1. As previously, the data was modelled to a linear quadratic function using a least squares fit. The model parameters were $\alpha = 0.085\text{Gy}^{-1} \pm 0.04$ and $\beta = 0.093\text{Gy}^{-2} \pm 0.004$.

The curve has the low alpha/beta ratio and broad shoulder associated with late responding normal tissues, before falling off quickly at doses beyond 2Gy. Although the error bars are reasonably small it is not possible to objectively
perceive any hypersensitivity at low absorbed doses. This would be shown by survival at low absorbed doses falling beneath the level described by a linear quadratic description of cell survival, before returning to “normal” linear quadratic behaviour at higher absorbed doses.

This type of response to ionising radiation has previously been modelled by an adapted version of the Linear Quadratic model termed the induced repair (IR) model [115].

$$SF = e^{-\alpha \left[ 1 + \left( \frac{\alpha_s}{\alpha} - 1 \right) e^{-\left( \frac{D}{D_c} \right)} \right] D - \beta D^2}$$

Equation 5.1 Induced Repair Model of Cell Survival

In this model $\alpha_s$ is the slope of the survival curve observed during the hypersensitive phase, whilst $D_c$ represents a threshold absorbed dose above which these effects no longer apply. An example of the model is provided for illustration in figure 5.2a in which $\alpha_s/\alpha$ equals 30 and $D_c$ is set to 1Gy. For the purposes of the illustration the values for $\alpha$ and $\beta$ derived from the PC-3 survival curve are used. The result is a clear difference in the nature of the survival curves. However, according to Joiner et al the $\alpha_s/\alpha$ ratio for PC-3 is approximately 6.5 [60]. If this value is used in the IR model, figure 5.4b is obtained and it becomes apparent why such an effect cannot be observed in the data presented. At this lower $\alpha_s/\alpha$ ratio the effect is much more subtle and it is clear that the clonogenic assay used lacks the precision to allow the characterisation of such small deviations from a standard model.

The measured survival curve compares well with other published data on the response PC-3 cells to irradiation [62, 116, 117]. The $\alpha/\beta$ ratio of $\sim$0.9Gy is much lower than most tumour types which are typically higher than 4Gy. However this compares reasonably well with clinical estimations of an $\alpha/\beta$ ratio equal to $\sim$1.5Gy in prostate carcinoma [118].
Figure 5.2 The effect of hypersensitivity on cells with a) a hypothetical $\alpha_s/\alpha$ ratio of 30 and b) an $\alpha_s/\alpha$ ratio of 6.5 as measured for PC-3 cell line [60].
5.3 **Response of PC-3 to Low Dose-Rate Exposure**

In the case of hypersensitive cell lines, the linear quadratic model does not predict the response to radiation at low absorbed doses. Therefore it is likely that such cell lines would fail to conform to the linear quadratic model, when exposed to low dose-rate irradiation.

PC-3 cells were cultured in 6 well plates and incubated with $^{90}$Y-EDTA in order to provide low dose-rate irradiation. Cells were treated at dose-rates ranging from 0.06Gy/hr to 1.1Gy/hr. Cells were treated for either 24, 48, 72 or 96 hours. For each timepoint, an unirradiated negative control aliquot of cells was also incubated. Response was measured using the clonogenic assay.

The results of this experiment are shown in figure 5.3 alongside the response of the cells to an acute absorbed dose of external beam radiation. The data points are grouped according to treatment time.

Initially, between 0-0.5Gy, the survival curves follow the shoulder of the acute exposure curve. Beyond 0.5-12Gy, it is possible to differentiate between the

![Figure 5.3](image.png)

**Figure 5.3** Response of PC-3 cells to low dose-rate $^{90}$Y-EDTA irradiation over 24, 48, 72 or 96 hours. Responses are shown in relation to the response of PC-3 cells to acute irradiation.
groups. The longer the treatment time (and therefore the lower the dose-rate for a given absorbed dose), the more effective the treatment. This inverse dose-rate effect is a notable departure from what might be expected from a classical dose-rate relationship. However, even the most effective treatment regime (T=96 hours) is not as effective as the acute single fraction dose delivery used to define the radiosensitivity of the cell line.

The data presented in figure 5.3 is grouped by common treatment times T. Therefore the higher absorbed dose points are equivalent to higher dose-rates. Beyond 12Gy, for those cells treated over 96 hours, there would appear to be a change in the slope of the survival curve, indicating decreased radiosensitivity per unit dose. Such a transition in the slope of the survival curves is also seen for cells treated over shorter periods of time, albeit at lower absorbed doses as the treatment time is reduced.

Although, the results clearly demonstrate an inverse dose-rate effect, it would appear that as the total radiation absorbed dose delivered increased beyond 20Gy, then a treatment time of 24 hours (higher dose-rate) is more effective than the equivalent absorbed dose delivered over 48 hours and 96 hours.

From these observations, it seems likely that cells are more sensitive to ionising radiation delivered below a threshold dose-rate. Above this dose-rate, radioresistance of the cells initially increases. However, as the dose-rate is then further raised, radioresistance decreases. In the next section, the Linear Quadratic model and the concepts of Biologically Effective Dose, are applied to this hypothesis.

According to the classical theory of radiation damage as either double or single strand breaks occur in DNA, a higher dose-rate should lead to additional single strand breaks in sections of DNA, already subjected to single strand breaks which have not had the time to repair, and consequently result in fatal damage to the cell. In the case of the data presented this is not so – although the acute irradiation is still the most damaging, the 96 hour treatment regime (lower dose-rate) is apparently more effective than the 24 hour treatment (higher dose-rate) and up to 5Gy is equally as effective as a single acute dose.
5.4 Application of the LQ Model to Inverse Dose-Rate Effect

In chapter 4, it was demonstrated how characterising the alpha and beta parameters of a given cell line made it possible to predict the response of that cell line to protracted low dose-rate irradiation by using the following equations.

\[ SF = e^{- (BED \times \alpha)} \]

\[ BED = D \times RE \]

\[ RE = 1 + \frac{2R_0\lambda}{(\mu - \lambda)(\alpha/\beta)} \frac{A - B}{C} \]

where

\[ A = \frac{1 - e^{-2\lambda T}}{2\lambda} \]

\[ B = \frac{1 - e^{-(\mu + \lambda)T}}{\mu + \lambda} \]

\[ C = 1 - e^{-\lambda T} \]

Equation 5.2 LQ Model Applied to Low Dose-Rate Irradiation

SF = survival; BED = Biologically Effective Dose; D = total absorbed Dose; RE = Relative Effectiveness; \( R_0 \) = Initial Dose-Rate; \( \lambda \) = the exponential decay constant; and \( \mu \) is the repair constant.

Using the values for \( \alpha \) and \( \beta \), determined in chapter 5.2 (0.085 and 0.093 respectively), the same equations were used to model the response of PC3 cells irradiated for 24 hours. As previously, a least squares fit of the experimental data to the adapted LQ model was used to find an appropriate value for the repair parameter \( \mu \). The results of the fit are shown in figure 5.4, alongside the clonogenic survival predicted by alternative values of \( \mu \). As shown previously, the value chosen for \( \mu \) has a significant effect on the degree of cell killing predicted. A value of 0.73hr\(^{-1}\) (equivalent to a half repair time of 0.95 hours) was chosen for further modelling of the response of the PC3 cells.
If the value of $\mu=0.73$ is used to predict survival to low dose-rate irradiation delivered over 48 and 96 hours, then the curves predict an increase in cell survival as the dose-rate at which a particular absorbed dose is reached decreases (see figure 5.5). However, when compared to the experimental data it can be seen that such a model clearly does not account for the behaviour observed. For each treatment time, the experimental data appeared to “undercut” the expected behaviour at lower absorbed doses before reverting towards the survival predicted by the model at higher absorbed doses. The difference between the predicted and the measured survival becomes increasingly large as the treatment time is increased. This data would therefore appear to support the hypothesis that below a critical dose-rate, PC3 cells are more sensitive to radiation. As the dose-rate rises above the threshold, the cells cease to behave in this hypersensitive manner and cell survival reverts to behaviour in keeping with the LQ model.
In order to explore this further, the concept of the Biologically Effective Dose (BED) was used to compare the different treatment regimes. The measured BEDs were compared with those predicted by the LQ model. (See figures 5.6a-d.) As the treatment time is increased, the discrepancy between the predicted BED and the measured BED becomes increasingly large. The longer the treatment time, the higher the absorbed dose at which the discrepancy is resolved. If the ratio of the measured BED to the predicted BED is plotted as a function of the initial dose-rate, rather than absorbed dose, then an interesting finding is observed (see figure 5.7). Although, the magnitude of the increased sensitivity to radiation is a function of the treatment time, there appears to be a common dose-rate (~0.5Gy/hr) beyond which there is no increased sensitivity to radiation.

