*University of Strathclyde*

Strathclyde Institute of Pharmacy and Biomedical

Science

# The Evaluation of Targeted Radionuclide Therapies and Radio Sensitising Agents in Malignant Melanoma

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A thesis submitted in fulfilment of the requirements for

the degree of Doctor of Philosophy

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# Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.



Signed:

Date:

# Acknowledgements

I would firstly like to thank my supervisor Dr Marie Boyd for allowing me to be a part of her group and for supporting the submission of this thesis. I would like to thank my second supervisor Dr Annette Sorensen for donating so much time and effort in trying to teach me science and Dr Anthony McCluskey for trying in vain to teach me combination index analysis!!

A huge thank you must go out to my long standing partner Katharina, who has truly been my rock over the course of this write up I genuinely could not have done this without you!

And finally, I dedicate this thesis to my parents and family, who never gave up hope and have lent the best support anyone could ever ask for.

# List of Abbreviations

| | |
|---|---|
| [$^{131}$I]MIBG | $^{131}$Iodine-labelled meta-iodobenzylguanidine |
| [$^{211}$At]MABG | $^{211}$Astatine-labelled meta-astatobenzylguanidine |
| µg | Microgram |
| µl | Microlitre |
| µM | Micromole |
| ATM | Ataxia Telengiectasia Mutated |
| ATR | Ataxia Telengiectasia and Rad3 related protein |
| bNAT | Bovine Noradrenaline Transporter |
| BZA | Benzamide |
| CMV | Cytomegalovirus Promoter |
| CO2 | Carbon Dioxide |
| dH2O | Distilled Water |
| DMI | Desmethylimipramine |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |

| | |
|---|---|
| DNA-PK | DNA Dependent Protein Kinase |
| DSB | Double-Strand DNA Break |
| dsDNA | Double stranded DNA |
| FACS | Fluorescence-Activated Cell Sorting |
| FCS | Foetal calf serum |
| FRAP | FKBP-Rapamycin-Associated Protein |
| g | Force of terrestrial gravity |
| Gy | Gray |
| H | Hours |
| H2AX | H2A Histone Family Member X |
| H2O2 | Hydrogen Peroxide |
| HCl | Hydrochloric Acid |
| HEPES | 4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid |
| HR | Homologous Recombination |
| HSV1716/NAT | Herpes simplex virus Glasgow strain 1716 NAT gene transfected |
| HTR | Human Telomerase RNA promoter |

| | |
|---|---|
| HTERT | Human telomerase reverse transcriptase |
| IκBα | Inhibitor of Kappa B |
| LET | Linear Energy Transfer Litre |
| MBq | Megabecquerel |
| mg | Milligram |
| MIBG | Iodine-131-Meta-Iodobenzylguanidine |
| Mins | Minutes |
| ml | Millilitre |
| mM | Millimolar |
| MMR | Mismatched repair |
| MRE11 | Meiotic Recombination 11 |
| MnSOD | Manganese Superoxide Dismutase |
| NaCl | Sodium Chloride |
| NAD+ | Nicotinamide adenine dinucleotide |
| NAT | Noradrenaline transporter |
| NBS1 | Nibin |
| ng | Nanogram |

| | |
|---|---|
| NFκB | Nuclear factor Kappa B |
| NHEJ | Non Homologous End Joining |
| NMSC | Non-melanoma skin cancers |
| NIS | Sodium Iodide Co-transporter |
| nm | Nanometre |
| $^{o}$C | Degrees centigrade |
| PARP-I | Poly(ADP-ribose)polymerase-1 |
| pATM | Phosphorylated ATM |
| PBS | Phosphate buffered saline |
| PFU | Plaque Forming Unit |
| PI | Propidium Iodide |
| RAD50 | DNA Repair Protein RAD50 recombinase |
| RAD51 | DNA Repair Protein RAD51 recombinase |
| RECIST | Response Evaluation Criteria in Solid Tumours |
| RIBBE | Radiation-Induced Biological Bystander Effect |
| ROS | Reactive Oxygen Species |
| RPA | Replication Protein A |

| | |
|---|---|
| RNA | Ribonucleic Acid |
| SD | Standard Deviation |
| SF | Surviving fraction |
| siRNA | Small Interfering RNA |
| SSB | Single-strand DNA break |
| ssDNA | Single Strand DNA |
| TNM | Tumour Node metastasis staging |
| TRT | Targeted Radionuclide Therapy |
| TRT | Targeted Radionuclide Therapy |

# Abstract

**Introduction:**
Malignant melanoma is highly resistant to conventional cancer therapies, characterised by a broad spectrum of radio resistance combined with a low response to chemotherapeutics. This state is compounded by inadequate dose delivery of conventional radiotherapy options. A targeted approach to radiotherapy exploiting native features of the melanoma cells such as melanin may in combination with radio sensitizing agents may improve the effectiveness of therapy towards the disease.

**Aims**:
The aims of this study were three-fold:

- To assess the effectiveness of the melanin binding radionuclide [$^{131}$I]MIP1145 in the treatment of malignant melanoma *in vitro* and *in vivo.*
- To investigate whether malignant melanoma cell lines and xenografts can be rendered susceptible to [$^{131}$I]MIBG radionuclide therapy via transfection in vitro with noradrenaline transporter (NAT) and via gene delivery *in vivo* with the HSV1716/NAT vector.
- To screen novel DNA repair and IKKβ Inhibitors in combination with X-Ray radiation to determine suitability for future targeted radiotherapy/drug combination therapy approaches.

**Results:**
[$^{131}$I]MIP1145 demonstrated accumulation and retention accompanied by considerable reductions in cell survival and tumour burden in melanotic melanoma cell lines and tumour Xenografts. Additionally, a modest uptake and cytotoxic effect of [$^{131}$I]MIBG was observed following transfection with the noradrenaline transporter *in vitro*. Xenografts bearing NAT transfected melanoma cells demonstrated tumour growth delay when treated with [$^{131}$I]MIBG. HSV1716/NAT Successfully delivered the NAT gene to melanoma tumours *in vivo* demonstrated high tumour specificity and tumour growth delay. The MRE11 inhibitor Mirin produced cytoxicity *in vitro* but not *in vivo* when combined with 2Gy X-ray radiation, reducing but not inhibiting γH2AX foci clearance post radiation treatment. The novel IKKβ Inhibitors SU567 and SU182 produce effects consistent with IKKβ inhibition and are cytotoxic to melanoma cell lines, enhancing 1Gy X-ray induced DNA damage and produce marked alterations in the cell cycle of treated cells.

**Conclusions:**
Melanoma tumours can be successfully targeted both endogenously and exogenously with radionuclide therapy. Investigations with novel radiosensitising agents identified that the MRE11 inhibitor Mirin produced a mild reduction in cell survival at drug concentrations as both as a single treatment and as a pre-treatment to 2Gy X-ray irradiation. Novel IKKβ inhibitors induce cytotoxity and enhance 1GY Xr-ray toxicity compared to X-ray treatment alone.

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# Chapter 1

# Introduction

## 1.1 *Introduction to cancer*

Cancer is a growing global epidemic, which poses an increasing public health risk to many populations worldwide. The most recent statistics from the World Health Organisation estimating that 14 million people worldwide were diagnosed with cancer in 2012 with an estimated mortality rate of 8.8 million in the same year (WHO 2015). New and effective therapies continue to be developed, with breakthroughs such as Tamoxifen and Herceptin for breast cancer and Cetuximab for colorectal cancer (Jonker, O'Callaghan et al. 2007) serving as examples of how new compound development can identify novel drug targets and significantly improve patient outcomes. Despite advances such as these for many forms of cancer, the incidence of malignant disease is expected to rise by 70% over the next 20 years (WHO 2015).

Transformation of normal cells and tissues to a malignant cancerous state is the result of a series of acquired fundamental changes to their physiology. These alterations can be categories into distinct “hallmarks of cancer” (Figure 1.1) which build on Carl Nording’s work of the 1950’s (Nordling 1953) and Alfred Knudson’s theories on retinoblastoma of the 1970’s (Knudson 1971) when the notion of cancer

development requiring multiple aberrations or 'hits' to the genome was first put forward.

Far from being a linear, step by step process, the development of malignant disease is a multifaceted and complex interplay between normal and dysplastic tissue whereby an affected cell diverges from a normal physiological state into a neoplastic condition described as "having growth which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimulation that evoked the change" (Willis 1961).

As part of this process, the mutation of proto-oncogenes, a group of genes which have the potential to induce a cancerous state when mutated into oncogenes, are frequently responsible for developing dysregulated and sustained proliferative signalling (Weinstein and Joe 2006). This background of genomic instability also affects genes responsible for suppressing tumour development. Loss of function of these 'tumour suppressor genes' enables rapidly proliferating neoplastic cells to escape normal grow suppression (Burkhart and Sage 2008) and apoptotic mechanisms to continue to excessively proliferate (Adams and Cory 2007, He, Rosen et al. 2007).

Weakly immunogenic neoplasms evade immune destruction (Kim R, Emi M et al. 2007, Teng, Jb; et al. 2008) and progresses to an altered metabolic state driven by the proliferative activity of oncogenes such as RAS and c-myc and the increasingly hypoxic conditions encountered as the tumour increases in size (DeBerardinis, Lum

et al. 2008). This leads to the development of lactate producing and glycolytic populations that enhance biosynthesis of cellular organelles (Vander Heiden and Thompson 2009) and the constitutive activation of angiogenic growth factors such as VEGF and FGF which, alongside the recruitment of immune and stromal cells including leukocytes, fibroblasts and endothelial cells (Bergers and Benjamin 2003, Shiao, Ganesan et al. 2011) produces a potent pro inflammatory, vascularising environment that promotes the growth and progression of the tumour towards a metastatic state.

Tumour metastasis occurs via one of three mechanisms, by spreading to surrounding lymphatic tissue, invading body cavities such as the peritoneum or most commonly, by breaching the surrounding venous and arterial systems. This facilitates the migration of tumour cells and the formation of new neoplastic sites in distant regions of the body (Weinberg 2007). Metastatic disease is the final stage of cancer development, the heterogeneity of both primary and metastatic tumours, often containing aggressive and highly evolved cells populations such as cancer stem cells which harbour considerable proliferative capability and therapeutic resistance presents a significant hurdle for effective treatment, and thus the development of novel cancer therapeutics must be a continual aim for the scientific and medical community.

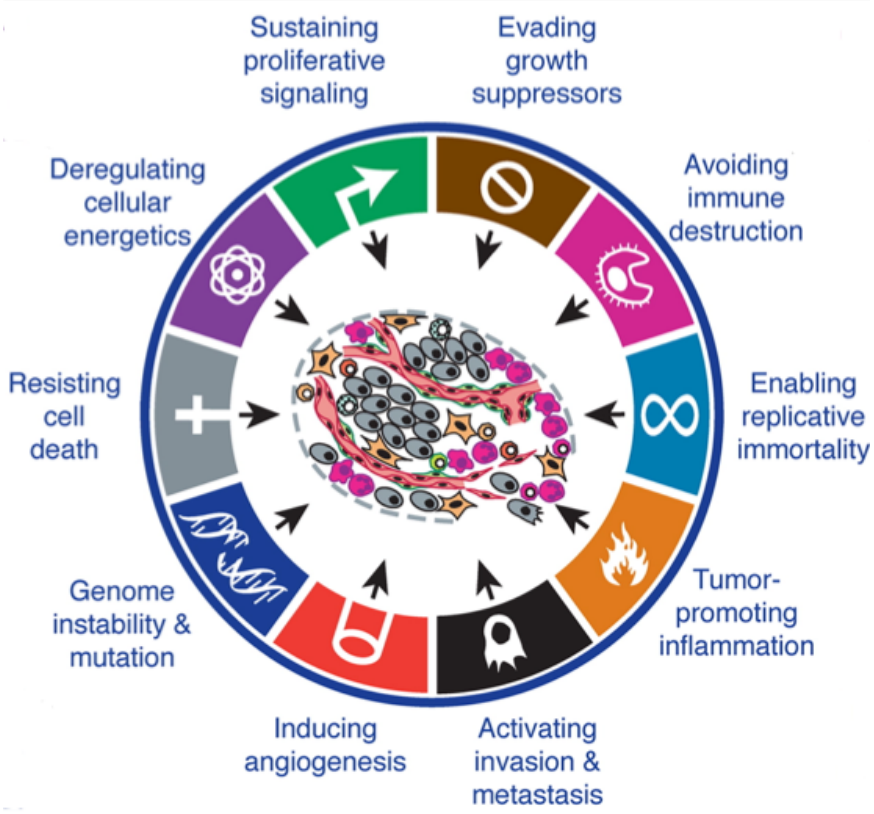


Figure 1-1 **The hallmarks of cancer.**

A cell must become self-sufficient and independent of external growth signalling and develop limitless replication potential whilst sustaining its own vasculature, become insensitive to anti-growth and apoptotic signals and acquire tissue invasive and metastatic characteristics to become cancerous. Image taken from Hanahan and Weinberg 2011.

## 1.2 *Melanoma*

The worldwide incidence of skin cancer has dramatically increased over the past two decades and represents a significant burden and health risk. Skin cancers are categorised as either a Malignant Melanoma, Basal cell carcinoma or Squamous cell carcinoma, the latter two are termed non-melanoma skin cancers (NMSC) and are the most common cancer in the Caucasian population (Alam, Nanda S Fau - Mittal et al. 2011). Basal cell and squamous cell carcinomas are considered mostly benign (Samarasinghe and Madan 2012), however malignant melanoma is a highly aggressive malignancy, with the global incidence of cutaneous malignant melanoma increasing dramatically in the last decade, representing 4% of new cancer diagnosis in the US and accounting for 1-2% of all malignant tumours throughout Europe (Jemal, Siegel et al. 2010).

Caucasian populations are known to be at higher risk of developing malignant melanoma with 20 times the incidence compared to Black and Asian populations, with occurrence more prevalent within female populations worldwide (Bataille and de Vries 2008). However European studies have shown that prevalence is equal between both genders (Bataille 2003, Markovic, Erickson et al. 2007). The site distribution of melanoma incidence differs depending on gender with males presenting malignancies along the trunk, and females presenting primarily on the legs and front torso, which matches the typical patterns of sun exposure for the genders (Markovic, Erickson et al. 2007), with women showing greater survival than men (Lideikaitė, Mozūraitienė et al. 2017).

## 1.3 *Diagnosis, Prognostic factors and staging of malignant melanoma.*

Melanoma is diagnosed using a selection of parameters most often termed as the ABCD or ABCDE criteria (Abbasi, Shaw et al. 2004). Observation of nevi demonstrating Asymmetry (A), Border irregularity (B), non-uniform Colour (C) and a Diameter greater than 6mm (D). This can be further expanded to consider the change of the nevi over time (Evolution and elevated surface – E) (Figure 1-2).