According to the data presented, no increase in biologically effective dose occurs beyond a threshold dose-rate of 0.5Gy/hr. Such an increase is most apparent for cells treated over longer periods of time. It would also appear that
such an increased sensitivity to radiation does not occur at the very lowest dose-rates.

Figure 5.6: Comparison of predicted vs measured Biologically Effective Dose for $^{90}$Y-EDTA treatment of PC3 cells over a) 24 hours; b) 48 hours; c) 72 hours. d) 96 hours.
In the case of the Induced Repair Model (equation 5.1), used to describe hypersensitivity in response to low single fraction doses of radiation, the $\alpha D$ term of the Linear Quadratic equation is modified to reflect the higher radiosensitivity displayed at low absorbed doses. A higher radiosensitivity could be due to a number of reasons – a higher than expected degree of physical damage; a greater propensity for cell death, due to changes in the activation of particular cell death pathways; or a change in the effectiveness with which damage is detected and repaired.

Since the physical nature of the ionising radiation is no different at low dose-rate, the first possibility of increased physical damage seems improbable. From the data presented, it would appear that all survival data falls somewhere in the region bound by the response to acute irradiation, and the continuous repair model defined by $\exp(-\alpha D)$, and that at no point is the delivery of low dose-rate irradiation more effective than single fraction dose delivery. Therefore a change in the efficiency of the repair process may be the most likely cause for the inverse dose-rate effect.

Figure 5.7 Ratio of actual BED to BED predicted by LQ model as a function of the initial dose-rate
If this were the case, then the Dale version of the LQ model could still be used as the basis for modelling response to low dose-rate irradiation. Under normal circumstances, the parameter $\mu$, is a constant used to describe a time-dependent repair process. In a hypothetical version of the model, which aims to describe the apparent biological outcomes presented above, it could instead be redesignated as a function of the initial Dose-rate, $D_0$. In the example shown below, the repair parameter is presented as a dose-rate dependent sigmoid function, describing a transition from no repair at low dose-rates to “normal” repair beyond 0.5Gy/hr.

![Graph showing dose-rate dependent repair parameter $\mu(D_0)$]

Figure 5.8 Threshold model of repair factor $\mu$

The curve is described by the following equation:

$$\mu(D_0) = \mu_{\text{min}} + \frac{\mu_{\text{max}}}{\left(1 + e^{-m(D_0-D_c)}\right)}$$

Equation 5.3 Dose-Rate dependent repair process

$D_c$ is the critical dose-rate at which $\mu$ has reached 50% of its maximum value, whilst $m$ is a parameter which controls the rate of change of the sigmoid function.
The result of this adaptation of the model is to describe a survival curve that initially follows the curve seen in response to acute single fraction irradiation, but then makes a transition to a survival curve in which the repair process is hampering cell kill. Figure 5.9 shows the sensitivity of the model to the shape of the sigmoid curve used to describe \( \mu \). It can be seen that parameters may be selected which result in a good fit of this adapted LQ model to the 96hr...
Application of the LQ model to a Hypersensitive Cell Model

experimental data (T=96 hours). However, the same parameters (m=20, Dc=0.5Gy/hr) do not result in such a good fit for the 48 and 72 hour exposures (see figure 5.10.)

![Graph of LQ model applied to experimental data](image)

Figure 5.10 Adapted LQ model applied to experimental data.

There are a number of limitations of this model which may explain this. Firstly, as with any version of the Linear Quadratic model, it is based upon a simplification of the biological response that may or may not be appropriate. Secondly, the extension of the model described in this chapter is based on the hypothesis that the repair process is a function of the initial dose-rate $D_0$. The main reason for making this assumption was mathematical, rather than biological – in this way $\mu$ is not a function of time and thus the solution to the LQ model still holds true. (See appendix 2.) It may be that a model in which $\mu$ is a function of the dose-rate at time $t$ would be a more appropriate form of the LQ model. Unfortunately it was not possible to derive an analytical solution to such a model. However, it was instead possible to make $\mu$ a function of the average dose-rate rather than the initial dose-rate. Since the average dose-rate of an exponentially decaying source is related to the treatment time $T$, this results in a better correlation between the experimental data and the proposed
model (see figure 5.11). In particular, the increase in BED relative to that predicted by the standard LQ model is now reduced with treatment time.

![Graph](image)

Figure 5.11 Further Adaptation of the LQ model applied to experimental data. In this case, $\mu$ is a function of the average dose-rate, rather than the initial dose-rate. ($m=18, D_0=0.31\text{Gy/hr}$)

An alternative to the proposed models, would be one in which the parameters defining radiosensitivity, $\alpha$ and $\beta$, were made a function of dose-rate instead of $\mu$. This would have the advantage of not constraining the effectiveness of low dose-rate irradiation to no more than that observed in response to acute single fraction irradiation. However, consideration of equation 5.2 shows that any such model would not result in a relative effectiveness that was also time dependent.

If estimations of cell survival are made based upon these extensions of the LQ model then some interesting effects are predicted. Figure 5.12a shows the predictions of cell survival for hypothetical treatments at different dose-rates based upon the version of the model in which $\mu$ is a function of initial dose-rate. For each curve shown there is a fundamental limit on the maximum absorbed dose achievable. This is due to the relatively short half-life of the $^{90}\text{Y}$. E.g. If the initial dose-rate is $0.25\text{Gy/hr}$ and this follows a monoexponential decay with a half-life of 2.67 days then at $T=\infty$ the absorbed dose delivered is $27.5\text{Gy}$. If the initial dose-rate is doubled to $0.5\text{Gy/hr}$ then the maximum absorbed dose
delivered is also doubled. However, it is striking to note that due to the inverse dose-rate effect the cell kill is higher at the lower applied dose-rate, even though the total absorbed dose absorbed is lower by a factor of 2.

Figure 5.12 Predictions of cell survival in response to $^{90}$Y delivered at varying initial dose-rates. A) $\mu$ is a sigmoid function of initial dose-rate. B) $\mu$ is a sigmoid function of the average dose-rate. 
Actual experimental data points are also shown.
The experimental data are in fact, better fitted by the alternative proposal, in which $\mu$ is a function of the average dose-rate (see figure 5.12b). Similar qualitative trends are predicted. Nonetheless, at high absorbed doses this model should be treated with caution. $\mu$ was made a function of average dose-rate to provide an approximate solution to an integration that could otherwise not be solved. As $T$ approaches infinity, this approximation is increasingly less appropriate, since for any dose-rate, the average will approach zero and thus, the predicted cell kill will be equivalent to that resulting from an acute exposure to radiation.
5.5 Biological Investigation of Inverse Dose Rate Effect

In chapter 5.4, a threshold dose-rate of 0.3-0.5 Gy/hr was identified (see figure 5.7). Below this dose-rate, PC-3 cells appeared to be more sensitive to ionising radiation than predicted by the Linear Quadratic model. The aim of this section was to explore the cellular response to irradiation in terms of molecular biology. In particular, pathways already associated in the literature with hypersensitivity to acute radiation exposure, were investigated in order to identify any molecular link between hypersensitivity and the inverse dose rate effect. Figure 5.13 illustrates some of the common signalling pathways which can occur following exposure to ionising radiation and ultimately lead to cell cycle arrest at the G2/M checkpoint. This checkpoint has been cited as one of the
principal explanations for hypersensitivity in response to single fraction irradiation [115]. In particular, the ATM and Chk2 pathways were selected for investigation using Western Blot techniques to identify levels of these proteins post irradiation. The hypothesis behind these experiments was that G2/M cell cycle arrest would only occur above a threshold dose-rate. Cells were aliquoted into 6 well plates as previously and treated with concentrations of $^{90}$Y-EDTA, such as to cause initial dose-rates of 0.05Gy/hr; 0.1Gy/hr; 0.2Gy/hr; 0.3Gy/hr and 0.5Gy/hr as well as a negative control population. Several such well plates were set up, to allow harvesting of cells at more than one time point. Cells were lysed and probed for specific proteins as described in chapter 2.3.4. As well as measuring the phosphorylated form of Chk2 (Ck2-p), antibodies for ATM and ATM phosphorylated at serine 1981 were also used. In addition cleaved PARP, a marker for apoptosis, was also probed for. For the purposes of comparison with a cell line already shown to lack an inverse dose-rate effect and known to readily go into G2-M arrest, HL60 cells were also treated and probed for Chk2-p. All blots were also probed for tubulin, to account for differences in total protein levels between samples.

In addition to using Western Blot techniques to probe for specific proteins, the Cell TiterGlo assay was applied to PC-3 cells treated with $^{90}$Y-EDTA in normal cell culture media. The cells were incubated in 96 well plates at dose-rates of 0.05, 0.1, 0.2, 0.3, 0.5 and 0.8Gy/hr. The aim of this was to measure the effects of dose-rate upon cell proliferation and cell death during irradiation. Measurements were made every 48 hours, until the negative control cells reached confluence and ceased to proliferate.

The results of the Western Blot investigations are summarised in tables 5.1 to 5.4. When HL60 cells were exposed to low dose-rate irradiation, then higher levels of phosphorylated chk2 protein were observed in response, indicating the activation of a G2-M cell cycle arrest as part of the overall response to irradiation. It would appear that the level of protein phosphorylation is approximately proportional to the applied dose-rate. A different type of relationship might be expected for a cell line that displays increased sensitivity to low dose-rate irradiation. i.e. in the case of PC-3 cells, one can hypothesise that phosphorylation of Chk2 is absent at dose-rates below a threshold dose-rate, such as that identified in chapter 5.4.
Initially this would appear to be the case (see table 5.2). At 3 hours post irradiation, no increase in Chk2 phosphorylation was seen at dose-rates less than 0.2Gy/hr. However, at 24 hours and beyond, Chk2 phosphorylation is seen in response to all dose-rates. i.e. although there would appear to be a threshold absorbed dose (~ 1Gy) below which cell cycle arrest at G2-M does not occur, there is no threshold dose-rate required for the activation of this pathway. It is also worth noting that, once activated, Chk2-p levels appear constant over time at least up to 72 hours.

<table>
<thead>
<tr>
<th>Initial Dose-Rate (Gy/hr)</th>
<th>0.5</th>
<th>0.3</th>
<th>0.2</th>
<th>0.1</th>
<th>0.05</th>
<th>0</th>
</tr>
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<tr>
<td>T=5 hrs</td>
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<tr>
<td>T=48 hrs</td>
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Table 5.1 Phosphorylated Chk2 Protein Levels in Irradiated HL60 Cells

<table>
<thead>
<tr>
<th>Initial Dose-Rate (Gy/hr)</th>
<th>0.5</th>
<th>0.3</th>
<th>0.2</th>
<th>0.1</th>
<th>0.05</th>
<th>0</th>
</tr>
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<tr>
<td>T=24 hrs</td>
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<tr>
<td>T=72 hrs</td>
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</tbody>
</table>

Table 5.2 Phosphorylated Chk2 Protein Levels in Irradiated PC-3 Cells

<table>
<thead>
<tr>
<th>Initial Dose-Rate (Gy/hr)</th>
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<th>0.3</th>
<th>0.2</th>
<th>0.1</th>
<th>0.05</th>
<th>0</th>
</tr>
</thead>
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<tr>
<td>ATM (T=24 hrs)</td>
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<td></td>
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<tr>
<td>Tubulin (T=24 hrs)</td>
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</tbody>
</table>

Table 5.3 ATM Protein Levels in irradiated PC-3 cells alongside Tubulin control levels
Chk2 is phosphorylated downstream of ATM. As table 5.3 illustrates, ATM is present in PC-3 cells. However, total levels of ATM do not change in response to radiation. Instead, ATM autophosphorylates in response to DNA damage [119]. Unfortunately, probing for the phosphorylated version of ATM did not result in detection of an observable signal.

Analysis of the changes in ATP levels following irradiation provide further support for an inverse dose-rate effect (see figure 5.14). At 48 hours, cell proliferation in PC-3 cells irradiated at dose-rates below 0.5Gy/hr appears to be significantly reduced relative to negative control samples as well as cells irradiated at 0.5Gy/hr and 0.8Gy/hr (p=0.039). At 96 hours and beyond, the proliferation of the cells irradiated at these higher dose-rates is arrested and would appear to be followed by cell death. This observation correlates with the assay of the cleaved PARP protein using Western Blotting (see table 5.4). Cleaved PARP, a marker for apoptosis was not observed until 72 hours in those cells treated at the higher dose-rates.

Taken together, these two sets of results lend support to the idea that below the proposed threshold dose-rate, cells undergo an early response to irradiation, whilst above this threshold there is a later delayed response to irradiation.

Figure 5.14 Change in relative cell viability during irradiation of PC-3 with Y-90 EDTA
### Initial Dose-Rate (Gy/hr)

<table>
<thead>
<tr>
<th>Initial Dose-Rate (Gy/hr)</th>
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<th>0.3</th>
<th>0.2</th>
<th>0.1</th>
<th>0.05</th>
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<table>
<thead>
<tr>
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<th>0.1</th>
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<th>0</th>
</tr>
</thead>
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<tr>
<td>T=72 hrs</td>
<td>![Image]</td>
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</table>

Table 5.4 Cleaved PARP Protein Levels in Irradiated PC-3 Cells
5.6 Discussion

In chapter 5.3, an apparent inverse dose-rate response to beta irradiation in the prostate adenocarcinoma cell line, PC-3, was described. This response is a contradiction of the version of the Linear Quadratic Model developed for low dose-rate irradiation by Dale.

The inverse dose-rate effect has previously been reported in the literature, particularly for prostate carcinoma cells. Deweese et al compared clonogenic survival at 0.25Gy/hr and 60Gy/hr in several malignant prostate cell lines including PC-3 [120]. In contrast to the results presented in this thesis, they found the low dose-rate irradiation to be more effective in terms of cell kill. The response of a range of cells, previously shown to exhibit hypersensitivity to low doses, in response to low dose-rate irradiation has also been investigated by Mitchell et al [62]. The cell lines included PC-3 as well as a glioblastoma cell line (T98G) and glioma cell line (A7). The experimental arrangements were different to those described in this thesis, in that cells were irradiated at continuous dose-rates within a water tank for much longer periods of time (up to 24 days). Media was changed only every seven days. Nonetheless, the authors observed an inverse dose-rate effect in all these cell lines, including PC-3. However, for all 3 cell lines, the surviving fraction of cells was always greater than that when exposed to a single fraction of high dose-rate irradiation.

An important question to ask, is to what extent could in-vitro demonstrations of hypersensitivity, or the inverse dose-rate effect, extrapolate into clinical benefit? Although hypersensitivity is a low dose phenomenon, it is relevant to note that EBRT is delivered by fractionating the total dose into daily doses of 2Gy. Furthermore, TRT can be considered to be a dose delivery based upon infinitely small dose fractions immediately followed by the next.

In external beam radiotherapy, predictions based upon modelling smaller and more frequent fractions of radiation have suggested that hypersensitivity may lead to better outcomes [121, 122]. However, experimental investigations of ultrafractionation, whereby radiation is delivered in fractions of ~0.5Gy, 2-3 times per day rather than conventional daily fractions of 2Gy, have delivered mixed results. Preclinical investigations, comparing ultrafractionation vs. conventional fractionation, of T98G and A7 xenografts in nude mice concluded
that conventional fractionation was more effective, despite the evidence of hypersensitivity observed in-vitro [123, 124]. However, a clinical trial of ultrafractionation in 31 patients with unresectable glioblastomas claimed an advantage over conventional radiotherapy, in terms of overall and progression free survival [125]. Further clinical evidence in favour of ultrafractionation was described by Harney et al. This group recruited patients with metastatic skin nodules and compared responses in randomly matched pairs of nodules, treated with either ultrafractionation or conventional fractionation [126].

Typical dose-rates for TRT have been reported as being in the range of 0.08 – 0.33Gy/hr [127]. i.e. the same order of magnitude as the dose-rates used for these in-vitro experiments. Despite this, there is rather less evidence regarding the clinical advantage of using lower dose-rates in the context of TRT. This is partly due to the limited cross over between tumour types demonstrating an inverse dose-rate effect (primarily glioma, glioblastoma and prostate cell lines) and the established radiopharmaceuticals available. However, in the case of prostate cancer, it is possible to compare brachytherapy treatments using $^{125}$I seeds implanted in the prostate bed, with external beam radiotherapy. If the dosimetry of each modality is compared, then the BED for brachytherapy with $^{125}$I is usually ~145Gy [128]. In the case of EBRT it is either the same or higher [102, 127], yet comparisons of the two treatment modalities consistently show improvement in biochemical tumour control using low dose-rate brachytherapy [130-132]. TRT is routinely applied as a palliative therapy for prostate cancer patients with bone metastases. A number of radiopharmaceuticals are available with half-lives ranging from 1.9 days to 50.5 days. However to date, comparisons of these options have consistently failed to demonstrate any difference in effectiveness between these radiopharmaceuticals [133, 134].