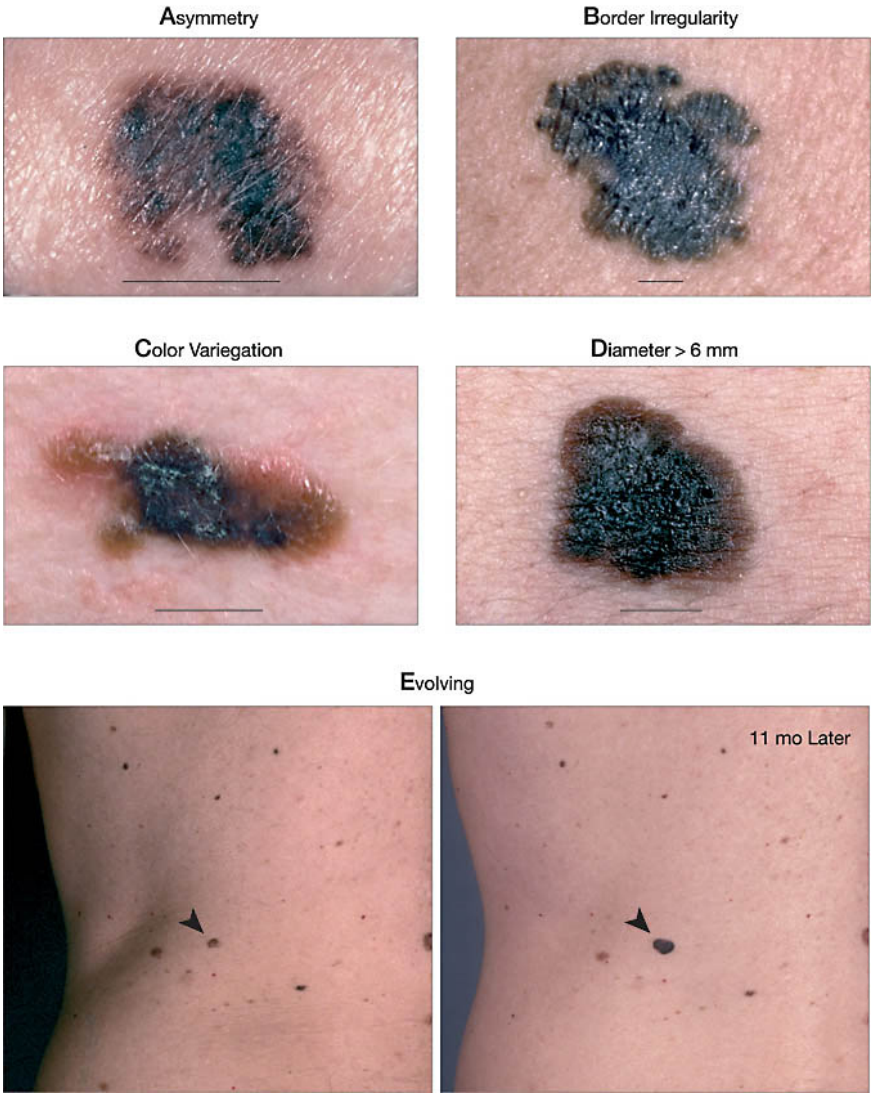


Figure 1-2 ABCDE Criteria for Melanoma Diagnosis

Melanoma is diagnosed using a range of parameters, Asymmetry (A), border integrity (B), colour variation (C), diameter (beyond 6mm) (D) and the change of the nevi over time (E). Taken from (Abbasi, Shaw et al. 2004).

If a potential malignancy is identified via these criteria a further examination is undertaken, involving dermoscopy and or biopsy techniques prior to an evaluation of prognosis of which several methods are employed, ranging from simple techniques such as the measurement of tumour thickness to molecular methods such as the identification of melanoma specific biomarkers (Healsmith, Bourke et al. 1994, Marghoob, Swindle et al. 2003). The most commonly accepted methods are presented below.

### 1.3.1 *Tumour thickness.*

Tumour thickness is one of the most reliable prognostic factors for malignant melanoma. It is, along with lymph node status and metastasis, utilised in the TNM staging system (Balch, Soong et al. 2001, Kim, Reintgen et al. 2002, Balch, Soong et al. 2004) .The determination of tumour thickness is achieved through use of the widely accepted Breslow's tumour thickness system, which involves measuring the tumour from the granular layer through to the deepest point of tumour invasion following resection of the primary tumour. Alternatively, the lesser-used Clark's levels can be used to determine tumour invasion by measuring the level of tumour invasion into the dermal layer (Figure 1-3).

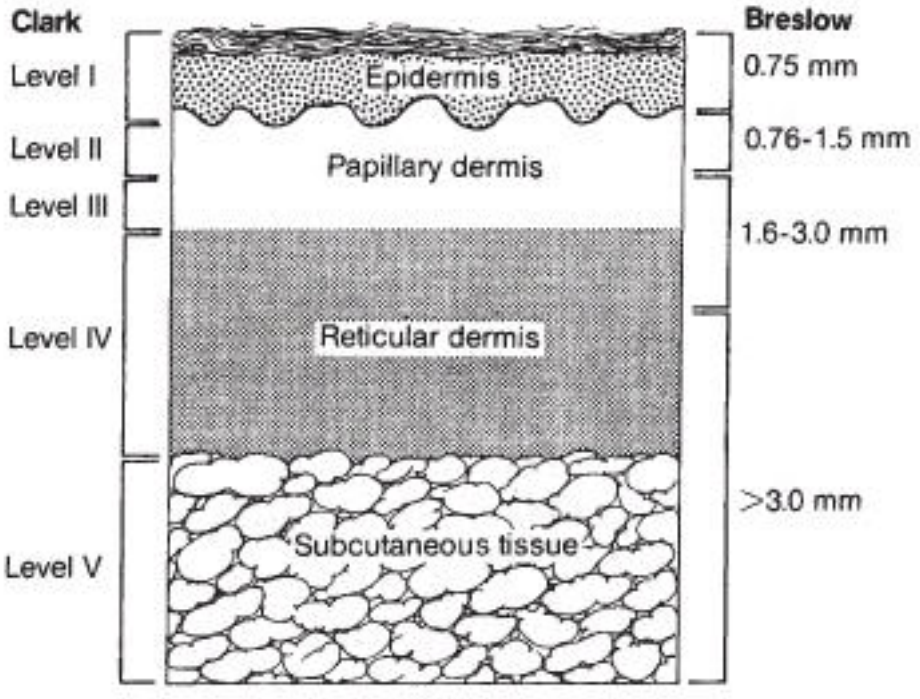


Figure 1-3 **Breslow's tumour thickness and Clarks levels.**

Breslows tumour thickness system measures tumour thickness from the granular layer. Clarks levels determine thickness through the dermal layers. image taken from (Pernick 2002-2009)

### 1.3.2 *Sentinel Lymph node status.*

The presence of metastatic bodies within the sentinel and surrounding lymph nodes of the primary tumour site are another important determinant of prognosis. Sentinel node biopsies (SNB) from patients with a primary tumour thicker than 1mm have shown metastatic bodies in 16-20% of cases. If disease is found in the sentinel lymph then a radical lymph node dissection is undertaken, whereby all of the surrounding lymph nodes are removed, as the likelihood of metastatic bodies being present in further lymph nodes increases by 20% if the sentinel lymph is compromised. The

presence of in-transit metastasis (cellular material from a tumour within the blood stream) is also included in the TNM staging, as it is considered an important indicator of the spread of metastasis throughout the lymphatic system (Balch, Soong et al. 2001, Balch, Soong et al. 2004).

### 1.3.3 *Distant Metastasis.*

Detection of metastasis distant to the site of the initiating primary tumour is a major indicator of patient survival. The prognosis for the patient becomes increasingly poor if the presence of visceral metastastic disease is detected (Ranieri, Wagner et al. 2006). Typically, visceral metastasis from malignant melanoma occurs in the lungs, bone tissue, central nervous system and liver. Detection of metastasis in the soft tissue such as surrounding skin layers and lymph nodes is associated with a less severe prognosis.

### 1.3.4 *Ulceration.*

Ulceration of the epidermis is strongly associated with tumour thickness. Between 20 and 60% of all primary melanomas ulcerate, with ulceration occurring at depth as well as at the surface of the primary site. Ulceration is associated with poor survival and disease recurrence so much so, that it can be employed as an independent indicator of survival (Melanoma Molecular Map Project 2010).

Several other factors including regression, age, lymphovascular invasion, tumour infiltrating lymphocytes, mitotic rate and histological type can also be used in prognosis, however they are not universally adopted into any prognostic system due to low instance or conflicting results (Balch, Gershenwald et al. 2009).

### 1.3.5 *TNM staging.*

The TNM staging system is the most widely accepted classification method for Malignant Melanoma. The system is dependent on several factors; tumour thickness (T) (as well as ulceration), sentinel node status (N) (including the presence of in-transit metastasis), and the presence of distant metastasis (M). This information is used to categorize the disease into four primary stages and nine sub-stages to determine a prognosis (Table 1-1) (Deichmann, Benner et al. 1999, Balch, Soong et al. 2001, Balch, Soong et al. 2004, Melanoma Molecular Map Project 2010, Zhuang, Scolyer et al. 2010).

<table>
<tr><th></th><th>T1a</th><th>T2a</th><th>T1b</th><th>T2b</th><th>T3a</th><th>T4a</th><th>T3b</th><th>T4b</th><th></th></tr>
<tr><td>N0</td><td>1a</td><td colspan="2">1b</td><td colspan="2">2a</td><td colspan="2">2b</td><td>2c</td><td>Stage 1-2</td></tr>
<tr><td>N1a</td><td colspan="2" rowspan="2">3a</td><td colspan="2" rowspan="2">3b</td><td colspan="2" rowspan="2">3a</td><td colspan="2" rowspan="2">3b</td><td rowspan="5">Stage 3</td></tr>
<tr><td>N2a</td></tr>
<tr><td>N1b</td><td colspan="2" rowspan="2">3b</td><td colspan="2" rowspan="2">3c</td><td colspan="2" rowspan="2">3b</td><td colspan="2" rowspan="2">3c</td></tr>
<tr><td>N2b</td></tr>
<tr><td>N2c</td><td colspan="8">3b</td></tr>
<tr><td>N3</td><td colspan="8">3c</td></tr>
<tr><td>M1a</td><td colspan="8" rowspan="3">4</td><td rowspan="3">Stage 4</td></tr>
<tr><td>M1b</td></tr>
<tr><td>M1c</td></tr>
</table>

Table 1-1 The TNM staging system.

Tumour thickness, sentinel node status and the presence of metastasis are used to predict clinical outcome. (Deichmann, Benner et al. 1999, Balch, Soong et al. 2001, Balch, Soong et al. 2004, Melanoma Molecular Map Project 2010, Zhuang, Scolyer et al. 2010)

Typically, Stage 1 melanomas and tumours in situ (Tis) have a 90-95% survival rate at 5 years However as the stages progress tumour thickness increases and the involvement of ulceration, nodal in transit cell clusters and metastasis are included within the staging, leading to the clinical outcome and survival diminishing significantly (Balch et al., 2004, Balch et al., 2001).

Stage 2 tumours have a varied outcome, as the presence and extent of ulceration is considered as well as tumour thickness. 5-year survival immediately drops to 78%

and can be as low as 45% if a tumour is found to be approximately 4mm in the presence of ulceration (Balch et al., 2004, Balch et al., 2001).

Stage 3 is determined by lymph node involvement. The severity is gauged through the number of nodes involved (eg. N1 for one node) and whether they present micro-metastasis (detected during pathological examination) or macro-metastasis, identified during surgical resection or detected outside of the lymph node capsule during pathological examination. (N1a for micro metastasis and N1b for macro metastasis.) 5 Year survival at stage 3 is 49% on average, with the worst possible combination (N3) consisting of 4 or more presenting nodes resulting on only 13% survival (Balch et al., 2004, Balch et al., 2001).

The 5-year survival by stage 4 is bleak, with a survival rate of only 9%, with only 6% of patients surviving past 5 years. The severity and sub-staging is determined by the presence of and position of distant metastasis. M1 indicates the presence of distant metastasis. With subcategories 1a,b and c indicating the position of the metastasis, either at the skin or subcutis and surrounding lymph nodes (1a), at the lung (1b) or further into visceral tissue (1c). The staging is automatically set at 1c if there is evidence of elevated lactate dehydrogenase (LDH) at any of the metastatic sites (Deichmann, Benner et al. 1999, Balch, Soong et al. 2001, Balch, Soong et al. 2004, Balch, Gershenwald et al. 2009, Melanoma Molecular Map Project 2010, Zhuang, Scolyer et al. 2010) .

## 1.4 *Genetics and Growth Characteristics.*

One of the hallmarks of melanoma is the highly defined pattern of tumour development and progression following a very specific growth pattern, with the tumour spreading radially from a nevus (skin mole) following initiation, before undergoing vertical growth and eventually developing increased vascularity allowing for clusters of cells to metastasize to distant sites (Figure 1-4).

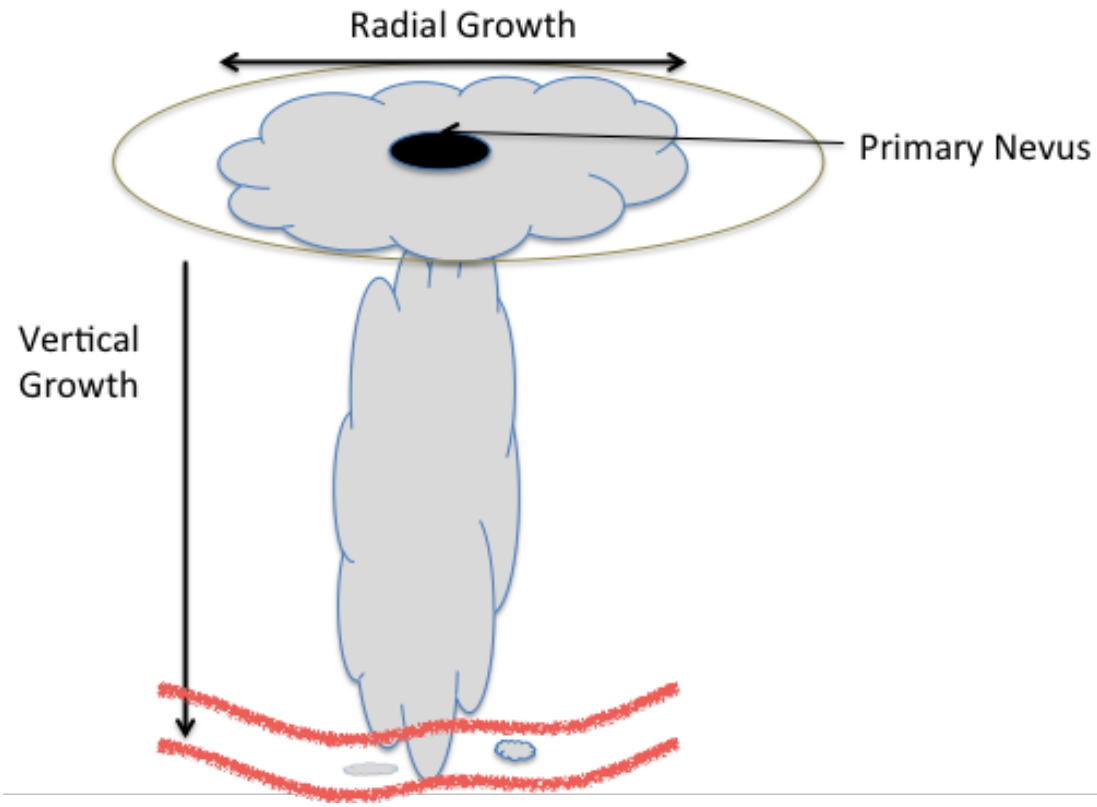


Figure 1-4 **Malignant melanoma growth characteristics**

Following tumour initiation, malignant cells grow radially across the surface of the skin before descending vertically into the dermal layers where cell clusters detach upon vascularization.

### *1.4.1 Initiation and Progression.*

The initiation and progression of melanoma tumours is well defined, with the majority of malignancies following a highly conserved pattern of mutation through a number of molecular pathways (Figure 1-4), leading to radial and vertical growth as well as metastatic spread. The primary risk factor associated with melanoma development is exposure to high intensity UV from primarily the UV-B wavelength of the ultra violet spectrum, typically associated with doses received in situations such as sunbathing in climates with higher than yearly average UV exposure as well as the use of artificial tanning lamps such as those utilised in sun beds (Kadekaro, Kavanagh et al. 2003, Cust, Armstrong et al. 2011).

There is increasing evidence that certain genetic dispositions may also leave individuals more susceptible to the development of melanoma. Mutations in genes such as CDKN2A and CDK4 are prevalent in as many as 25% of familial melanoma. (Bataille 2003) CDK4 is a significant regulator of G1 phase cell cycle progression via regulation of the Rb signalling pathway and CDKN2A codes for 2 proteins, p16 and p14ARF which act not only with the Rb pathway but also as a stabiliser of p53, governing apoptosis. Indeed mutations of both RB and p53 associated pathways has been heavily implicating in many primary tumours (Sherr and McCormick 2002, Burkhart and Sage 2008) however some studies have shown familial mutations in these regions to be rare, with estimates of penetrance in the population of approximately 0.01% (Bataille 2003).

Within the wider population, proteins within the mitogen-activated protein kinase pathway (MAPK) are heavily implicated in melanoma tumour development; 90% of both benign and malignant neoplasms have been shown to exhibit genetic aberrations in both NRAS, a G-protein which signals in response to mitogen receptor activation (c-kit) and BRAF, a serine/threonine kinase commonly phosphorylated by NRAS. Aberrations of this nature result in constitutive activation of the MAPK pathway, promoting aberrant growth and differentiation within melanocytes (Benjamin, Melnikova et al. 2007, DeLuca, Srinivas et al. 2008, Fecher, Amaravadi et al. 2008).

Further, acquisition of mutations to NRAS and/or BRAF have been linked to loss of function of the PTEN tumour suppressor gene, the protein product of this gene has protein and lipid phosphatase activity which are responsible for the proteins involvement in both G1/S cell cycle control and tumour suppression (Wu et al., 2003,). Inhibition or loss of PTEN typically signifies the advancement of a nevi towards radial and vertical growth via deregulation of the cell cycle, as well as activation of AKT and anti-apoptotic proteins prior to metastasis (Tsao et al., 2000 Shao and Aplin, 2010,) which is followed by the expression of motility and pro angiogenic factors such as VEGF, MMP2 and FGF, enabling metastatic spread (Tsao, Zhang et al. 2000, Wu, Goel et al. 2003, Benjamin, Melnikova et al. 2007, Govindarajan, Sligh et al. 2007, Melnikova and Bar-Eli 2008, Madhunapantula and Robertson 2009, Shao and Aplin 2010).

## 1.5 *Current treatments.*

Current clinical options such as surgery are effective if the melanoma is detected early, however, chemotherapy and immunotherapy are employed in later stage malignancies, typically stage 3 and 4 tumours (according to the Breslow index) (Martinez and Otley 2001; Garbe, Eigentler et al. 2011), as the chances of metastasis in this cohort are greatly increased (Keilholz, Punt et al. 2005, Giblin and Thomas 2007, Gogas, Kirkwood et al. 2007). If metastasis is detected, the mean patient survival is only 6-9 months (Gogas, Kirkwood et al. 2007). This poor patient survival rate highlights the current lack in effective treatment for malignant melanoma and emphasizes the need to develop more effective patient therapies.

### *1.5.1 Chemotherapy agents.*

Late detection of melanoma usually results in metastasis, and as such a variety of chemotherapeutic agents have been utilised in the treatment of metastatic melanoma with limited success.

#### 1.5.1.1 *Dacarbazine.*

Dacarbazine (DTIC) is currently viewed as the benchmark treatment for melanoma and is the only cytotoxic drug to be approved by the FDA for the treatment of melanoma (Lui, Cashin et al. 2007). DTIC is a non-cell cycle specific prodrug, which is

converted via cytochrome P450 in the liver to form 5-[3-hydroxymethyl-3-methyl-triazen-1-yl]-imidazole-4-carboxamide (HMMTIC) and 5-[3-methyl-triazen-1-yl]-imidazole-4-carboxamide (MTIC) (Reid, Kuffel et al. 1999). DTIC is administered intravenously with a half-life of approximately 40 minutes (Marchesi, Turriziani et al. 2007), creating toxicity via the generation of methyl adducts to the $O^6$, $N^3$ or $N^7$ purine bases of DNA, resulting in disruption of the $O^6$- guanine –alkyl- transferase (MGMT) and Mismatch repair (MMR) DNA repair enzymes, this results in inaccurate cellular replication, as well as inhibition of DNA repair, resulting in tumour cell apoptosis (Turriziani, Caporaso et al. 2006, Gogas, Kirkwood et al. 2007, Lui, Cashin et al. 2007, Marchesi, Turriziani et al. 2007)

There has been a multitude of studies and clinical trials into the efficacy of Dacarbazine for the treatment of melanoma since its introduction, however despite the compounds popularity and continued therapeutic use, the effectiveness of DTIC is highly variable (Chapman, Einhorn et al. 1999). As a single agent Dacarbazine is administered at standard doses of between 150 mg/$m^2$ and 250 mg/$m^2$ as daily treatments over 5 days, has consistently demonstrated response rates of between 12 and 25%, many of these only being partial responses (Breithaupt, Dammann et al. 1982, Eggermont and Kirkwood 2004, Keilholz, Punt et al. 2005, Schadendorf, Ugurel et al. 2006, Gogas, Kirkwood et al. 2007, Lui, Cashin et al. 2007). The mechanisms for resistance to Dacarbazine have only recently begun to be elucidated. In vitro studies have shown that treatment with Dacarbazine can result in the overexpression of Interleukin IL-8, VEGF as well as NFKB, which may facilitated resistance to cytoxic compounds, with studies demonstrating enhanced tumour growth *in vivo* following treatment (Lev, Ruiz et al. 2003, Lev, Onn et al. 2004). The drug is currently being

investigated for melanoma treatment in combination with other known anticancer agents such as Cisplatin to explore the compound effectiveness as a combination therapeutic (Guven, Kittler et al. 2001) (Keilholz, Punt et al. 2005)

#### 1.5.1.2 *Temozolomide.*

Temozolomide (TMZ) is structural and functional relative of DTIC, acting as an alkylating agent TMZ is activated at physiological pH conditions rather than via the P450 cytochrome, and is capable of passing through the blood brain barrier, making it a viable treatment option for brain metastasis. The compound is orally administered with plasma concentrations peaking at 30-90mins following ingestion. Typically, a therapeutic dose of 200mg/$m^2$ per day is followed for a 5-day cycle (Marchesi, Turriziani et al. 2007). A recent review by Quirt, Verma et al. 2007 collated and reviewed results from 21 phase I and II trials as well as 2 randomized phase III and 3 randomized phase II trials involving TMZ in comparison to DTIC, including combinations with interferon**α** and thalidomide. The study concluded that TMZ is comparable to DTIC in terms of response rate and survival, producing higher response rate as part of a combination with other agents. However this improved patient response did not translate into a significant increase in over-all survival (Quirt, Verma et al. 2007). These results indicate that although TMZ may prove a viable alternative to DTIC for the treatment of melanoma, particularly in cases of brain metastasis, it is not yet demonstrating proven melanoma cures either as single agent or in combination with other chemotherapeutics and thus emphasizing the need for novel and effective new treatment modalities.

## 1.6 *Drug Combinations.*

Due to the low response rates by melanoma patients for both Dacarbazine and Temozolomide, chemotherapy combination therapy is widely used in an effort to improve patient survival. A range of compounds have shown modest single agent efficacy in clinical trials, this has subsequently been included in combination therapies in order to improve patient outcome.

Of these compounds, platinum-based drugs such as cisplatin and carboplatin achieve slightly improved patient responses when compared to Dacarbazine alone. cisplatin achieves a 15% partial response rate as a single compound and carboplatin 19%. As part of novel combination therapy regimes with WR-2721, a thiol derivative with potential host protective benefits. Cisplatin has achieved much higher responses rates (mostly partial) of ~53%, however phase II clinical trials of this combination resulted in unacceptable levels of toxicity in the renal and gastrointestinal systems as well as ototoxicity (Guven, Kittler et al. 2001)

Taxanes (Paclitaxil), nitrosurea compounds (carmustine/fotemustine) and alkaloids (Vinblastine) all show similar patient responses as single agents to dacarbazine and platinum compounds. More promisingly, a Paclitaxil derivative (Abraxine) (Nyman, Campbell et al. 2005) has shown a 27% response rate in patients during a phase II clinical trial (Gogas, Kirkwood et al. 2007, Bhatia, Tykodi et al. 2009).

Double combinations of these compounds with Dacarbazine did not however yield particularly high responses (10-20%) and as a consequence, triple and quadruple regimens have been developed, such the CVD (cisplatin, vinblastine and dacarbazine) and the Dartmouth regimen (4 drug dacarbazine, cisplatin, carmustine, and tamoxifen) (Bhatia, Tykodi et al. 2009).

Phase II trials of the CVD regimen demonstrated an encouraging 40% response rate in advanced melanoma patients. (Legha, Ring et al. 1996). However, phase III trials of CVD failed to determine a statistically significant difference in response rate to Dacarbazine alone. (Gogas, Kirkwood et al. 2007) Trials involving the use of CVD and Immunotherapy improved complete responses to 22% when used as a sequential therapy (Legha, Ring et al. 1996).

The Dartmouth regimen also produced encouraging phase II results, 46% of late stage melanoma patients responded to therapy with mostly partial responses. However as with CVD, Phase III trials failed to determine any significant advantage for using the regimen over Dacarbazine (Chapman, Einhorn et al. 1999, Gogas, Kirkwood et al. 2007).

Despite the use of these agents both singularly and in combination, patient responses and survival remain low. Patient side effects can also be quite severe ranging from fatigue to alopecia and myelosuppression and neuropathy (Legha, Ring et al. 1996, Gogas, Kirkwood et al. 2007, Bhatia, Tykodi et al. 2009). It is evident from these results that more effective chemotherapy regimens are urgently required.

## 1.7 ***Radiotherapy.***

The apparent radio-resistant nature of melanoma tumours is well documented, with initial observations of resistance dating back to dermatological studies from the 1920's (MacKee GM 1921), prompting a reluctance to utilise radiation therapy for melanoma tumours. This has resulted in a prolific acceptance of resistance, despite a lack of clinical or biological evidence for this reasoning.

In point of fact, recent clinical reviews and *in vitro* studies are challenging the dogma and demonstrating that melanoma tumours do in fact respond to radiation therapy when utilised appropriately (Stevens and McKay 2006, Khan, Khan et al. 2011). When considering the apparent resistance of the tumours to radiation, it is considered that a number of factors may play a role in the persistence of the disease. For example, studies using mouse xenografts have noted that the frequency of metastasis was higher in tumours which have been subjected to sub lethal doses of X-ray radiation, finding that radiation induced hypoxia results in up regulation of the Urokinase-Type Plasminogen Activator Receptor, commonly recruited in metastastic disease (Rofstad, Mathiesen et al. 2004), which may account for the aggressive recurrence of the tumour.

Also, it has been noted that the anti-apoptotic pathways within melanoma such as those involved in the NFκB, PI3K-AKT and COX-2 pathways are highly active, resulting in greater levels of survival following radioactive assault (Johnson, Ivanov et al. 2008) which is compounded by the inherent expression of repair genes involved in double strand repair such as RAD51, RAD52, BRCA1 and BRCA2 (Jewell, Conway et al. 2010).

There are also inherent difference and variability in tumour population, some cells may be expressing melanin, which is a known radioprotective mechanism, and there is an increasing body of evidence demonstrating that highly resistant progenitor cells, commonly referred to as cancer stem cells, may also have involvement in initiation and recovery following irradiation (Kinnaert, Morandini et al. 2000, Fang, Nguyen et al. 2005, Pajonk, Vlashi et al. 2010).

These observations serve to highlight the potential benefits for further study of radiation therapy for melanoma, particularly in respect to the delivery of lethal doses to a tumour mass and the use of radiosensitisers to abrogate resistance pathways to better overcome potential resistance mechanism which may be present within the tumour burden.

## 1.8 *Novel Targeted radiotherapy methods for malignant melanoma.*

Targeted radiotherapy presents a novel potentially effective approach to malignant melanoma therapy, whereby a specific molecular characteristic of tumour cells are exploited as a way of introducing a variety of radionuclides conjugated to tumour specific carrier (radiopharmaceuticals) into the tumour cells in a highly tumour specific manner, allowing for a concentrated accumulation of a radiotherapeutic dose.

Tumour seeking compounds utilised in targeted radiotherapy can be conjugated to a variety of radionuclide's which are selected based upon the desired decay emission. This is typically either an $\alpha$ particle, $\beta$ particle or Auger electron emitter which are considered to bear the most clinical benefit vs X-ray irradiation (Ward 1988). The amount of energy deposited by each of these particles over a given distance is termed the linear energy transfer (LET), stated in terms of electron volts (keV) per unit of distance ($\mu$m) (Pearson, Jan et al. 2012). The higher the LET, the more damage a particle will do over a shorter distance. This information is vital when considering the target cell type, size of tumour and carrier molecule as each of these factors can have an impact on the amount of radioactivity delivered.

For example, $\alpha$ particles such as 211 Astatine ($^{211}$At) carry a high LET (100 keV/ $\mu$m) but can only travel a short distance (50-80$\mu$m), making them best suited to imaging and treating micrometastases using a carrier molecule that will deliver the particle within the cell membrane (Zalutsky and Pozzi 2004, Mulford, Scheinberg et al. 2005). Similarly, $\beta$ particles such as 131 Iodine ($^{131}$I) have a lower LET of 0.2 keV/$\mu$m but travel a much larger distance (0.8mm) making them better suited to larger solid tumours (Gudkov, Shilyagina et al. 2016), and auger electron emitters which have a moderate LET of 4-25 keV/$\mu$m but are extremely low energy with a path length of (0.6-17$\mu$m) such as 123 Iodine ($^{123}$I) (Behr, Sgouros et al. 1998) are best suited for delivery methods that reach the nucleus (Donoghue and Wheldon 1996, Pouget, Lozza et al. 2015). Table 1-2 details these radionuclides, their decay particles and clinical applications.

| Radionuclide | Decay Particle | Particle Range | LET | Half Life | Clinical Application |
|---|---|---|---|---|---|
| $^{123}$I | Auger Electron | 0.6-17um | 4-25 keV/μm | 13 hours | Imaging/Therapy |
| $^{131}$I | β Particle | 0.8mm | 0.2 keV/μm | 8 days | Therapy |
| $^{211}$At | α Particle | 50-80um | 100 keV/μm | 7 hours | Imaging/Therapy |

Table 1-2 Decay Particles of radionuclides

Radionuclides with differing LET values are selected based upon the desired clinical application. Information was taken from (Zalutsky and Pozzi 2004, Gudkov, Shilyagina et al. 2016) and (Behr, Sgouros et al. 1998)

### *1.8.1 Melanin and its potential as a therapeutic target.*

The selection of a cellular target that will facilitate the entry of radionuclides into the cell is vital to targeted radiotherapy treatments. In the case of melanoma, melanin production and the molecular machinery involved in melanogenesis are increasingly popular targets (Farmer, Gidanian et al. 2003) due to melanomas relatively unique ability to synthesize the pigment throughout tumorigenesis (Lazova and Pawelek 2009). Which is due to the genetic alterations that take place throughout melanoma progression, where by mutations of the BRAF$^{V600E}$ and NRAS (Kadekaro, Kavanagh et al. 2003, DeLuca, Srinivas et al. 2008) proto-oncogenes results in elevated melanogenesis via ERK1/2 induced constitutive activation of the melanogenesis regulating gene MITF (Goding 2000).