A version of the Linear Quadratic model which can account for inverse dose-rate effects was described in chapter 5.4. In this adaptation of Dale’s model of response to an exponentially decaying dose-rate, the repair parameter $\mu$, is a function of either the initial dose-rate, or the average dose-rate over the treatment time. This model is based upon a number of assumptions. Primarily, a change in the efficiency with which DNA is repaired is assumed to be the underlying mechanism behind the inverse dose-rate effect. Also, the increased
radiosensitivity is based upon the premise of a threshold dose-rate below which, repair processes are deficient.

It is assumed by most authors that the inverse dose-rate effect is a consequence of the hyper-radiosensitivity effect and that they share the same underlying biological mechanism [56, 60, 115, 135]. Explanations of hyper-radiosensitivity at low absorbed doses are centred on the inactivation of the G2-M cell cycle checkpoint at low absorbed doses of radiation.

Two distinct checkpoints at G2-M have been described [136]. The first relates to G1 and S phase irradiated cells, which will accumulate in G2 as a result of the checkpoint. A second checkpoint relevant to cells irradiated in G2 is thought to be behind hyper-radiosensitivity. This checkpoint has been described as being activated by ATM at a threshold absorbed dose of \(~0.4\text{Gy}\) which matches the threshold absorbed doses observed in hypersensitive cell lines [135-137]. Indeed, in a study of synchronised V79 Chinese Hamster cells, hypersensitivity was found to be confined to G2 phase cells only [138]. Cells lacking functional ATM have also been shown to have an increased sensitivity to low dose-rate irradiation [140, 141].

Although experimental evaluation of ATM-p(ser1981) levels could not be achieved, Checkpoint2 kinase is one of the downstream events initiated by ATM activation in response to DNA damage that ultimately leads to cell cycle arrest between G2 and M phase. Phosphorylation of Chk2 has been previously demonstrated in PC-3 cells [140] and linked to the G2 specific cell cycle arrest described above [143]. Thus if Chk2-p is considered a surrogate marker for ATM phosphorylation, the results presented in this thesis suggest that the ATM-Chk2 pathway is not activated in irradiated PC-3 cells until a threshold of \(~0.6\text{Gy}\) is reached. Once activated, the levels of Chk2-p are absorbed dose independent. Both of these observations are in keeping with the descriptions of hypersensitivity. However, there is no threshold dose-rate below which this pathway is not followed, suggesting that inverse dose-rate effects may not necessarily be explained by a failure to prevent damaged cells passing into mitosis. This is in contrast to the findings of Collis et al [108], who as well as observing a higher clonogenic cell kill in PC-3 and DU-145 (another prostate cancer cell line) treated to 2Gy at 0.1Gy/hr compared to 45Gy/hr, also
observed a lack of ATM-p(ser1981) in DU145 cells treated at the lower dose-rate.

In fact, a dose-rate dependence of Chk2-p is seen in HL60, whereby lower levels are observed at low dose-rates. As shown in chapter 4, this cell line does not appear to have an inverse dose-rate effect. However the changes in Chk2-p over time do correlate with investigations by Vavrova et al into this cell line [106]. They showed continued accumulation in G2 at 0.2Gy/hr whilst cells irradiated at 0.1Gy/hr moved through G2 or underwent apoptosis by 48 hours. Essentially this would suggest that a dose-rate dependent step function in the activation of G2/M cell cycle arrest is not a signature of the inverse dose-rate phenomenon.

There are further reasons why the assumed biological mechanism given to explain hypersensitivity to acute radiation may not be the underlying cause of the inverse dose-rate effect. In the extension of the LQ model, $\mu$ is changed as a function of dose-rate. This parameter describes the repair rate of single strand breaks in DNA. Although ATM is an effector of DNA repair, it has been associated primarily with the repair of double strand breaks [144-146]. An alternative explanation may lie with Poly(ADP-ribose) polymerase (PARP), a protein associated with single strand repair [147, 148]. The relevance of PARP to hypersensitivity is less well understood, but PARP inhibition has been shown to sensitise cells to radiation at low absorbed doses [149]. In the results presented cleaved PARP, a surrogate marker for apoptosis, was not detected until 72 hours after treatment had started, and not detected at all at dose-rates below 0.3Gy/hr. However, it is not possible to draw definitive conclusions regarding PARP’s role as an effector of DNA repair as a function of dose-rate. A similar absence of apoptotic activity in hypersensitive cells (glioma U118MG and colon HT29) exposed to low dose-rate irradiation was observed by Carlsson et al [150].

In conclusion, the existence of an inverse dose-rate effect has been confirmed in the PC-3 cell line. Moreover, the Linear Quadratic model has been extended to describe the situation whereby the rate of repair of single strand breaks is a sigmoid function of dose-rate. Such a model correctly predicts the existence of
an inverse dose-rate effect. Nevertheless, the underlying mechanism of such an effect remains unclear.

For further validation of this model, it would be important to repeat the experiments described in this chapter with further cell lines also known to be associated with hypersensitivity. The clinical relevance of the inverse dose-rate phenomena to TRT should also be investigated using preclinical animal models alongside appropriate radiopharmaceuticals.
6 Bystander Effects

6.1 Introduction

The concept of the bystander effect was introduced in chapter 1.3.7, whereby cell death can be elicited in non-irradiated cells, due to interactions with neighbouring irradiated cells. Demonstrations of the effect fall into two categories, either by using media transfer experiments or by using alpha particle microbeams to target individual cells. The extent to which these effects are clinically important in either EBRT or TRT remains unclear. In the case of EBRT, the absorbed dose distribution at the microscopic level is relatively homogenous. i.e. any irradiated cell receives the same absorbed dose as its neighbours. Thus, the effect of any potential bystander effect is included in the measured overall response to irradiation. In the case of TRT treatments, there is a greater tendency for absorbed dose distributions to be heterogeneous at the microscopic scale, especially if low energy beta emitters or even alpha emitters are used. Therefore it is reasonable to postulate that a better understanding of the spatial propagation of bystander effects may be necessary to fully interpret dose-response relationships. Furthermore, the investigations by Boyd et al into bystander effects elicited by cells targeted with radionuclide labelled MIBG demonstrating a greater toxicity of media from $^{131}$MIBG treated cells (up to 70% cell kill) compared to media from EBRT treated cells (maximum 40% cell kill) suggest an important difference in the bystander response at different dose-rates. Thus, the bystander effect may be of particular importance in understanding the response of cancer cells to TRT and may not be well modelled by current versions of the Linear Quadratic.

The aim of this chapter was to identify a cell line exhibiting a bystander effect in response to EBRT, which could also be specifically targeted with an appropriate radiopharmaceutical. The cell line would be used as the basis for a series of preliminary media transfer experiments to investigate the bystander effect in TRT. In order to investigate the spatial propagation of the bystander effect, it was also desirable that the cell line could be manipulated to form spheroid aggregations of cells.
6.1.1 Cell Selection
Two breast carcinoma cell lines, BT-474 and T47D, were identified as potential candidates on the basis that they had previously been shown to aggregate into spheroids if grown in appropriate conditions [151]. In order to successfully radiolabel these cells, their respective receptor expression was considered. From the literature BT-474 was thought to express the HER2 receptor [67] but not the HER1 (EGFR) receptor [152]. Conversely it would appear that T47D cells express EGFR receptors but not HER2 [153]. The advantage of seeking to target HER2 and EGFR, was that commercial immunotherapy treatments that target these receptors are available, namely Herceptin and Cetuximab. However, only Herceptin could be sourced for use in these experiments. Therefore, it was decided to proceed with investigation of the BT-474 cell line only.

Before carrying out a series of clonogenic assays on irradiated BT-474 cells, a test experiment was carried out using the ATP viability assay. Media was transferred from flasks of BT-474 cells irradiated using EBRT to 10Gy, to unirradiated flasks of cells at 4 hours post irradiation and 48 hours post irradiation.