This enhanced melanin expression is coupled with the presence of aberrant melanosomes within many melanotic tumours, which leak melanin and the by-products of melanogenesis into the cytoplasm (Fruehauf and Trapp 2008).

#### 1.8.1.1 *Melanogenesis.*

Human melanocytes generate melanin within the melanosome, A specialised vesicle, which provides short term storage for synthesized melanin and contains the metabolic machinery required for melanin synthesis (Sulaimon and Kitchell 2003).

The amino acid tyrosine is the primary and rate limiting substrate in melanogenesis. Tyrosine undergoes a twostep enzymatic process via tyrosinase, initially generating Dopa and resulting in the production of Dopaquinone (Borovanský J. 2011).

Dopaquinone is further converted to either Dopachrome or Cystienyldopa, at which point Dopachome is further processed via the tryp1 enzyme to eventually form the darkly pigmented Eumelanin. Cystienyldopa is produced as a result of the interaction of glutathione with the dopaquinone molecule, leading to the production of lightly pigmented Pheomelanin (Figure 1-5).

As melanoma progresses tryp1 activity is eventually lost due to MITF suppression (Salti, Manougian et al. 2000), shifting the ratio of eu/pheomelanin in favour of pheomelanin, resulting in further oxidative stress.

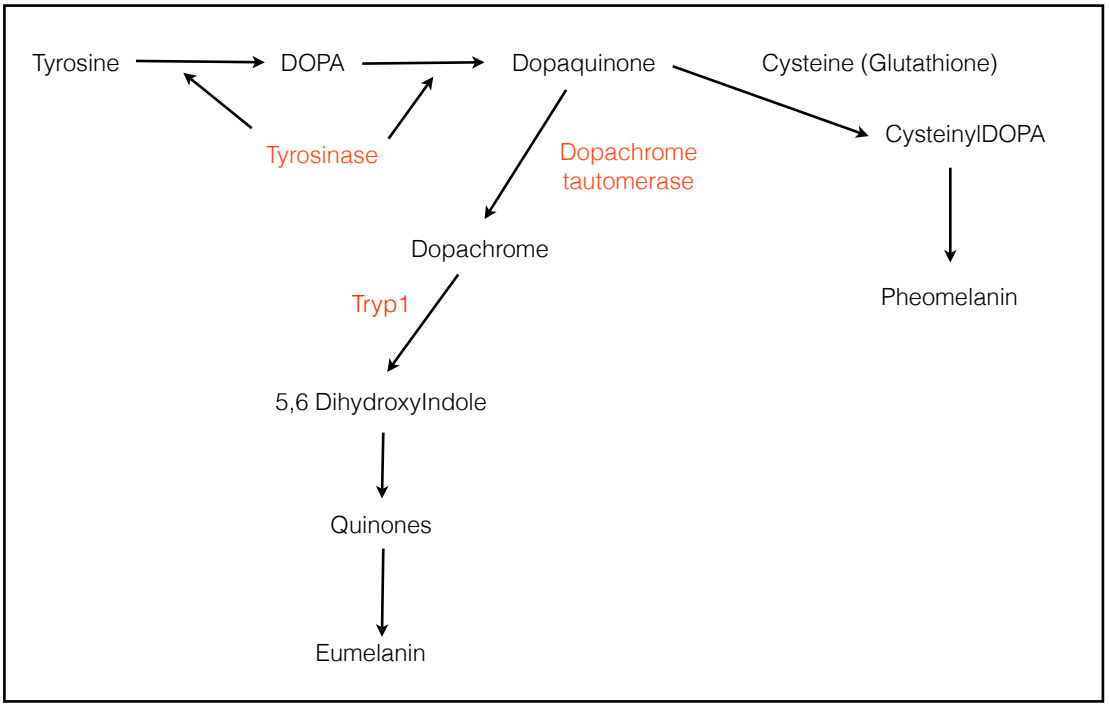


Figure 1-5 **Melanin production.**

The amino acid tyrosine is used as a starting material by melanocytes to generate either the darkly pigmented Eumelanin or lightly pigmented Pheomelanin. As melanoma progresses to a metastatic state tryp1 activity can be lost, shifting melanin production in favour of Pheomelanin.

### 1.8.1.2 *Properties of Melanin.*

Melanin is a pigmented polymer generated by tyrosine oxidation. It has long been associated with the photoprotective role of melanocytes and there are two forms of melanin produced eumelanin and pheomelanin. Eumelanin in particular demonstrates the potential to absorb and dissipate 99.9% of UV radiation in the form of heat (Meredith and Riesz 2004).

### 1.8.1.3 *Eumelanin.*

The darkly pigmented Eumelanin is typically produced in larger quantities than pheomelanin and is believed to confer the most protective benefit to the cell compared to pheomelanin (Prota 2000). Demonstrating antioxidant activity and the ability to scavenge reactive oxygen species and other excitable molecules (Meredith and Sarna 2006).

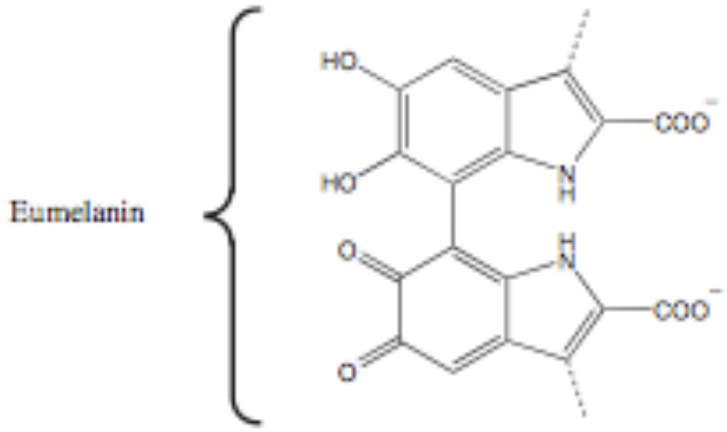


Figure 1-6 A schematic of eumelanin.

The eumelanin molecule consists of a high number of indole groups Taken from Riley 2003

### 1.8.1.4 *Pheomelanin.*

Pheomelanin is lightly pigmented, requiring reduced glutathione and cysteine for synthesis (Ando, Kondoh et al. 2007). In contrast to eumelanin, pheomelanin is thought to contribute to UV induced damage, demonstrating photolysis upon UV exposure, generating super oxide anions and hydroxyl radicals (Chedekel, Smith et al. 1978). pheomelanin also interacts with oxygen, zinc and copper; further generating

hydroxyl and superoxide anions (Farmer, Gidanian et al. 2003) which is thought to contribute to tumour progression (Gidanian, Mentelle et al. 2008). The ratio of pheomelanin rises as melanoma progresses (Hearing 2000), as the tyrosinase 1 (TRYP1) enzyme function is lost, and eumelanin production stops, with many tumours losing the capacity to produce melanin entirely (Fang, Tsuji et al. 2002). Interestingly, Benzamides demonstrate a higher ionic binding capacity to pheomelanin, due to the high number of carboxyl groups present (Labarre, Papon et al. 2002).

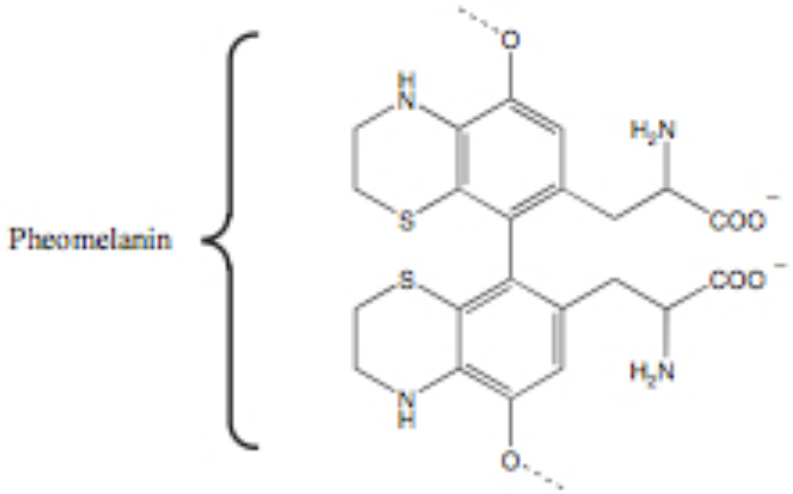


Figure 1-7 The structure of Pheomelanin.

Taken from Riley 2003.

## 1.9 Targeting melanin with radiopharmaceuticals.

Melanin contains many anionic carboxyl groups, aromatic rings and quinone structures which facilitate the binding of cationic compounds such as amines and metals via a complex combination of ionic bonding, hydrophobic interactions and Van Der Waals attractions, which are not yet fully understood (Larsson 1993, Bridelli, Ciati et al. 2006). However, this inherent binding capability makes melanin an attractive

target when considering the treatment of melanoma tumours with radiotherapy in the form of targeted radiopharmaceuticals.

One of the earliest reported melanin binding compounds investigated is Methylene Blue, a polycyclic phenothiazinium dye with a high binding affinity to melanin (Potts 1964) and structure which enables conjugation to radionuclides (Blower, Clark et al. 1997). A variety of radionuclides have been conjugated to methylene blue with the use of $\alpha$ particle emitter $^{211}$Astatine, and the Auger emitter $^{125}$I conjugated versions of the compound first being reported in animal models by Link, Brown et al. 1989 who noted a maximum accumulation of the both $^{221}$At and $^{125}$I forms after 1hr of exposure in murine B16 melanoma cells, localising in the melanosomes surrounding the nucleus. When translating this to a therapeutic setting *in vivo* the $^{221}$At methylene blue form demonstrated significantly higher reduction of lung tumour number when compared to the $^{125}$I form, where it was noted that the differences in emission (auger electon vs. $\alpha$ particle) had resulted in a much higher therapeutic efficacy, reducing the tumour burden by 97%. Following further investigation using human xenografts $^{221}$At methylene blue has also demonstrated considerable tumour growth delay in both cutaneous tumours, lymph node and micro metastasis (Link and Carpenter 1990, Link and Carpenter 1992).

The $\beta$ emitter $^{131}$Iodine has also been conjugated to methylene blue (Sobal, Rodrigues et al. 2008). Bio distribution studies in human melanoma patients using $^{131}$I methylene blue reported a rapid 90% decline in detectable radioactivity in blood samples but a 4 fold increase in retention by metastatic brain tumours vs the levels

seen in normal surrounding tissue (Link, Costa et al. 1996). Further bio distribution data showed that within 2.5 minutes the highest organ uptake observed was in the liver, kidneys and thorax. Both liver and thorax cleared the radioactivity rapidly within 1.5 hrs with kidney clearance occurring over 24 hrs. These results were encouraging and $^{131}$I methylene blue alongside the $^{211}$Astatine conjugated variant was reported to have entered phase 1 clinical trials in 1999 (Link 1999).

More recently it has become apparent that methylene blue acts as a monoamine oxidase inhibitor (MOA inhibitor) and can induce significant toxicity within the central nervous system following intravenous injection in combination with serotonin reuptake inhibitors (SSRI) (Gillman 2010, Gillman 2010, Stanford, Stanford et al. 2010, Gillman 2011). These findings, along with a recent report by the CDC into the number of people being prescribed SSRI's demonstrating a 400% increase in usage within the United States between 1994 and 2008 (Laura A. Pratt 2011) may pose a significant hurdle in developing methylene blue based radiopharmaceuticals for use in the general population.

The observation that melanotic tumours accumulate melanin in the extracellular space surrounding the tumour mass either as a result of melanin being distributed by a cellular process or via the release of melanin from necrotic cells has been reviewed by Dadachova, Nosanchuk et al. 2004 who have developed a melanin binding murine generated antibody (6D2) for the targeting of melanoma *in vivo*. (Dadachova, Revskaya et al. 2008) The 6D2 antibody has been radiolabelled with the $\beta$ emitter $^{188}$ Rhenium and assessed in both eumelanotic (MNT1) and pheomelanotic (A2058) nude

mouse models, demonstrating tumour specific retention of the antibody, the studies noted significant tumour growth delay following administration of 0.5-1.5 mCi of $^{188}$Re-6D2 conjugated antibody in A2058 xenografts and 1.5mCi in MNT1 xenografts (Dadachova, Nosanchuk et al. 2004, Dadachova, Revskaya et al. 2008).

In a study which targets melanin directly, the use of a radio labelled quinoxaline salt, termed ICF01012, which was conjugated with the auger emitting isotope Iodine-125 and the β emitting isotope Iodine-131 for imaging and therapy respectively was reported (Bonnet-Duquennoy, Papon et al. 2009) . Quinoxaline is a derived from N-(2-diethylaminoethyl)-2-iodobenzamide ($BZA_2$), member of the benzamide compound family (BZA) which are noted for high retention in melanotic cells. Chezal et al., 2008,)

It is considered that benzamide molecules such as $BZA_2$ and Quinoxaline most likely binding as a result of the high aromatic ring structures of both compounds interacting, uptake and release studies using synthetic tyrosine-melanin have indicated that the ionic interactions involving the positively charged BZA compounds and the previously described anionic carboxyl groups of melanin, as well as hydrophobic interactions between BZA and melanin involve two distinct modes of binding, the higher levels of carboxyl groups within pheomelanin may increase susceptibility to ionic interactions with BZA compounds (Kadekaro et al., 2003 Labarre et al., 2002). For this reason, alongside low patient toxicity BZA are often conjugated to iodine-125 and used as tracers for stage IV Melanoma. (Michelot, Moreau et al. 1991, Michelot, Moreau et al. 1993, Moreau, Michelot et al. 1995)

Bonnet-Duquennoy et al., 2009's investigation used *In vivo* nude mouse xenograft models of the human M4beu melanotic metastatic tumour line alongside C57BL/6J mouse B16F0 non-metastatic and B16Bl6 metastatic tumour mouse melanoma xenografts with the aim of determining the bio distribution and cellular localization of ICF01012. Using secondary ion mass spectroscopy (SIMS) the study demonstrated co-localization of [$^{125}$I]ICF01012 with melanin polymers within B16F0 cells, with retention being observed up to 8 days post exposure. Additionally, therapeutic assessment of [$^{131}$I]ICF01012 using the human M4beu xenograft model demonstrated significant tumour growth delay vs. untreated controls following 2 doses of [$^{131}$I]ICF01012.

Further to this, C57BL/6J mouse xenografts demonstrated significant tumour growth delay in radiopharmaceutical treated mice and an absence of lung metastasis in the [$^{131}$I]ICF01012 treated B16B16 metastatic model when compared to untreated controls. later comparisons of the uptake and therapy potential of ICF01012 in human melanotic M4Beu and SK-MEL-3 tumour cell lines to human amelanotic A375 and M4Dau tumour cell lines, foundthat [$^{125}$I]ICF01012 was only retained in the melanotic lines M4Beu and SK-MEL-3 both *in vitro* and *in vivo* as nude mouse xenografts. [$^{125}$I] ICF01012 was seen to be unretained *in vitro* and undetectable *in vivo* in both the A375 and M4Dau xenograft models (Bonnet, Mishellany et al. 2010).

Therapy studies published in the same paper demonstrated a high susceptibility of both SK-MEL-3 and M4Beu nude mouse xenografts to [$^{131}$I]ICF01012 administration,

noting in the SK-MEL-3 model that tumour doubling times were delayed from 10.8 ± 2.4 days to 18.1 ± 3.3 days following a single dose of the compound. This was compared to no observed difference in tumour growth dynamics in the M3Dau xenograft model using the same single dose schedule, serving to demonstrate potential of $^{131}$I conjugated benzamides for the treatment of pigmented melanoma tumours (Bonnet, Mishellany et al. 2010) .

### *1.9.1 [$^{131}$I]MIP-1145.*

$^{131}$I Benzo[1,3]dioxole-5-carboxylic acid[4-(2-diethylamino-ethylcarbamoyl)-2-iodo-5-methoxy-phenyl]-amide (termed $^{131}$I MIP1145 and named 'Solazed' commercially) was initially identified by Schering Plough (Germany) following a large scale screen of novel benzamides as having a comparatively higher melanin binding affinity than other molecules in the benzamide family.

This compound has been further developed by Molecular Insight Pharmaceuticals (Boston) where (Joyal, Barrett et al. 2010) describe that [$^{131}$I]MIP-1145 bound *in vitro* with high affinity and retention to SK-MEL-3 melanotic tumour cells, with 43% of total compound administered retained vs. 13% retained in the equivalent A375 amelanotic cell cultures. This evidence of a preference for melanotic tissue was further consolidated when the nude mouse xenograft model of SK-MEL-3 demonstrated considerable retention of [$^{131}$I]MIP-1145 at 5.91 $\pm$3.94 %ID/g 24hrs

post inoculation vs. 0.04 ±0.02 %ID/g in A375 xenografts at the same time point (Joyal, Barrett et al. 2010).

A considerable reduction in tumour growth was observed following dosing schedules of 1 inoculation of [$^{131}$I]MIP-1145 a week over 1,2 or 3 weeks . At 35 days post inoculation 1 dose of [$^{131}$I]MIP-1145 resulted in a 79% reduction in tumour volume vs. the saline controls. This effect was further exemplified in the 2 dose and 3 dose groups which saw tumour regression rates of 11% and 40% respectively at day 35 (Joyal, Barrett et al. 2010).

Further dosimetry in rhesus monkeys noted the dose limiting organ to be the lower large intestine and doses of up to 16GBq as tolerable, whilst mouse xenograft results suggested that the anticipated effective dose to humans would be 2.5GBq/m$^2$. This dose would result in 13.3Gy exposure to the dose limiting large intestine, 3.3 times less than the regulated 45Gy limit and recommended [$^{131}$I]MIP-1145 be put forward for stage 3 and 4 melanoma clinical trials. The use of cytotoxic radionuclides, conjugated to melanin binding compounds such as the benzamides [$^{131}$I]MIP-1145 and [$^{131}$I]ICF01012 represents a novel and potentially highly effective method of exploiting melanin in the treatment of melanotic melanoma with targeted radiotherapy (Joyal, Barrett et al. 2010).

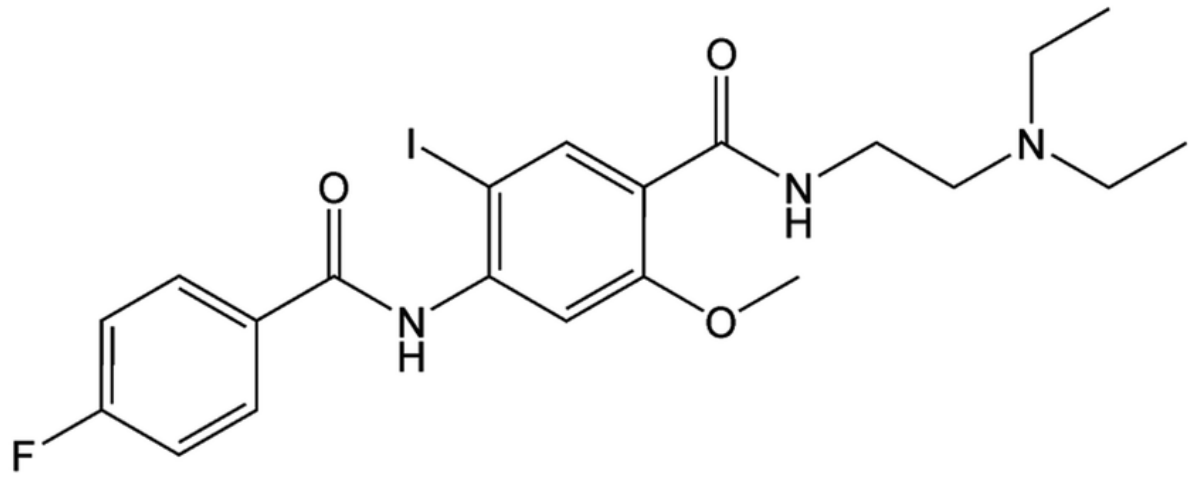


Figure 1-8 **The structure of $^{131}$I Benzo[1,3]dioxole-5-carboxylic acid[4-(2-diethylamino-ethylcarbamoyl)-2-iodo-5-methoxy-phenyl]-amide($^{131}$IMIP1145).**

$ABZA_2$ analogue, which has demonstrated a high binding affinity for Melanin. Taken from (Joyal, Barrett et al. 2010).

## 1.10 *The use of gene therapy to render tumour cells amenable to TRT.*

Although the above mentioned studies have shown that benzamide compounds are effective in targeting melanotic malignant melanoma, a proportion of malignant melanoma tumours are known to be amelanotic (Gualandri, Betti et al. 2009) with patient data showing they are incapable of accumulating benzamide compounds (Larisch, Schulte et al. 1998). In cases such as these the utilisation of gene therapy techniques may provide a viable method of rendering melanoma cells amenable to targeted radiotherapy, regardless of melanin status (Wheldon, Mairs et al. 1999)

### *1.10.1 MIBG and the Noradrenaline Transporter.*

Metaiodobenzylguanadine (MIBG) is an analogue of noradrenaline (Smets, Loesberg et al. 1989) amenable to labelling with radionuclide isotopes of iodine and readily taken up by the noradrenaline transporter (NAT), which is typically over expressed by tumours of neural crest origin (Streby, Shah et al. 2015) [$^{131}$I]MIBG has been used clinically for the treatment of the neural crest tumours neuroblastoma and phaeochromocytoma (as reviewed by Troncone and Rufini 1997).

Gene manipulation studies involving radiolabelled MIBG and the NAT gene have demonstrated the potential for MIBG therapy in tumour types that do not typically express the NAT gene. In 1999 our labs first reported successful [$^{131}$I]MIBG uptake and cytotoxicity following plasmid transfection of the bovine noradrenaline transporter gene (bNAT) into the non-NAT expressing glioma line UVW under the control of the RSV rous sarcoma virus promoter (pNAT+) (Boyd, Cunningham et al. 1999). A 15 fold increase in [$^{131}$I]MIBG uptake in UVW/NAT+ cells was observed compared to UVW/NAT+ cells treated with desmethylimipramine (DMI), a noradrenaline transporter agonist. [$^{131}$I]MIBG exposure did not result in uptake in either untransfected UVW and antisense (pNAT(-)) transfected controls. Moreover, survival fractions were found to be reduced in a dose dependant manner in both UVW/pNAT+ monolayer and spheroid models no effect on survival was seen the equivalent UVW or pNAT(-) system.

It was later reported by Boyd, Mairs et al. 2001 that complete clonogen sterilisation of UVW/pNAT+ spheroids occurs at 7Mbq/ml [$^{131}$I]MIBG. This effect was amplified by maintaining spheroid structure 48hrs post treatment prior to plating for clonogenic assay, where 6Mbq/ml [$^{131}$I]MIBG was required to elicit the same response citing a significant cross fire effect relating to the $\beta$ emitting $^{131}$Iodine. Building upon this Boyd *et al.* found that pNAT driven by the human telomerase promoters hTERT and hTR, which are known to be expressed in low levels in normal tissue, yet constitutively active in various malignancies (Härle-Bachor and Boukamp 1996, Tahara, Yasui et al. 1999, Boyd, Mairs et al. 2001) resulted in successful uptake of [$^{131}$I]MIBG when transfected into the UVW glioma line at approximately 70% of the efficiency of the previously reported RSV promoter driven NAT plasmid.

Subsequently, spheroid models of RSV/HTERT/hTR transfected UVW cells were studied to compare each promoter and the effectiveness of the $\beta$ emitter [$^{131}$I]MIBG and $\alpha$ emitter [$^{211}$At]MIBG. UVW/NAT cells driven by the RSV promoter produced a 25-fold increase in uptake compared to DMI controls, hTR and hTERT promoter driven NAT uptake was 68% and 56% lower than RSV respectively (Boyd, Mairs et al. 2004). All transfected cell lines were reported to successfully uptake both [$^{131}$I]MIBG and [$^{211}$At]MIBG.

Clonogen sterilisation of the spheroids (considered 0.1% survival) was seen to be three times more efficient using [$^{211}$At]MIBG with sterilisation achieved using 1.5 and 0.004MBq in the RSV promoter, 8.5 and 0.0075MBq in the hTR promoter and 9 and

0.008 MBq in the hTERT promoter of [$^{131}$I]MIBG and [$^{211}$At]MIBG respectively (Boyd, Mairs et al. 2004).. Notably, radiopharmaceutical treatments involving a novel mosaic spheroid model consisting of either 5% or 10% transfected cells demonstrated greater than anticipated growth retardation, which resulted in complete spheroid sterilisation with a 5% transfected population using the RSV and hTERT promoter, an induction of the radiation induced bystander effect (RIBBE) where by the use of radionuclides results in cytoxicity of non-target adjacent cells both via direct interaction with decay particles and through interactions with previously irradiated cells (Mothersill and Seymour 2001).

Clinical applications of [$^{131}$I]MIBG as a single agent are yet to prove curative, with therapy frequently centred around palliation (Gaze and Wheldon 1996, Streby, Shah et al. 2015). Greater efficacy is seen when [$^{131}$I]MIBG is administered as a multi-modal therapy in conjunction with chemotherapeutics, prolonging patient survival (Gaze and Wheldon 1996, Castel, Canete et al. 2000, Mastrangelo, Tornesello et al. 2001). Our labs have shown that combining [$^{131}$I]MIBG with agents such as the topoisomerase 1 inhibitor Topotecan and the PARP inhibitor PJ34, which interfere with DNA replication and repair pathways and act as radiosensitisers (Miyamoto, Huang et al. 2000, Hirai, Shirai H Fau - Fujimori et al. 2012), produces significant inhibitory effects on tumour growth. Additionally, [$^{131}$I]MIBG/Topotecan combinations enhanced cytotoxicity in NAT expressing SK-N-BE(2c) and UVW/NAT cell lines and xenograft models beyond the effectiveness of [$^{131}$I]MIBG treatment and topotecan treatments alone, where simultaneous treatment of the compounds was found to cure UVW/NAT tumour baring nude mice (McCluskey, Boyd et al. 2005).

Further investigations including [$^{131}$I]MIBG, topotecan and the Poly ADP ribose polymerase (PARP) - 1 inhibitor PJ34 demonstrated that combinations with topotecan did not induce high levels of toxicity, although following simultaneous administration the drug interaction was found to be synergistic in UVW/NAT and SK-N-Be(2c) cell lines after combination index analysis. The simultaneous administration of [$^{131}$I]MIBG/ topotecan/PJ34 was found to be the most effective treatment schedule in UVW/NAT cells, with all treatment schedules proving synergistic and highly effective in SK-N-Be(2c) cell lines. *In vivo* this was found to translate to triple combinations of [$^{131}$I]MIBG/ topotecan/PJ34 delaying tumour growth more effectively than either [$^{131}$I]MIBG or topotecan as both single agents and in combination (McCluskey, Mairs Rj Fau - Tesson et al. 2012).

The potential for using gene therapy in conjunction with [$^{131}$I]MIBG therapy in neuroblastoma cell lines that do not natively express the noradrenaline transporter was shown by Cunningham, Boyd et al. 2000 who transfected bNAT within a pcDNA3 expression vector into SK-N-MC that do not natively express the noradrenaline transporter. This was confirmed to uptake [$^{131}$I]MIBG *vs* untransfected controls, though this uptake was shown to be 67% less efficient than the native NAT expressing SK-n-BE(2c) cell line. Spheroid growth investigations found that a 4mbq/ml dose of [$^{131}$I]MIBG elicited growth delay in all NAT expressing cell lines with a reduced growth delay noted in the transfected cell line (14.6 days delay) *vs* SK-N-BE(2c) (17.3 days delay). These previous investigations will serve as a model for the following study, using radionuclide therapy to target natively expressed melanin as a

single agent and in conjunction with novel radio sensitising compounds in order to determine their effectiveness for the treatment of malignant melanoma.

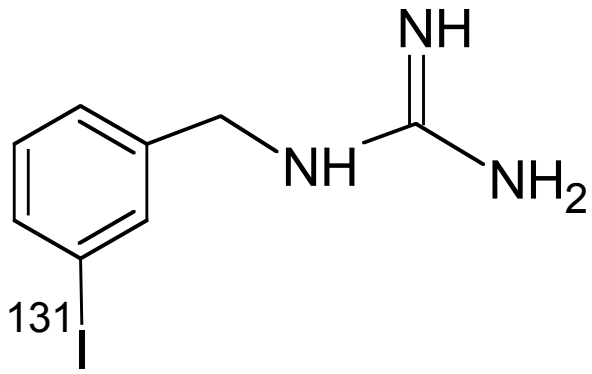


Figure 1-9 **[$^{131}$I] meta-iodobenzylguanidine ([$^{131}$I]MIBG).**

[$^{131}$I]MIBG is a structural analogue of bretylium and guanethidine, both of which are noradrenergic neurone blockers. It is selectively transported into and concentrated within neuroadrenergic tissue by the noradrenaline transporter (NAT) (Mairs, Fullerton et al. 2005).

### *1.10.2 Modified Herpes simplex-1 Virus (HSV).*

The development of viral and immune based therapies for malignant melanoma has progressed rapidly over the past decade. Modified Herpes Simplex -1 viruses (HSV-1) have been highlighted as some of the most promising agents for cancer therapy due to a broad tropism, episomal cyclolytic replication cycle and well characterised large genome. This has led to the development of selectively replicating oncolytic Herpes simplex viral vectors (oHSV) which have been studied as both single and combination agents in a variety of cancers, such as ovarian cancer (Benencia, Courreges et al. 2005), Glioma (Quigg, Mairs et al. 2005), and Melanoma (Randazzo, Bhat et al. 1997, Klatzmann, Cherin et al. 1998).

Modified HSV was initially introduced as a possible therapy option for malignant Glioma by (MacLean, ul-Fareed et al. 1991) currently a large variety of modified HSVs are available, one such variant HSV1716, bares deletions in both copies of the RL-1 gene, encoding the ICP34.5 neurovirulence factor (Shah, Benos et al. 2003) In wild type HSV-1 the ICP34.5 protein enables the reactivation of protein synthesis in non-dividing cells following protein kinase R (PKR) mediated shutdown of the translation machinery via dephosphorylation of eukaryotic initiation factor $\alpha$ (eIF2$\alpha$), allowing translation to proceed unhindered (Varghese and Rabkin 2002). Therefore, HSV1716 having lost ICP34.5, is unable to reinitiate translation becoming replication incompetent in non-dividing cells. Replication is restored when infecting tumour cells baring constitutive activation of the MAPK Kinase (MEK) pathway, which inhibits the

activation of PKR in response to stress or viral infection, allowing the continuation of viral replication in the absence of ICP34.5 (Smith, Mezhir et al. 2006).