![Figure 6.1 Initial testing of BT-474 for bystander effect. ATP viability assay of cells treated with media transferred from BT-474 treated with 10Gy EBRT.](image)
The results of this experiment (see figure 6.1) suggested that a bystander effect can be observed. No difference was observed between the effects of media transferred 4 hours post irradiation and that transferred 48 hours post irradiation. This would suggest that the effectors of cell death are released by cell cultures relatively quickly and that they persist for at least 48 hours.
6.2 Response of BT-474 to EBRT
Adherent BT-474 cells were grown as described in chapter 2.1. For delivery of acute irradiation, the $^{137}$Cs Irradiator was used (chapter 2.2). Cell survival was assessed using a clonogenic assay using Crystal Violet staining (chapter 2.3.) A total of 18 25cm$^2$ flasks of BT-474 cells were prepared. Of these, 12 (including two negative controls) were irradiated with absorbed doses of up to 10Gy. The remaining 6 were designated as recipient cultures for bystander experiments.

6.2.1 Direct Irradiation
6 of the 12 irradiated flasks were incubated for 48 hours before being seeded for clonogenic assay.

![Graph showing cell survival curve for BT-474 cells in response to single fraction EBRT.]

The cell survival curve for BT-474 in response to acute single fraction irradiation is shown in figure 6.2. As previously, the data was modelled to a linear quadratic function using a least squares fit. The model parameters were $\alpha = 0.221\text{Gy}^{-1} \pm 0.01$ and $\beta = 0.018\text{Gy}^{-2} \pm 0.001$. Thus $\alpha/\beta = 12\text{Gy}$. Of note are the relatively large error bars, compared to the previous results obtained with
the crystal violet clonogenic assay presented in chapter 5. The error bars reflect the variation in the number of colonies formed within triplicate sets of measurements. The data shown is the result of two independent experiments. The alpha/beta ratio is larger than that observed when HL60, MOLT4 and particularly PC-3 cells were irradiated. However, this magnitude of alpha/beta ratio is more typical of most solid tumour types.

6.2.2 Bystander Response
At 24 hours post irradiation, media was aspirated from the 6 flasks designated as recipient cultures. This was replaced with media from the 6 EBRT irradiated flasks (including a negative control) not destined to be seeded for clonogenic assessment. After further incubation for 24 hours, these recipient cell cultures were seeded for assessment of clonogenic survival at the same time as the remaining set of 6 irradiated flasks. The effect of the transferred media is shown in figure 6.3a. A Mann-Whitney test revealed a significant difference between the number of control colonies grown and the number of colonies resulting from the recipient cells (p<0.05). The maximum cell kill (~50%) was caused by media transferred from the cells irradiated with the lowest absorbed dose, 0.5Gy. Beyond this radiation absorbed dose, the cell kill elicited by the transfer of media remained independent of the absorbed dose delivered to the irradiated donor cells (40-45%). One of the most striking aspects of the observed results is the fact that for low absorbed doses, the bystander effect appears to be more toxic than direct irradiation. This might suggest that a by-product of irradiation may provide a protective effect against the toxins that are responsible for the bystander effect in media transfer experiments.
In order to confirm these observations, the experiment was repeated on a separate occasion. Although, the same response to direct irradiation (i.e. the standard cell survival curve) was observed (see figure 6.2) a less striking bystander response was observed (see figure 6.3b). In this case, the difference between the number of control colonies and the number of recipient colonies was not significant (p=0.1). However, in the case of this 2nd experiment, the initial number of cells per flask was less than in the first case by a factor of 2.
(as determined by the relative number of colonies grown in the negative control plates.) It could therefore be argued that the higher number of irradiated cells in the first experiment led to a higher concentration of toxins in the transferred media, which was subsequently more effective against the recipient cell cultures.
Figure 6.3 The effect of media from irradiated BT-474 cells on unirradiated BT-474 cells. A) First independent experiment. B) 2nd independent experiment. The LQ response to single fraction EBRT is shown for comparison.

Figure 6.4 Summary of Bystander Effect from EBRT irradiated BT-474 cells
6.3 Dose Response of BT-474 Cells Exposed to LDR

6.3.1 Treatment with $^{90}$Y-EDTA

Prior to investigating any bystander effect caused by cells exposed to low dose-rate beta emissions, a study to establish the response to direct irradiation at low dose-rates was carried out.

In chapter 6.2, the response to EBRT was established. The higher alpha/beta ratio of 12 means that in principle, the response is more linear than for other tissues over the range of doses applied and hence the effects of fractionation may be less significant than for tissues or cells with a lower alpha/beta ratio. Therefore, BT-474 cells were treated over either 24 or 96 hours, since it was anticipated that if the LQ model was predictive of the dose-response relationship, then changes in dose-rate would have less effect than in other cell lines such as the PC-3.

As previously, cells were irradiated with $^{90}$Y-EDTA in a 2.5ml volume of cell culture. At either 24 hours or 96 hours, the radioactive media was aspirated and cells were diluted into fresh media for assessment of clonogenic survival.

![Figure 6.5 Response of BT-474 to $^{90}$Y-EDTA](image)
The results of these experiments are shown in figure 6.5. Over the range of 0.5Gy – 10Gy, the observed survival in response to $^{90}$Y-EDTA is generally closely matched to that seen in response to EBRT. It is possible that at lower absorbed doses up to 5Gy, the efficacy of the treatment is greater than with EBRT. However, this may be due to experimental uncertainties associated with the clonogenic assay as mentioned in the previous section. At 20Gy, no surviving colonies were observed for cells irradiated over 24 hours, whilst a single colony (out of 3 triplicate wells) was measured in the case of cells irradiated over 96 hours. The data points therefore represent the maximum possible survival at 20Gy, but it not possible to define precise clonogenic survival at 20Gy. In terms of dose-rate, a significant difference between treatments over 24 hours vs 96 hours was not observed ($p=0.37$).

As previously, the precise predictions of Dale’s version of the LQ model applied to an exponentially decaying dose-rate at higher absorbed doses, are dependent on the value assigned to the repair parameter $\mu$. In figure 6.6, two alternative possibilities are presented. In one case, $\mu = 0.035$ equivalent to a repair time of 20 hours. This provides a better fit to the data but makes use of a theoretically unsound repair time which is much longer than that normally assumed of 1-2 hours. If a repair time of 2 hours ($\mu=0.35$) is used instead, then a possible interpretation of the results is that cells are more sensitive to TRT than predicted by LQ parameters derived from response to EBRT (see figure 6.6b). On the other hand, it can also be seen that the reproducibility of the clonogenic response is poor and that therefore this interpretation should be treated with caution.
Figure 6.6 Linear Quadratic model applied to $^{90}$Y-EDTA treatment of Bt-474 cells. Results are shown for an assumed repair time of A) 20 hours and B) 2 hours.
6.3.2 Treatment with $^{90}$Y-DOTA Herceptin

In order to carry out media transfer experiments to investigate potential bystander effects, an alternative to $^{90}$Y-EDTA was required, since a requirement of such studies is that the media transferred is itself free of radioactivity.

Therefore the procedure described in chapter 2.2.4 was developed to label $^{90}$Y to DOTA conjugated Herceptin. The hope was that $^{90}$Y-DOTA-Herceptin would specifically bind to the HER2 receptor expressed on BT-474, thus providing localised low dose-rate irradiation at the same time as removing at least some of the $^{90}$Y-DOTA-Herceptin from the cell culture media.

As well as a bystander response, the result of direct exposure to this radiopharmaceutical was also measured, since it was thought that the interaction of the Herceptin antibody with its receptor may have the potential to change the observed response of the cells to increasing absorbed doses of radiation.

The immunotherapeutic effect of the radio-labelled antibody was controlled by adding an amount of unlabelled DOTA-Herceptin to the $^{90}$Y-DOTA-Herceptin, such that the total amount of antibody was consistent across all wells, regardless of the amount of radioactivity added. A positive control treatment consisting of cold antibody only was included in the experimental set up (as well as a negative control). Clonogenic survival results were calculated relative to the positive control group in order to ascertain the effects of the radiation alone. The activity added ranged from 0.3MBq to 6MBq.

In practice, not all of the added $^{90}$Y-DOTA-Herceptin was taken up by the BT-474 cells. In order to ascertain the concentration of radioactivity remaining in the cell culture media, 150μl samples were taken (in triplicate) and counted on the gamma counter against an $^{90}$Y standard derived from the original stock of $^{90}$YCl. The amount of radioactivity bound to the cells was measured by sampling the cell culture during the process of seeding cells for the clonogenic survival assay. After cells in the 6 well plates were trypsinised and resuspended in 5ml cell culture media, 150μl samples were taken and counted against the $^{90}$Y standard.
Hence, calculations of absorbed dose were made by summing the relative contributions of the activity bound to the monolayer as well as that from activity uniformly distributed in the media (see chapter 3.3.2 for details).