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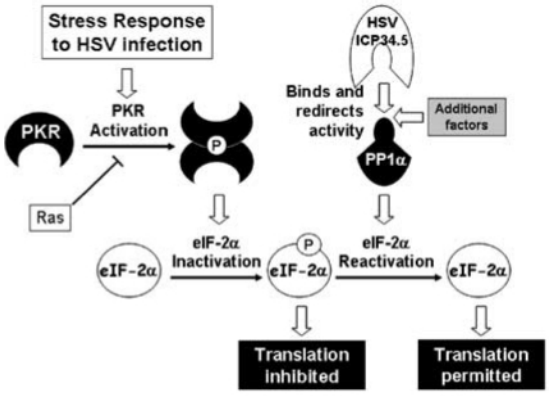



Figure 1-10 **Herpes simplex reactivation of translation machinery**

The host cell activates the PKR protein in response to the stress of infection; this deactivates the translation machinery, with the aim of halting viral replication. However, the ICP34.5 protein produced by HSV reactivates the translation machinery, allowing viral replication to proceed (Shah, Benos et al. 2003).

Further modification of the HSV1716 vector to express the Noradrenaline transporter facilitates the uptake of MIBG radionuclides by infected tumour cells (Figure 1-11). This virus (HSV1716/NAT) has been studied in conjunction with MIBG in glioma lines, the combination was found to enhance cell kill when compared to virus/ radiation alone. The virus was constructed using a CMV IE promoter driven NAT/IRES/EGFP 5.4kb DNA fragment inserted into an RL.del/pGEM3zf(-) vector with the addition of multiple cloning sites. CMV/NAT/IRES/EGFP/PolyA clones were then co transfected onto BHK21/C13 cells alongside HSV17+ viral DNA to generate viral plaques containing the HSV1716/NAT vector (Quigg, Mairs et al. 2005).

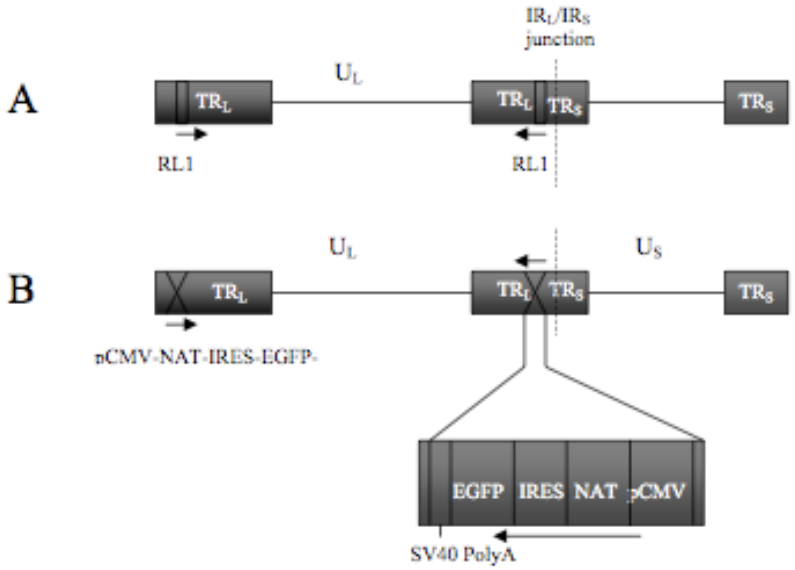



Figure 1-11 **Structure of wild-type and modified Herpes Simplex vector genome**

(A) Aschematic view of the wild type HSV virus. (B) Represents the HSV1716/NAT vector, where the γ34.5 locus has been deleted and replaced with the NAT gene as well as a CMV promoter to drive expression and GFP (green florescence protein) for imaging. Figure adapted from (Quigg, Mairs et al. 2005).

A previous pilot study by (MacKie, Stewart et al. 2001) which involved the injection of HSV1716 into the Melanoma lesions of 5 patients with stage IIII Melanoma, demonstrated that the virus injected at $10^3$ plaque forming units flattened palpable masses, with multiple injections resulting in tumour necrosis. The application of immunohistochemistry to tumour and normal cells indicated that viral replication was confined to the tumour site.

## 1.11 ***Emerging Therapies.***

Our growing understanding of the genetic, physiological and immune characteristics of malignant melanoma has enabled the development of novel therapeutic agents demonstrating greater efficacy and patient personalization than conventional chemotherapeutics. A range of the more promising therapies are discussed below.

### *1.11.1 BRAF and Multi-Kinase Targeted Therapy.*

BRAF mutations are among the most commonly identified in melanoma patients(Rubinstein, Sznol et al. 2010) and it is due to this high prevalence that drugs attempting to specifically target B-RAF are under development and in clinical trials and most recently entering clinical trials.

#### *1.11.1.1 Sorafenib.*

Sorafenib is a bi-aryl urea which has been specifically designed to target b-RAF and c-kit mutations by binding to the ATP active site of kinase with activity also being documented in angiogenic pathways and those involved in tumor progression, including vascular endothelial growth factor receptors 2 and 3 (VEGFR-2, VEGFR-3) as well as platelet derived growth factor b (PDGFR- b). It is unsurprising, due to this variation in active targets that Sorafenib has demonstrated a wide variety of responses in nude mouse xenograft models (Queirolo and Acquati 2006). The specificity of Sorafenib may however prove problematic in future patient trials, as the drug is unspecific in its affinity for BRAF, affecting both wild type and mutated

versions of the BRAF gene, potentially impacting on healthy tissue expressing wild type BRAF as well as malignant cells expressing mutated versions of the gene.

Sorafenib has been assessed both as a single agent and as a component of combination therapies with temozolomide, paclaxtaxcil, carboplatin and dacarbazine. Phase II studies with a cohort of 37 late stage melanoma patients involving the compound alone have shown that when used as a single agent at 400mg twice/daily, Sorafenib demonstrated no significant benefit to late stage melanoma patients,regardless of Braf mutation status, although the dose used produced favourable toxicity results and palliation in certain subjects (Eisen, Ahmad et al. 2006).

Combination studies with Sorafenib are however now showing more success. Phase II temzolomide studies in combination with Sorafinib are producing favorable responses in late stage melanoma patients with brain metastases(Gogas, Kirkwood et al. 2007, Amaravadi, Schuchter et al. 2009). In a recent study administering a combination of Dacarbazine, (200mg twice a day) with of sorafenib and 1000mg/m-2 of Dacarbazine produced disease stabilization in 37% of treated patients (Eisen, Marais et al. 2011). However when studied in combination with Carboplatin and Paclataxil, Sorafenib did not improve the effectiveness of the Carboplatin/Paclataxil therapy (Hauschild, Agarwala et al. 2009). Most recently Sorafenib has entered Phase 1 trials in combination with Bortezomib, however objective responses are yet to be documented (Sullivan, Ibrahim et al. 2015).

#### *1.11.1.2 Vemurafenib*

More recently, the BRAFV600e inhibitor Vemurafenib has emerged as an encouraging potential therapy, inducing responses in almost 50% of stage IV melanoma patients during phase 1 and 2 clinical trials (ref). By comparison, Dacarbazine achieved a 5% response rate during the same study. 6-month survival for Vemurafenib was 20% higher than Dacarbazine with 84% of patients getting what? Be specific and clear even if it means reiteration surviving up to the 6-months post treatment, lending weight to the targeting of BRAF V600e as a potential therapeutic. However it should be noted that further investigations into Vemurafenib concluded that patient survival past 6 months (long term survival) was not improved with Vemurafenib therapy versus the Dacarbazine control (Chapman, Hauschild et al. 2011). As of 2016, Vemurafenib has been approved as a therapy for malignant melanoma.

Although the results with both Serafenib and Vemurafenib in what patients are encouraging, it must be considered that the major limitation to the application of therapies directed towards a genetic target and the studies associated with them, is that these therapies will apply to only those patients expressing the target mutation, limiting the capacity of these compounds and others like them for wide spread clinical use.

### *1.11.2 Immunotherapy*

One of the most promising and rapidly emerging areas of melanoma therapy research is in the development of immunotherapies. Historically malignant melanoma has proven responsive to cytokine immunotherapy such as the use of interferon alpha 2b (IFN-α2b) as an adjuvant therapy following surgery (Anaya, Xing et al. 2008). When administered intravenously at 20 mU/m2 for 5 days a week over a 4 week period before being reduced to 10 mU/m2 for 3 days week for 11 months IFN-α2b has been shown to improve recurrence free survival by 42% over 5-years (37% for IFN-α2b patients versus 26% for observation patients) and increasing overall survival by 24% over the same period (46% for IFN-α2b patients versus 37% for observation patients) (Kirkwood, Strawderman et al. 2016).

Similarly, high dose IL-2 has also been investigated for use in late stage melanoma patients in a randomized phase 3 trials, both as a single agent and as a combined therapy with current general chemotherapy options such as dacarbazine and cisplatin, as well as INFα). Clinical studies determined that although promising as a single agent and in combination with INFα, the addition of IL-2 to chemotherapy regimens did not significantly improve outcome or patient survival in the melanoma study group (Keilholz, Punt et al. 2005, Gogas, Kirkwood et al. 2007).

#### *1.11.2.1 Antibody therapies*

Therapeutic use of monoclonal antibodies modulating t-cell activity and host immune recognition.against melanoma specific antigens is a highly promising area. The cytotoxic T -lymphocyte antigen-4 CTLA4 and the programmed cell death receptor -1 (PD-1) pathways are expressed during melanoma progression (Shah, Chien et al. 2008)). Both attenuate t-cell responses to the tumour with the PD-1 ligand PD-L1 acting to mask the tumour from immune clearance (Kaunitz, Cottrell et al. 2017).

Clinical trials with the anti-CTLA4 antibody Ipilimumab improved T- cell responses to patient malignancies and improved over all survival (10.1 months and more recently in combination with Dacarbazine 11.2 months (Robert, Thomas et al. 2011) in non-resectable stage 3 and 4 patients, who had not responded to previous therapeutic intervention (Hodi, O'Day et al. 2010), following these highly encouraging results Ipilimumab was approved as a melanoma therapy in 2015.

PD-1 pathway therapeutics such as the , the PD-1 antibody nivolumab have demonstrated even greater patient responses and survival rates with few side effects than ant-CTLA-1 monoclonal antibodies (6.9 months progression free survival vs. 2.8 months for iplimumab). Combination therapies of nivolumab and ipilimumab are even more promising – with combined responses reaching 58% and a mean survival of 11.5 months (Seidel, Otsuka et al. 2018).

## 1.12 *Novel NF-κB inhibitors.*

The NFKβ protein family regulates gene expression within the immune system in response to stimulation and pro inflammatory cytokines secreted by the likes of microbes and viruses as well as gene expression in involved in apoptosis and cellular growth. In the inactive form, NFKβ is bound via its nuclear localization sequence to IKB in the cytoplasm, activation of the complex is achieved through ubiquitination and proteasomal degradation of IKB, which frees NFKβ for nuclear translocation.

NFKβ presents itself as an attractive target for melanoma therapy as NFKβ binding has been observed (specifically the RelA nuclear translocating protein) at an elevated level in melanoma patients, both in early and late stages of the disease (Amiri and Richmond 2005). Constitutive activation of NFKβ has been attributed to AKT activation, RAS/RAF activation as well as P16/INK4A mutations and P14/ARF mutations. UV irradiation has also shown to be a factor contributing to elevated NFKβ (Amiri and Richmond 2005, Ueda and Richmond 2006, Cooper and Bowden 2007). It is for these reasons and the wide spread influence of NFKβ that effective inhibitors of the protein may prove significant in melanoma therapy.

## 1.13 *Inhibitors of DNA repair.*

DNA repair pathways are thought to be a major factor in melanoma drug resistance, with many genes associated with repair being over expressed in malignant melanoma. A recent study by Jewell, Conway et al. 2010, on patient primary melanoma samples demonstrated to statistically significance the up-regulation and over expression of key genes in double strand DNA repair and metabolism including RAD51 and TOP2A (Jewell, Conway et al. 2010). These genes are heavily involved with homologous recombination repair following a double strand break assault and have been associated with poor prognosis and relapse following overexpression or mutation not only in melanoma but also in other aggressive tumour types (Lamy, Fina et al. 2011, Wang, Chai et al. 2011). The study also highlighted genes involved in Nucleotide excision repair (eg.XPA), base excision repair (XRCC1) miss match repair (MLH1) and non-homologous end joining (XRCC5).

In light of the heavy involvement of genes associated with homologous recombination repair, targeting of this pathway and the proteins associated with it, in combination therapy with agents that induce double strand breaks is a logical avenue of investigation.

Mre11 is an integral protein in the DNA double strand break repair pathway. Mre11 acts to phosphorylate the PI3K-like protein ATM. Phospho-ATM is heavily involved in the activation of the DNA double strand break repair mechanism.

As activation of the DDSB repair pathway is dependent on ATM phosphorylation, it is theorised that inhibitors of proteins such as Mre11, may prove to be of use as radio-sensitizing agents. One such agent, 'Mirin' inhibits the exonuclease activity of Mre11 and thus prevents Mre11 phosphorylation of ATM. This, in theory would diminish the cells ability to repair double strand breaks induced by radioactive assault (Hakem 2008, Kelley and Fishel 2008, Kuroda, Fujiwara et al. 2010, Smith, Tho et al. 2010).

## 1.14 ***Aim of this Study.***

- To assess the effectiveness of the melanin binding radionuclide [131I]MIP1145 in the treatment of malignant melanoma in vitro and in vivo.
- To investigate whether malignant melanoma cell lines and xenografts can be rendered susceptible to [131I]MIBG radionuclide therapy via transfection in vitro with noradrenaline transporter (NAT) and via gene delivery in vivo with the HSV1716/NAT vector.
- To screen novel DNA repair and IKKβ Inhibitors in combination with X-Ray radiation to determine suitability for future targeted radiotherapy/drug combination therapy approaches.

# Chapter 2

# Materials and Methods

## 2.1 *Cell culture conditions*

All tissue culture was performed in a laminar flow cabinet with vertical flow under aseptic conditions.

The human melanoma cell lines A375 and A2058 (Boussemart, Malka-Mahieu et al. 2014) were obtained from ATCC. cell lines were cultured in high glucose DMEM 11960 (Gibco/Invitrogen Ltd, Paisley UK) and supplemented with fetal bovine serum (10% v/v; Autogen Bioclear,Mile Elm Calne, Wiltshire,UK/ Biosera Ltd, The Broyle Ringmer East Sussex UK), Penicillin/Streptomycin (100U/ml;Gibco/Invitrogen Ltd), Fungizone (2ug/ml;Gibco/Invitrogen), L-Glutamine (2mM; Gibco/Invitrogen Ltd) and Sodium Pyruvate (100mM Gibco/Invitrogen Ltd). Cultures were seeded at a density of $2X10^5$ and incubated at $37^0$C and 5% $CO_2$ in $75cm^2$ treated tissue culture flasks (Iwaki tissue culture flasks with double sealed caps, distributed by sterilin UK). Cells were sub-cultured twice per week once monolayers became 70-90% confluent following detachment by the addition of trypsin (0.05%; Gibco, Invitrogen Ltd).

The mouse melanoma cell line B16-F10-LUC2 (Overwijk and Restifo 2001) cell line was a gift from Dr Christine Dufes at Strathclyde university and cultured in RPMI 1640 52400 (Gibco, Invitrogen Ltd) at $37^0$C and 5% $CO_2$ and supplemented with fetal bovine serum (10% v/v; Autogen Bioclear,Mile Elm Calne, Wiltshire,UK/ Biosera Ltd, The Broyle Ringmer East Sussex UK), Penicillin/Streptomycin (100U/ml;Gibco/Invitrogen Ltd), Fungizone (2ug/ml;Gibco/Invitrogen) and L-Glutamine (2mM; Gibco/Invitrogen Ltd). Cultures were seeded at a density of $2X10^5$ cells and incubated at $37^0$C and 5% $CO_2$ in $75cm^2$ treated tissue culture flasks (Iwaki tissue culture flasks with double sealed caps, distributed by Sterilin UK). Cells were sub-cultured twice per week once monolayers became 70-90% confluent following detachment by the addition of trypsin (0.05%; Gibco, Invitrogen Ltd).

The SK-MEL-3 human melanoma cell line (Fogh, Fogh et al. 1977) was cultured in McCoy's 5a Medium Modified 26600-023 (Gibco, Invitrogen Ltd) at $37^0$C and 5% $CO_2$ and supplemented with fetal bovine serum (15% v/v; Autogen Bioclear,Mile Elm Calne, Wiltshire,UK/ Biosera Ltd, The Broyle Ringmer East Sussex UK), Penicillin/Streptomycin (100U/ml;Gibco/Invitrogen Ltd), Fungizone (2ug/ml;Gibco/Invitrogen) and L-Glutamine (2mM; Gibco/Invitrogen Ltd).

The malignant glioma cell line UVW (Mackie, Freshney et al. 1988) was maintained in Eagle minimum essential medium (Invitrogen) with 10% (v/v) fetal bovine serum, Penicillin/Streptomycin (100 U/mL), Fungizone (2 mg/mL), and Glutamine (2mM).

Cultures were seeded at a density of $2x10^5$ cells per plate and incubated at $37^0$C and 5% $CO_2$ in $75cm^2$ treated tissue culture flasks (Iwaki tissue culture flasks with double sealed caps, distributed by Sterilin UK). Cells were sub-cultured twice per week once monolayers became 70-90% confluent, following detachment by the addition of trypsin (0.05%; Gibco, Invitrogen Ltd).

Long term storage of cell stocks was achieved by continual liquid nitrogen immersion; $1x10^6$ cells were suspended in 1ml of freezing medium (8mls of cell culture media as specified for each line, additional supplementation of 1 ml of fetal bovine serum and 1ml of DMSO) and aliquot into cryo-vials before being cooled gradually over night to $-70^0$C prior to liquid nitrogen storage. Recovery was achieved by rapid reheating to $37^0$C in warm water before being suspended in an addition 5mls of cell media and seeded into a $25cm^2$ tissue culture flask (Nunc plastics). Culture flasks were incubated for 24hrs at $37^0$C and 5% $CO_2$ prior to the replacement of the media with 5mls of standard media in order to remove the remnants of the freezing buffer.

## 2.2 *Characterisation of cell lines.*

The cell lines utilized in this study were subject to characterization via the study of plating efficiency, determination of doubling time, and radio-sensitivity. Periodic reassessment of these characteristics was carried out throughout the course of the project in order to monitor changes in phenotype and to rule out cross contamination

throughout the study. The cells were also genetically profiled in house to verify origin and designation.

### *2.2.1 Plating Efficiency*

The plating efficiency (P.E) of a given cell line is determined by the number of distinct colonies formed following seeding of a defined number of cells. For the purposes of this study a viable colony is described as one consisting of 50 cells or more. A P.E is calculated and expressed as a percentage through application of the formula:

No. Of colonies formed/No. Of cells seeded*100.

In order to assess the plating efficiency of each cell line cells from each culture are removed from the stock flasks by treatment with a 0.05% (w/v) solution of Trypsin-EDTA in PBS (Invitrogen), the cell are subsequently counted via haemocytometer and seeded in triplicate into 35-mm Petri dishes (Nunc plastics) in triplicate at 250,500,750 and 1000 cells respectively. The cells were then incubated for 10-14 days or until visible colonies are seen, the colonies are then fixed in methanol: PBS (50:50) and stained with 10% Gram's Crystal Violet solution (BDH). Clusters containing 50 or more cells are scored by eye as colonies. Plating efficiency is determined by dividing the number of colonies counted by the number of cells seeded.

### *2.2.2 X-Irradiation.*

External beam Ionising radiation was utilized throughout this study, X-Ray irradiation was achieved using the X-RAD 225 X-Ray processor, (Precision X-Ray Inc. Connecticut USA). A dose rate of 2.2Gy/min was reached at a distance of 50cm from the X-Ray source.

## 2.3 ***Radio sensitivity to external beam irradiation: Clonogenic survival following X-ray irradiation***

Assessment IS of the melanoma cell lines inherent radio sensitivity was achieved by determination of the survival fraction of a given cell line following exposure to low LET X-ray radiation. Survival is defined as the ability of a cell line to maintain clonogenicity following assault and therefore a clonogenic assay was performed following radiation exposure in order to assess the survival fraction, with radio sensitivity being described at the surviving fraction of cells following 2Gy irradiation (SF2)

In order to assess the clonogenic survival of the A375, A2058 and B16-F10-Luc2 lines, cells were cultured until 70% confluent prior to treatment, seeded in 25cm$^2$ flasks at initial concentrations of 2.5x10$^5$ cells/flask in 5mls of medium.

Flasks were irradiated at room temperature in 1ml of culture medium with 0.5Gy, 1Gy, 2Gy, 4Gy, 6Gy, 8Gy, 10Gy and 20Gy. Each experiment also included an untreated 0Gy Control. 24hrs following irradiation, all cells were detached from the flasks as described in and the single cell suspension counted Cell concentration was subsequently determined using a haemocytometer count with a known concentration of cells (250 cells/ treatment) seeded in triplicate in 5mls of medium onto 60mm cell culture plates (Nunc plastics). Cells were incubated at $37^0$C and 5%$CO_2$ for up to 10 Days to enable colony formation of surviving cells. Once colonies had formed, media was removed and colonies fixed with 100% methanol prior to staining with 10% Gram's Crystal Violet solution. Colonies consisting of 50 cells or more were subsequently scored as viable, with survival fraction being calculated as the number of colonies produced vs the number of cells seeded per plate following correction for plating efficiency.

## 2.4 *$^{131}$IMIBG studies.*

### *2.4.1 MIBG uptake studies.*

A375 and A375/NAT cells were seeded in six-well plates at an initial density of $1.5x10^5$ cells per well and cultured for 48 h. MIBG incorporation was measured by incubating the cells for 2 h with 7 kBq of $^{131}$I MIBG, (GE Healthcare,UK). Nonspecific uptake was measured in the presence of 1.5 mm desme- thylimipramine (DMI) (Sigma-Aldrich,UK). After incubation, medium was removed, the cells were washed with PBS and radioactivity was extracted using two aliquots of 10% (w/v) trichloroacetic acid.

The activities of the extracts were then measured in a gamma-well counter. Uptake was expressed as c.p.m. per $10^5$ cells.

### *2.4.2 NAT Gene Transfections.*

As melanin expression by melanoma cells is not consistent within a tumour population, it may prove advantageous to introduce a transgene to facilitate the selective uptake of radiopharmaceutical [$^{131}$I] Meta-iodobenzylguanidine **(**$^{131}$I MIBG) (Figure 1-9). Radiolabelled MIBG is a Noradrenaline analogue, which has been successfully used in the palliative treatment of Neuroblastoma, as Neuroblastoma tumours natively the express the Noradrenaline transporter, which facilitates selective uptake of [$^{131}$I] MIBG. Previous studies have shown that [$^{131}$I]MIBG can also be applied to other tumour types following successful transfection of the noradrenaline transporter gene into glioblastoma (Garaventa, Guerra et al. 1991, Gaze and Wheldon 1996, Boyd, Cunningham et al. 1999, Fullerton NE 2004, Mairs, Fullerton et al. 2005)

and bladder and prostate cancer cell lines (Fullerton, Boyd et al. 2004, Fullerton NE 2004) producing successful uptake of MIBG and demonstrating potential therapeutic benefit and as such transfection procedures were carried out as described below.

In order to undertake studies using $^{131}$IMIBG in melanoma cell lines stable transfection of the Noradrenaline Transporter (NAT) was undertaken. Bovine NAT cDNA was inserted into the EcoR1 site of the eukaryotic expression vector pSG-5

(Stratagene, Cambridge,UK) which was provided by Dr Michael Bruss and Professor Heinz Bonisch (University of Bonn) and was subcloned into the pREP9 episomal expression vector as previously described (Boyd, Mairs et al. 2002)

A375, A2058 and SK-MEL-3 cell lines were seeded in six well plates at a density of $1.5x10^5$ cells/well forty-eight hours prior to transfection. Once cells had reached 70% confluency, wells were treated as follows using Effectein transfection reagent (Quiagen, UK) according to the manufacturer's instructions.

## 2.5 ***Compound preparation and treatment.***

### *2.5.1 Mirin*

Mirin Stock solutions (Tocris UK) were suspended in Dimethly Sulfoxide (DMSO) at a concentration of 10mM and added to 1ml of cell medium at micro molar doses in $25cm^2$ tissue culture flasks (Nunc UK) for *in-vitro* studies. Mirin was prepared as a suspension in water and administered via oral gavage at a concentration of 50mg/kg during *in-vivo* studies. lyophilised stock powder was stored at room temperature whilst suspended stock solutions were stored in a refrigerator at $4^0C$.

### *2.5.2 Strathclyde University (SU) In house compounds*

All in house SU compounds were provided by Professor Simon McKay (University of Strathclyde). Stock solutions were suspended in either distilled water or DMSO at a

concentration of 10mM. Micro molar doses were added to 1ml of tissue culture medium in order to facilitate treatment of cultures in 25cm$^2$ tissue culture flasks.

## 2.6 *H2AX Foci expression and cell cycle analysis.*

Assessment of the amount of double strand DNA damage and subsequent induction of repair following ionizing radiation and drug treatments was achieved through FACs analysis of cells stained for γH2AX foci, which are reported to rapidly accumulate at the site of damage following insult. (Paull Tt Fau - Rogakou, Rogakou Ep Fau - Yamazaki et al. 2000)

Treated samples were prepared as a single cell suspension and pelleted via centrifugation at 1200rpm for 5 minutes and washed twice in PBS before being suspended in 2mls of 70% EtOH and stored at -20$^0$C. Cell pellets were subsequently washed and permeablised using the Upstate permeabilisation solution (Upstate) prior to incubation with 2.0ug/ml of Ser139 γH2Ax antibody (Millipore, UK). Samples were analysed for levels of γH2AX foci expression using the BD FACSaria flow cytometry cell sorter. Additionally, alterations in cell cycle and cell cycle arrest following ionizing radiation and drug treatment were also analysed; samples were collected and stored following the same methodology as for γH2AX foci expression. Pellets were washed and incubated with (5mg/ml) Propidium Iodide. (Bio-Rad) for 1hr prior to analysis on the BD FACS aria flow cytometry cell sorter.

## 2.7 *Radiation studies*

### *2.7.1 No-Carrier-Added Synthesis of $^{131}$I-MIBG*

Chemicals were purchased from Aldrich Chemical Co. High- performance liquid chromatography–grade solvents were obtained from Rathburn Chemicals. Carrier-free sodium $^{131}$I was purchased from GE Healthcare. No-carrier-added $^{131}$I-MIBG was synthesized by electrophilic iododesilylation of trimethylsilylbenzylguanidine, as described previously (Hunter and Zhu 1999, Sorensen, Mairs et al. 2012).

### *2.7.2 MIP-1145 Preparation*

[$^{131}$I]MIP -1145 was manufactured by Molecular Insight Pharmaceuticals, Boston, USA as described by (Joyal, Barrett et al. 2010) briefly, the MIP-1145 precursor N-(2-diethylamino-ethyl)-4-(4-fluoro-benzamido)-2-methoxy- benzamide was added in a molecular ratio of 1:1.2 to thallium trifluoroacetate TI (TFA)3 in trifluoroacetic acid (TFA) brought to a final volume of 0.3ml with the addition of a 50% acetic acid, 50% TFA mix, which was incubated at room temperature prior to the addition of 2.2GBq of of Na$^{131}$I prepared in 0.1M NaOH.

The reaction mix was then diluted in a formulation buffer consisting of 6%PEG-400, 2% EtOH, 6% ascorbic acid and 3% Sodium Gentisate at pH 4.4 before purification and analysis via liquid Chromatography (RP-HPLC). The resulting final concentration of material collected was 1.48MBQ/ml, which upon filtration yielded a final product of 95% radiochemical purity and specific radioactivity as high as 55.5TBq/nmol.

## 2.8 ***Viral vector studies***

### *2.8.1 HSV Vector production*

The HSV particles were produced in accordance with the protocol as described by Quigg *etal* 2005. Briefly, The NAT Gene was subcloned into the multiple cloning site of the pIRES2-EGFP vector (BD Biosciences Clontech, UK), the CMV IE Promoter subsequently cloned the pIRES2-EGFP vector upstream of the NAT Gene. The Resulting CMV/NAT/IRES/EGFP/PolyA inserts were linearised using SspI and co-transfected with HSV17+ DNA, isolated from the HSV1716 HSV Glasgow strain 17+, on to 80% confluent BHK21/C13 cells using the CaPO4 transfection method. Fluorescent, recombinant, viral plaques were purified and a stock (designated HSV1716/NA T) was grown and titrated in BHK21/C13 cells (Quigg, Mairs et al. 2005).

### *2.8.2 Biodistribution of HSV1716/NAT*

An assay for the presence of infectious HSV in mice bearing SK-MEL-3 tumours was performed by homogenizing tissue samples 3 and 7 d after HSV1716/NAT injection. Briefly, tissue samples were homogenized using an Omni TH-02 homogenizer in 1 mL of PBS before titration on BHK21/13C cells as described previously (Harland and Brown 1998).

Immunohistochemical analysis for the presence of HSV was performed on 3-mm-thick paraffin sections of tissue samples from mice bearing UVW and SK-MEL-3 tumours using a monoclonal antibody recognizing an unspecified epitope of HSV1 strain Stoker (Dako). Sections were pre-treated by microwaving for antigen retrieval. Bound antibody was visualized using the enVision system (Dako). Tissue sections were imaged using a Zeiss Axioplan 2 microscope and ISIS software (Metasystems) for capturing images. Negative controls were sections from tumours treated with PBS.

## 2.9 ***Western Blotting Analysis.***

### *2.9.1 Reagent Preparation (1litre).*

1x Running buffer was made by diluting 50mls 20x MOPS SDS Solution (Invitrogen, uk) in 950mls $dH_2O$ and stored at room temperature. Similarly, 1x transfer buffer working solution was made by diluting 50mls of 20x transfer buffer (Invitrogen, uk)

in 100mls MeOH and 850mls $dH_2O$. NaTT buffer containing 2.42g Tris HCL, 8.77g NaCL, and 300ul TWEEN 20 was diluted in 1L $dH_2O$ as a working solution.