The results shown in figure 6.7 reflect two independent experiments. In the original experiment (marked in red), after adding $^{90}$Y-DOTA-Herceptin to BT-474 and placing them on rollers for two hours, the cells were spun down and supernatant (together with the unbound $^{90}$Y-DOTA-Herceptin) removed prior to adding fresh media and resuspending and incubating the cells for 72 hours. Samples of the removed supernatant were assayed for radioactivity against a standard in order to calculate the amount of unbound radiopharmaceutical. In practice this unbound activity represented up to 98% of the total activity added to each sample. Hence, the range of absorbed doses covered is relatively small compared to previous experiments.

Therefore in the second experiment unbound activity was not removed in order to extend the range of absorbed doses applied. Hence, the absorbed dose to cells resulted from both radioactivity bound to the cells as well as unbound radioactivity in the cell culture media. The presence of unbound radioactivity in the media implies a saturation of the HER2 receptors on the cells. However, the total uptake of activity into cells was ten times greater on the second occasion in comparison with the first experiment. As described in chapter 2.2.4,
the labelling procedure was optimised to maximise the specific activity. Nonetheless, although the labelling procedure used was consistent on each occasion, there was a clear difference in the resulting uptake of $^{90}\text{Y}$-DOTA-Herceptin by BT-474 cells.

Relative to the negative control cells the positive control cells formed 75% fewer colonies as a result of the Herceptin alone. Since the total amount of antibody was kept consistent over all doses, figure 6.7 shows the effect of the radiation alone. Thus it could be seen that the response appeared to broadly follow that seen in response to $^{90}\text{Y}$-EDTA, although the effect at higher absorbed doses was not so toxic. Again, cell survival observed at low absorbed doses appeared to be lower than had previously been observed in response to a single fraction dose of EBRT.

### 6.3.3 Bystander Response (from 90Y-DOTA-Herceptin)

The original reason for using a radio-labelled antibody to investigate bystander responses was that specific uptake of the radiopharmaceutical could result in cell culture media that was free of radioactivity and could be transferred to recipient cell cultures. However, as described above complete uptake of the $^{90}\text{Y}$-DOTA-Herceptin could not be achieved.

Therefore an affinity chromatography technique using columns of Protein A beads was used to filter the media for transfer and specifically remove the radio-labelled Herceptin antibody (see chapter 2.4.4).

$^{90}\text{Y}$-DOTA-Herceptin was added to aliquots of BT-474 cells (designated as donor cells) at activities ranging from up to 6MBq as described in chapter 6.3.2. At the same time, a set of recipient cell cultures were set up. After 24 hours incubation, the media from the donor cultures was passed through a Protein A column until no further activity could be extracted. Media from the recipient cell cultures was aspirated and replaced with the filtered “bystander” media. The recipient cells were subsequently incubated for a further 48 hours before being prepared for clonogenic assay. Media from these cells was sampled and assayed for radioactivity concentration as previously described in order to calculate the direct dose to these cells from the transferred radioactivity.
Samples of the recipient cells were also assayed for radioactivity resulting from bound $^{90}$Y-DOTA-Herceptin.

In response to the transferred media from irradiated BT-474 cells, a toxic bystander effect was not observed (see figure 6.8a). The hypothesis that the toxic effects of the transferred media, would closely match the toxicity of direct irradiation with $^{90}$Y-DOTA-Herceptin could not be confirmed. Although there was some loss of clonogenic survival, it correlated with the LQ response which would be anticipated from direct irradiation as a result of $^{90}$Y radioactivity which could not be extracted from the transferred media ($r^2 = 0.33)$.

### 6.3.4 Bystander Response (from 90Y-DTPA-YAML568)

A bystander response could not be elicited by transferring the media from $^{90}$Y-DOTA-Herceptin irradiated BT-474 cells to untreated BT-474 cells. In order to rule out an inhibitory effect of the Herceptin antibody, it was decided to repeat the experiment using a non-specific antibody, YAML568, which could be labelled with $^{90}$Y and used to irradiate BT-474 in a similar fashion to $^{90}$Y-EDTA, but subsequently filtered from the media using the affinity chromatography techniques already developed for working with $^{90}$Y-DOTA-Herceptin.

Aliquots of BT-474 cells were prepared in 6 well plates (2.5ml media per plate) as for previous experiments. Donor and recipient aliquots were taken from the same parent flask. The donor aliquots were treated with activities up to 13MBq (~25Gy). After 24 hours the media was removed from the donor cultures and passed through Protein L columns. The filtered media was used to replace the media from the recipient cell cultures. After a further 48 hours, the recipient cell cultures were set up for clonogenic assay. As before the activity concentration in the transferred media was measured and used to calculate the direct dose to the recipient cells.

As with irradiation with $^{90}$Y-DOTA-Herceptin, media transferred from $^{90}$Y-DTPA-YAML568 irradiated BT-474 cells, did not result in an observable bystander effect (see figure 6.9).
Figure 6.8 Bystander Effect of Media transferred from $^{90}$Y-DOTA_Herceptin treated BT-474 cells and passed through Protein A Column. Clonogenic Survival plotted as a function of A) Dose to the Donor Cell Cultures and B) Dose to the Recipient Cell Cultures as a result of residual radioactivity in transferred media.
Figure 6.9 Bystander Effect of Media transferred from $^{90}$Y-DTPA-YAML568 treated BT-474 cells and passed through Protein L Column. Clonogenic Survival plotted as a function of A) Dose to the Donor Cell Cultures and B) Dose to the Recipient Cell Cultures as a result of residual radioactivity in transferred media.
6.4 Discussion

The breast adenocarcinoma cell line BT-474 was characterised for radiosensitivity to single fraction irradiation using the LQ model. An $\alpha/\beta$ ratio of 12Gy was measured. This measured response to gamma irradiation is of the same magnitude as found by other investigations using this cell line [154]. Furthermore, the analysis of clinical treatments of breast cancer by Guerrero et al has confirmed the relatively high $\alpha/\beta$ ratio in-vivo [155]. This higher ratio is more typical of solid tumours. From the LQ model it follows that over the ranges of absorbed doses considered, the BT-474 cells are less affected by fractionation or changes in dose-rate, since the $\alpha D$ term will dominate to a greater extent than for cell lines with a lower $\alpha/\beta$ such as HL60 or MOLT-4. This is reflected in the clonogenic survival of BT-474 in response to $^{90}\text{Y}$-EDTA. Up to absorbed doses of 10Gy, there is no appreciable difference between the survival of cells treated over 24 hours or 96 hours. Neither treatment is significantly less effective than the single fraction EBRT. In order to achieve a best fit of the LQ survival to the experimental data a repair time of 20 hours was suggested. Such a figure is substantially more than the values typically assumed and indeed more than the figure of 1 hour calculated by Guerrero et al [155]. However, when a repair time of 2 hours was assumed then the measured cell survival was generally 50% less than that predicted. It is debatable whether this was a result of experimental uncertainties in the determination of the alpha and beta parameters, or whether these results imply an additional cell kill as a result of the bystander effect.

The bystander effect can be defined as the response of a non-irradiated cell brought about by the targeting of its neighbour with irradiation (or indeed any other type of stress). The clinical relevance of this phenomenon is debatable – in the case of EBRT the radiation absorbed dose to neighbouring cells is likely to be effectively identical. Even for dose distributions resulting from Beta emitting radiopharmaceuticals such as $^{90}\text{Y}$ or $^{131}\text{I}$, this is likely to be the case. It may only be particularly relevant for Alpha or Auger emitting radiopharmaceuticals.

There would appear to be two distinct mechanisms for bystander effects [156]. In the first case direct cell to cell communication may occur between irradiated
cells and their neighbours via gap junctional intercellular communication (GJIC). Separate studies have shown the range of these effects may be between 40mm to 1mm from the site of the radiation energy deposition [157, 158]. However GJIC is generally a property of normal cells, which is not present in cancer cells [159]. (As a consequence, therapies aimed at restoring GJIC have been proposed as a means of sensitising cancer cells to existing treatments [160, 161].) The second mechanism for the bystander effect is the release by irradiated cells of various cytokines, such as interleukin-6, interleukin-9 and tumour necrosis factor α [156].

It is this mechanism which underlies the toxicity of transferred media from irradiated cells to non-irradiated cells. Toxicity from transferred media was observed when BT-474 cells were exposed to media from irradiated BT-474 donor cells, using both an ATP assay as well as the clonogenic assay. As in previous studies, the effect appeared most significant at low absorbed doses, but beyond 1Gy was dose independent [162, 163]. A potentially significant difference between these previous studies and those results presented is the apparent increased toxicity of the bystander effect in comparison with direct irradiation at absorbed doses <2Gy. Previous analyses have made the assumption that the direct effect of ionising radiation and the bystander effect were additive. These observations of BT-474 cells would indicate that irradiation of cells may trigger an inhibitory response against any cytokine factors released into media. i.e. the effects are not additive.

The association of bystander responses with low absorbed dose in particular, has prompted the hypothesis that bystander effects could be of further significance to cells treated with higher absorbed doses delivered at lower dose-rates. This was one of the main findings of the investigations of Boyd et al [65] in which alpha, beta or Auger emitting radionuclides were used to radiolabel MIBG and used to treat cells transfected with the Noradrenaline Transporter (NAT) gene. Clonogenic survival was measured in response to direct irradiation as well as to transferred media. The effect of the media from cells treated with $^{131}$I-MIBG (a beta emitter) was found to closely follow that of direct irradiation and no plateau was observed. This finding is in contrast with the lack of any observable bystander response to the media from BT-474 cells.
treated with $^{90}$Y-DOTA Herceptin or $^{90}$Y-DTPA-YAML568. It is possible to hypothesise that, due the finite amount of radioactivity that could not be removed from the transferred media, this discrepancy may be due to the protective effect of low absorbed doses of radiation implied by the results observed in response to EBRT. It is also possible that the Protein-L affinity columns removed any toxic bystander effect mediators from the culture media. Other investigations of bystander effect arising from cells irradiated using radionuclides have also yielded negative results. Whilst Kishikawa et al. observed a bystander response (both in-vivo and in-vitro) from LS147T colon carcinoma cells targeted with the thymidine analog $^{125}$I-5-iodo-2'-deoxyuridine ($^{125}$IdUrd), the use of $^{123}$IdUrd was found to have a stimulatory effect [164].

Differences in Linear Energy Transfer (LET) have been proposed as a potential explanation for variations in the observed bystander effect in response to irradiation with different radionuclides [165]. For example, Anzenburg et al. investigated bystander responses in both DU145 prostate carcinoma cells and AG01522 skin fibroblasts, when DU145 cells were irradiated with either X-rays or alpha particles [166]. A (clonogenic) bystander response was observed in the AG01522 cells when the DU145 were exposed to low LET X-ray radiation but not in response to high LET alpha radiation. However, a difference in LET is less likely to explain the different bystander response of BT-474 to EBRT vs $^{90}$Y irradiation. The LET of electrons arising from $^{137}$Cs and $^{90}$Y irradiation are $\sim 1 \text{keV}/\mu\text{m}$ and $0.26 \text{keV}/\mu\text{m}$ respectively, compared to 60-100keV/μm in the case of alpha emitters [47, 167, 168]. Furthermore, Anzenburg et al. found that neither irradiation with X-rays or alpha particles elicited a bystander response in the DU145 cells themselves. i.e. as well as consideration of the type of radiation, a better understanding of the biological response to factors released by irradiated cells is required to predict the significance of these effects on cancer (and normal) cells.

The dosimetry results for a spheroid of cells with surface bound radiopharmaceutical presented in chapter 3.4 gave rise to an alternative experimental approach to investigating bystander effects. It was noted that different sized spheroids, which bound $^{90}$Y radioactivity in proportion to their surface area, would have the same dose volume histogram and hence the
same theoretical fraction of surviving cells. It could be hypothesised that any differences in cell survival could be due to the finite propagation of a bystander effect through different sized spheroids. Such an approach would include GJIC effects as well as cytokine release, and could provide an experimental basis for evaluating any future mathematical models of low LET bystander effects. (A number of models have already been developed for analysing high LET bystander effects [169, 170, 171].) Unfortunately, uptake of $^{90}\text{Y}$-DOTA-Herceptin by BT-474 cell spheroids sufficient to cause clonogenic cell kill could not be achieved.

Nonetheless, the treatment of BT-474 monolayers with $^{90}\text{Y}$-DOTA-Herceptin yielded interesting results. Herceptin is the commercial name of Trastuzumab, a monoclonal antibody with affinity for the Her2 receptor expressed by 20-25% of breast cancer tumours. It is used for treatment of metastatic breast cancer and may also be used as part of first line treatment of early breast cancer [172]. Herceptin promotes cell cycle arrest (particularly during G1) and apoptosis by reducing the signalling of MAPK and PK13 pathways [173]. Radio-labelled Herceptin has been proposed by several groups as a novel therapeutic radiopharmaceutical [174-178].

When BT-474 cells were exposed to $^{90}\text{Y}$-DOTA-Herceptin the clonogenic response to irradiation relative to a positive control was the same as that observed in response to $^{90}\text{Y}$-EDTA. i.e. despite the possibility of competing molecular signalling pathways in response to both irradiation and Herceptin, the LQ model was still able to predict the additional cell kill effect of increasing the absorbed radiation dose.

In conclusion, a cell line demonstrating a bystander effect was identified. It’s response to low dose-rate irradiation was observed to conform to that predicted by the LQ model. However, there are some limitations to the experimental design that must be considered. The media transfer experiments involving $^{90}\text{Y}$ failed to elicit a bystander effect in BT-474. In order to rule any potential filtration of toxins by the Protein-L affinity columns, the method could be validated using the cell lines previously demonstrated by Boyd et al to exhibit a bystander response to low dose-rate irradiation.
7 Concluding Remarks

The version of the Linear Quadratic model developed by Dale [104] has been shown to accurately predict the response of a range of cell lines to low dose-rate irradiation using radionuclides. This confirmation of the validity of the model is particularly important, since it is increasingly being applied to evaluations of dose-response in the clinical setting [179],[38]. Such approaches should therefore lead towards an improved correlation between dosimetry and treatment outcome, both with respect to tumour control as well to unwanted side effects.

An exception to this case has been the PC-3 prostate carcinoma cell line, which displayed an inverse dose-rate effect counterintuitive to the response predicted by the LQ model. This increased sensitivity to low dose-rate irradiation may be explained by modelling the repair parameter \( \mu \) as a sigmoid function of dose-rate rather than as an absolute constant. In cell lines displaying a conventional dose-rate response, the LQ model was predictive of response.

In addressing the question of whether the LQ model is able to address clonogenic survival, a number of secondary issues have also been investigated. A number of reasons why the radiobiology of TRT may differ to that of EBRT have been previously raised in the literature, in particular the role of apoptosis as a route to cell death in response to low dose-rate treatment; the question of whether low dose hypersensitivity will translate into low dose rate hypersensitivity; and whether bystander effects play a more significant role in response to low dose-rate irradiation.

Apoptosis is undoubtedly an important part of the response of cells to radiation, and it has been shown in this thesis and by others that treatment with low dose-rate irradiation can indeed elicit this response. However, it may not be the case that apoptosis is a mode of cell death that should be specifically associated with one method of radiation delivery over another.

Low Dose Hypersensitivity is a phenomenon that may well be a marker of tumour types that will not follow the predictions of the LQ model as well as being more sensitive to TRT. However, whilst cells displaying LDH have been
shown to exhibit an inverse dose rate effect, the threshold activation of the G2/M cell cycle checkpoint which has been cited as being the underlying cause of LDH does not appear to be the defining basis for increased sensitivity to low dose rate hypersensitivity. Whatever, the underlying cause of the inverse dose-rate effect, the limitations of the LQ model in in-vitro investigations of certain cell lines, should be considered when future work is extended to in-vivo models. It is harder to draw firm conclusions regarding the relative importance of the bystander effect. In the case of the BT-474 breast carcinoma investigated, preliminary investigations using media transfer experiments have suggested that irradiation may promote protection against the cytokine mediated toxicity of the bystander effect. If this were generally the case, then the significance of the bystander effect may be limited to higher LET particles such as Auger electrons or alpha particles, whose ranges do not extend beyond single cell diameters. Thus, the LQ model appeared to provide a reasonable prediction of dose-response whether to $^{90}$Y-EDTA or to $^{90}$Y-DOTA-Herceptin. However, the lack of bystander response observed in response to either treatment with $^{90}$Y-DOTA Herceptin or $^{90}$Y-DTPA-YAML568 was in stark contrast to the results of Boyd et al, who observed significant effects using $^{131}$I-MIBG. Unfortunately the “dose” associated with the $^{131}$I-MIBG was expressed in units of radioactivity concentration rather than absorbed dose, making direct comparison of response to EBRT and TRT challenging. It therefore remains an open question as to whether a bystander effect elicited by TRT leads to a higher cell kill than predicted by the Linear Quadratic model. A continuing question is to what extent the three radiobiological phenomena discussed are interrelated. For example, several authors have investigated whether increased levels of apoptosis are associated with Low Dose Hypersensitivity. Marples et al found no correlation between LDH and apoptosis [61], as did Chanda et al [180]. In the case of this thesis, the results would also tend to suggest no correlation between apoptosis and the inverse dose-rate effect. The HL60 and MOLT-4 cell lines were both highly prone to apoptosis, but showed a conventional relationship between dose-rate and radiation sensitivity. The PC-3 cell line investigated in chapter 5 which displayed an inverse dose-rate effect did appear to be prone to apoptosis.
However, only those cells exposed to dose-rates above the threshold dose-rate identified were positive for apoptotic cell death, indicating that if anything apoptosis and increased sensitivity to low dose-rate radiation were mutually exclusive.

Inverse correlation has also been demonstrated between the bystander effect and Low Dose Hypersensitivity [181]. This would also appear to be the case with BT-474 – a cell line which demonstrated a clear bystander effect (at least in response to EBRT) but which also followed a conventional dose-rate response relationship.

In drawing conclusions on dose-response relationships the reliability of accurate dosimetry has been of fundamental importance. Monte Carlo simulations of electron interactions using PENELOPE were validated against reference data. The only previously published data on the dosimetry of radionuclides in cell cultures used convolution of analytical expressions of the dose point kernel to calculate doses. In comparison with the PENELOPE dosimetry, differences of up to 20% were noted. Such margins of error could have a significant effect on the interpretation of comparisons of response to EBRT and TRT.

As with any investigation, there are certain limitations which need to be considered. Although, the calculation of absorbed dose, resulting from the application of radioactivity has been carried out to a high degree of accuracy, the calculation of absorbed dose relies upon the accuracy with which the amount of radioactivity itself has been measured. In general, a figure of ±5% is typical of the accuracy of radionuclide calibrators.

Furthermore, the panel of cell lines used in this thesis was relatively narrow compared to the vast array of tumour cell lines available for study, and it should not necessarily be assumed that the results observed will translate to all cell lines. In particular, further work could be carried out with a second cell line displaying LDH to confirm the validity of the threshold repair extension of the LQ model.

It should also be noted that this work concentrated exclusively on beta emissions, both from the point of view of dosimetry and biological effect.
Development of both alpha and auger emitting radiopharmaceuticals continue and this work does not address the validity of the LQ model in these situations. Neither can it be assumed that results observed in-vitro, will also be observed in-vivo. The main measure of response used in this thesis has been the percentage of clonogenic cells surviving. A natural extension of the in-vitro experiments carried out may be to investigate the ability of the LQ model to predict Tumour Control Probability (TCP) as well as Normal Tissue Complication Probability (NTCP) in pre-clinical animal models of particular cancer types.

In addition, the methods and approaches taken in this thesis to explore general aspects of cancer cell response to TRT, should perhaps be incorporated into the development of new radiopharmaceuticals, in order to better select the approaches that may be most likely to result in a clinically effective treatment.

In conclusion, the validation of the LQ response is particularly timely as radiobiological modelling based upon the LQ formalism is increasingly being advocated to augment the analysis of dose-response outcomes in clinical TRT [27, 182]. A minor drawback affecting the implementation of the LQ model in TRT may be the requirement for prior knowledge of the repair times associated with a particular tumour type. However, with tumour types of high $\alpha/\beta$ ratio in particular, a standard value of 1-2 hours should suffice.

One of the remarkable aspects of the success of the LQ model is its basis on relatively simple concepts of the biological response to irradiation. In spite of the subsequent advances in the understanding of the many and various biochemical pathways that occur in response to ionising radiation, the LQ model has successfully underpinned the optimisation of radiation dose delivery in EBRT. There would appear to be no reason why the same approach should not be followed with respect to Targeted Radionuclide Therapy.
8 References


References

References


136. Krueger, S.A., et al., Transition in survival from low-dose HYPER-RADIOSENSITIVITY to increased radioresistance is independent of activation
References


Appendix 1

Derivation of S-Factor from ev per primary particle to mGy/MBq-s

$E_{ev}$ is the energy per primary particle in units of eV

$E_J$ is the energy in SI units of Joules.

$D$ is the absorbed dose per primary particle in SI units of Gy.

$m$ is the mass of the geometric volume in SI units of kg

\[ E_J = E_{ev} \times 1.6 \times 10^{-19} \]

\[ D = \frac{E_J}{m} \]

The branching ratio of $^{90}Y$ is 1. Therefore the dose per primary particle is equivalent to the dose per unit of cumulative activity (in Bq-s).

\[ S - \text{factor} (mGy/MBq - s) = D \times 10^3 \times 10^6 \]
Appendix 2
Solution to Incomplete Repair Model for finite treatment time T.

Relative Effectiveness can be expressed as follows (in any situation) [103]

\[
RE = 1 + \frac{\text{Type}_B \text{ Damage}}{\text{Type}_A \text{ Damage}}
\]

In the situation of a monoexponential dose-rate,

\[
\text{Type}_A \text{ Damage} = \alpha D
\]

\[
\text{Type}_B \text{ Damage} = 2\beta \times \left[ \int_0^T dt \cdot \hat{D}(t) \int_0^t dw \cdot \hat{D}(w)e^{-\mu(t-w)} \right]
\]

\[
= 1 + \frac{2}{D_T(T)} \times \left[ \int_0^T dt \cdot \hat{D}(t) \int_0^t dw \cdot \hat{D}(w)e^{-\mu(t-w)} \right]
\]

\[
= 1 + \frac{2}{D_T(T)} \times D_0^2 \int_0^T dt \cdot e^{-\lambda t} \int_0^t dw \cdot e^{-\mu(w)}
\]

\[
= 1 + \frac{2}{D_T(T)} \times D_0^2 \int_0^T dt \cdot e^{-\lambda t} \int_0^t dw \cdot e^{-(\lambda-\mu)w} e^{-\mu t}
\]

\[
= 1 + \frac{2}{D_T(T)} \times D_0^2 \int_0^T dt \cdot e^{-\lambda t} \times e^{-\mu t} \left[ -e^{-(\lambda-\mu)w} \right]_0^t
\]
\begin{align*}
&= 1 + \frac{2}{D_{T}(T)\left(\frac{\alpha}{\beta}\right)} \times D_{0}^{2} \int_{0}^{T} dt \cdot e^{-\lambda t} \times e^{-\mu t} \left(1 - e^{-(\lambda - \mu) t}\right) \\
&= 1 + \frac{2}{D_{T}(T)\left(\frac{\alpha}{\beta}\right)} \times \frac{D_{0}^{2}}{(\lambda - \mu)} \int_{0}^{T} dt \cdot e^{-\lambda t} e^{-\mu t} e^{-\lambda t - \mu t} \\
&= 1 + \frac{2}{D_{T}(T)\left(\frac{\alpha}{\beta}\right)} \times \frac{D_{0}^{2}}{(\lambda - \mu)} \int_{0}^{T} dt \cdot e^{-(\lambda + \mu) t} - e^{-2\lambda t} \\
&= 1 + \frac{2}{D_{T}(T)\left(\frac{\alpha}{\beta}\right)} \times \frac{D_{0}^{2}}{(\lambda - \mu)} \times \left[ e^{-2\lambda t} - e^{-(\lambda + \mu) t} \right]^{T} \\
&= 1 + \frac{2}{D_{T}(T)\left(\frac{\alpha}{\beta}\right)} \times \frac{D_{0}^{2}}{(\lambda - \mu)} \times \left( \frac{e^{-2\lambda t}}{2\lambda} \right) \left[ e^{-(\lambda + \mu) t} \right]^{T} \\
&= 1 + \frac{2}{D_{T}(T)\left(\frac{\alpha}{\beta}\right)} \times \frac{D_{0}^{2}}{(\lambda - \mu)} \times \left( \frac{1 - e^{-(\lambda + \mu) t}}{(\mu + \lambda)} - \frac{1 + e^{-2\lambda t}}{2\lambda} \right)
\end{align*}