### *2.9.2 Sample preparation and blotting.*

Cell lines treated with BMS, SU182 and SU567 (as described under SU drug preparation) were collected by addition of 150ul per flask of lysis buffer consisting of parp, PMSF,NaCl, Triton X, $ddH_2O$ and protease inhibitor cocktail Cat no. 535142 (Calbiochem). Cells were incubated for 1-3 minutes at $37^0$C and removed into a 1.5ml Eppendorf tubes via cell scraper and immediately stored at $-80^0$C. Protein concentration was quantified using the Pierce BCA Protein Assay Kit (thermo scientific – Cat no. 23227) according to the manufacturer's instructions. 20ug of total protein along with a 10kb ladder was electrophoresed though a polyacrylamide gel immersed in 1x MOPS SDS running buffer in a volume of 10ul, at 200v for 1hr using a novex precast gel cassette (NuPAGE BIS-TRIS gel, NUPage electrophoresis system ,Invitrogen, UK) before being transferred in 1x transfer buffer to a Nitrocellulose membrane (Whatman Protran BA85) at 30 volts for 1hr.

The nitrocellulose membranes were incubated in blocking buffer (3% BSA, 25mM Tris –HCL pH 7, 0.8% NaCl, 0.05% TWEEN-20). on a rocking platform over night at $4^0$C.

### *2.9.3 Antibody staining*

***Primary antibodies***

For primary antibody staining, nitrocellulose membranes were incubated in 5mls 0.3% BSA/ NaTT blocking buffer over night on a rocking plate at $4^0$C with either mouse monoclonal anti IKKαantibody (diluted to 1:1000) Calbiochem UK, Rabbit monoclonal Anti IKKβantibody (diluted to 1:1000) (Abcam UK), Anti p65 antibody (diluted to 1:1000) (rabbit monoclonal) (Santa Cruz, Europe) or Anti Phospho p65 (diluted to 1:1000) (rabbit monoclonal) (Cell signaling technologies) and washed 6x 15mins in NaTT buffer.

***Secondary antibodies***

Secondary antibody staining was carried out using eitherPeroxidase conjugated Anti Mouse (1:1000) and anti Rabbit (1:1000) (Jackson immunological research). Nitrocellulose membranes were incubated in 5mls 0.3% BSA/NaTT, Plus antibody for 1hr on a rocking plate at $4^0$C and washed 6x 15mins in NaTT buffer prior imaging.

The proteins of interest were visualized using enhanced chemiluminescence reagents (ECL Detection reagent, Amersham UK) following the manufacturers instructions. The amount of transferred protein from a given sample was quantified by densitometry of the X-ray films from each membrane and normalized on comparison with the intensity of the p65 control samples. Band intensity levels were determined using Image J software (NIH)

## 2.10 *HT Super-oxide dismustase activity Assay (SOD)*

MnSOD activity and total SOD protein content was determined using the HT Superoxide dismustase Assay (Trevigen). Cells were isolated as per the manufacturers instructions and stored at -80$^0$C prior to the initial protein content being determined using the Pierce BCA Protein Assay Kit (Thermo Scientific – Cat no. 23227).

## 2.11 *In vivo studies.*

### *2.11.1 Experimental Animals*

Six-week-old female congenitally athymic nude mice of strain MF1 nu/nu were obtained from Charles River. All In vivo experiments were performed in accordance with the U.K. Coordinating Committee for Cancer Research guidelines on experimental neoplasia in animals (Workman, Aboagye et al. 2010)

In order to prepare cell lines for injection into the flanks of nude mice, cells were maintained in T75 tissue culture flasks under the standard culture conditions for each cell line, 37$^0$C at 5% $CO_2$. Cells were cultured to 70% confluency before being detached etc. cells washed with PBS to remove any FCS and medium. Cells were passaged through an 18 gauge needle to ensure a single cell suspension and cells prepared in a solution of PBS at 3x10$^6$/cell/200microliters. Each mouse received a

subcutaneous injection of between 3 and $5x10^6$ cells, depending on the study. Experimental procedures were initiated between 7 and 14 days post injection, to enable the establishment of tumour xenografts at a size of approximately $100mm^3$. Treatment groups were randomized and placed in the biohazard containment facility in line with local safety regulations as pertinent to the natural of the study. Animals were measured and bodyweight monitored daily for signs of potential toxicity. Evaluation of animals for signs of distress as carried out according to standard guidelines (Morton 1985). Animals whose tumours reached 1,900 $mm^3$ were euthanized.

### *2.11.2 [$^{131}$I]MIP1145 Pilot study.*

Sk-Mel3 Melanoma xenografts were established in nude mice following the procedures outlined under in vivo studies, above. 1.25 GBq/Kg of [$^{131}$I]MIP1145 and/or 80mg/Kg of Dacarbazine (Sigma Aldrich) were administered intravenously on days 7, 14, and 21.

## 2.12 ***HSV1716/NAT [$^{131}$ I] MIBG Pilot study.***

### *2.12.1 Tumor Therapy*

Mice with tumors of approximately 100 mm3 diameter were randomized into treatment groups of 12 mice each. Groups of animals received 1 · 105, 1 · 106, or 1 · 107 PFU of either HSV1716 or HSV1716/NAT diluted in 50 mL of PBS by intratumoral

injection. Control animals received injection of 50 mL of PBS. Virus injection was followed by intraperitoneal injection of 10 MBq of [$^{131}$I]MIBG simultaneously with or 24 h after virus or PBS injection.

To determine the efficacy of systemic administration HSV1716/NAT on tumour growth, groups of animals received an intravenous injection of 1 · 107 PFU of HSV1716/NAT diluted in 100 mL of PBS or PBS alone, followed by an intraperitoneal injection of 10 MBq of [$^{131}$I]MIBG 24 h after virus or PBS injection.

Subcutaneous tumours were measured with calipers immediately before treatment and every 2 or 3 d thereafter. On the assumption of ellipsoidal geometry, diameter measurements were converted to an approximate volume by multiplying half the longest diameter by the square of the mean of the 2 shorter diameters. Mice whose xenograft volume reached 1,900mm3 were euthanized. For every animal, relative tumour volume (volume at any time point divided by volume immediately before treatment) was plotted against time, and the area under the time–volume curves was determined by trapezoidal approximation. The area under the time–volume curves was used as a measure of treatment effectiveness for the purpose of comparison between groups. For animals that were euthanized because of fast tumour growth, before the termination of the experiment, the tumour volume curve was extrapolated at constant volume from the time of euthanasia.

## 2.13 ***Data analysis.***

Graphs and statistics were produced the Graphpad Prism graphing package (Graph pad Software La Jolla, CA USA).

# Chapter 3

# The effects of external beam and targeted radiotherapies on melanoma cell survival *in vivo* and *in vitro.*

## 3.1 *Introduction*

Since the early 1920's malignant melanoma has been commonly regarded as a highly radio resistant disease (MacKee GM, 1921) and as a result is used as a therapy option in as low as 1% of melanoma diagnoses (Delaney, Barton et al. 2004). However, recent research and clinical observations have demonstrated a spectrum of radioresistance within melanoma tumours (Stevens and McKay, 2006; Khan et al., 2011). This has led to researchers and clinicians reassessing the potential of radiotherapy for the treatment of malignant melanoma and external beam radio therapy has since been successfully deployed in early stage melanoma patients. Despite the renewed promise of radiotherapy however, the use of conventional treatment methods remains largely palliative in metastatic disease (Barker and Lee 2012).

Targeted radionuclide therapy offers several distinct advantages over conventional external beam therapy, where applications are designed to offer high specificity to the tumour site resulting in a reduced systemic toxic dose delivered to the patient as

well as enabling the delivery of therapeutic doses of radiation to metastatic sites, which may otherwise be missed by external beam treatments. In order to utilise target radiotherapy however, a malignancy must first express an ideally native targetable molecular characteristic. This has been achieved in several tumour types such as with the targeting of the sodium iodide symporter NIS with radio-iodide in thyroid tumours (Carvalho and Ferreira), the noradrenaline transporter enabling MIBG uptake in neuroblastoma (Mairs 1999) and [$^{131}$I]MIP 1095 targeting PSMA in prostate cancer (Eder, Eisenhut et al. 2013, Tesson, Rae et al. 2016).

Melanocytes, the dermal cell type from which Malignant Melanoma develops, generate the pigmented biopolymer melanin in response to Ultraviolet radiation induced damage. Melanin contains carboxyl and phenolic hydroxy groups that readily bind to metal and organic amines (Larsson 1993, Prota 2000). This trait is partially maintained in melanoma tumours, containing both pigmented and non-pigmented cells (Gualandri, Betti et al. 2009). The presence of melanin provides an opportunity to target melanoma tumours using compounds with high melanin binding affinity to deliver targeted radiotherapy (Bonnet-Duquennoy, Papon et al. 2009). However, the presence on amelanotic cells may leave a proportion of the tumour mass unresponsive to this form of targeting and thus the use of gene therapy as a means of introducing molecular features that render melanoma which do not express melanin amenable to targeted radiotherapy warrants investigation.

Chapter 3 - The effects of external beam and targeted radiotherapies on melanoma cell survival in vivo and in vitro.

## 3.2 *Aims*

- Characterise the behaviour of the A375, A2058 and B16-F10-Luc2 melanoma cell lines.
- Ascertain the effects of X-ray radiation as a single agent on each cell line.
- Determine whether the cell lines are amenable to treatment with $^{131}$IMIBG radiopharmaceutical.
- Explore the potential of melanin as a native target for radiotherapy.

## 3.3 *Plating Efficiency.*

The Plating Efficiency (PE) was determined as a percentage from the number of cells seeded on to a tissue culture dish that have progressed and formed colonies (Table 3-1). PE experiments have determined that a seeding density of 250 cells per plate is optimum for all cell lines as shown in table 1-2.

| **Cell Line** | **Plating Efficiency** |
|---|---|
| **A375** | 0.84 (±0.12) |
| **A2058** | 0.78 (±0.03) |
| **B16-F10-Luc-2** | 0.85 (±0.06) |
| **A375/CMV-NAT** | 0.91 (± 0.08) |

Table 3-1 Plating efficiencies (P.E) of the A375, A2058, B16-F10-Luc-2 and A375-CNV/NAT cell lines.

Cells were seeded at a density of 250 cells/plate. ± represents the standard deviation of the mean.

## 3.4 *Results of melanoma cell line transfections.*

In order to enable targeting of the melanoma cell lines with $^{131}$IMIBG, each cell line was transfected with three individual plasmids carrying the bovine noradrenaline transporter gene (bNAT) cDNA under the constitutive control of different promoters

in order to determine the most efficient inducer of [$^{131}$I]MIBG uptake. The promoters, Cytomegalovirus promoter (CMV) and the catalytic protein subunit and RNA telomerase promoters, HTERT and HTR respectively, were chosen after previous studies from our labs have shown that these elements are effective at inducing [$^{131}$I]MIBG uptake following transfection (Cunningham, Boyd et al. 2000, Boyd, Mairs et al. 2001)

Transfection experiments were performed and followed by uptake studies using 7 kb [$^{131}$I]MIBG in order to determine whether the transfection had been successful, which would result in fold increases in [$^{131}$I]MIBG uptake.

## 3.5 *Results of [$^{131}$I] MIBG uptake studies in NAT transfected A375 melanoma cell lines.*

The A375 cells transfected with the NAT gene driven by the CMV promoter demonstrated a statistically significant 17.5 fold increase in [$^{131}$I]MIBG uptake compared to the same cells treated with Desmethylimipramine (DMI), a selective monoamine uptake inhibitor which competatively blocks [$^{131}$I]MIBG uptake in NAT expressing cells ($(p<0.0001)$ compared by paired t-test). A375 Cells transfected with the NAT gene driven by the HTR and HTERT promoters did not demonstrate a statistically significant active accumulation of [$^{131}$I]MIBG ($p>0.05$). Figure 3-1 shows the [$^{131}$I]MIBG uptake capacity of transfectant A375 cell lines in the presence and absence of DMI.

It is often observed that CMV promoter activity diminished with both passage number and after recovery from liquid nitrogen and therefore stocks of A375-CMV/NAT cells were banked in bulk at early passage, with uptake capacity checked at regular intervals in order to mitigate this effect.

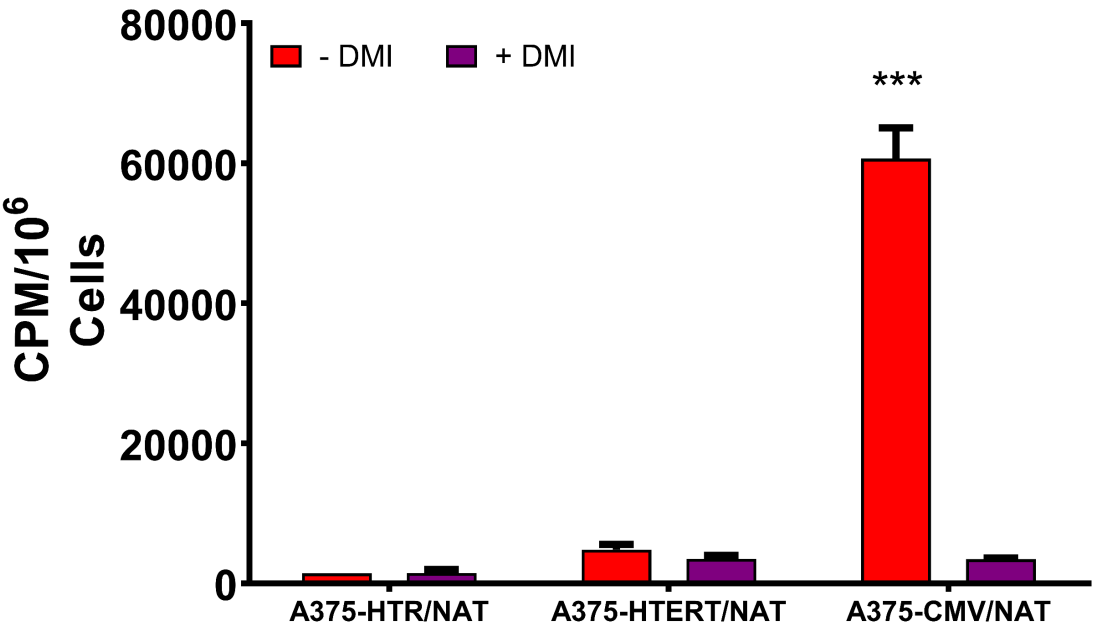



**Figure 3-1 Uptake of [$^{131}$I]MIBG in the A375 -HTR/NAT,A375-HTERT/NAT and A375-CMV/NAT transfected cell lines.**

Uptake is expressed as counts per minute (CPM) per 106 cells. Desmethylimipramine (DMI) inhibits the specific uptake of [$^{131}$I]MIBG by the noradrenaline transporter. Experimental data is representative of the mean of three experiments performed in triplicate, error bars are indicative of standard deviation (SD). The difference in uptake between the -DMI and +DMI treated samples only reached statistical significance in the A375-CMV/NAT cell line, with no significant uptake in either the A375-HTR/NAT or A375-HTERT/NAT lines. (***$p \leq 0.0001$; paired t-test).

## 3.6 *Results of [$^{131}$I] MIBG uptake studies in NAT transfection A2058 melanoma cell lines*

A2058 cells were transfected with the NAT gene driven by the HTR, HTERT and CMV promoters respectively, demonstrating a statistically significant 6.7 fold increase in [$^{131}$I] MIBG uptake in the A2058-CMV/NAT cell line compared to the same cells treated with Desmethylimipramine (DMI) ($p<0.0001$). A2058 Cells transfected with the NAT gene driven by the HTR and HTERT promoters did not demonstrate a statistically significant active accumulation of [$^{131}$I]MIBG ($p<0.05$). Figure 3-2 shows the [$^{131}$I] MIBG uptake capacity of transfectant A2058 cell lines in the presence and absence of DMI.

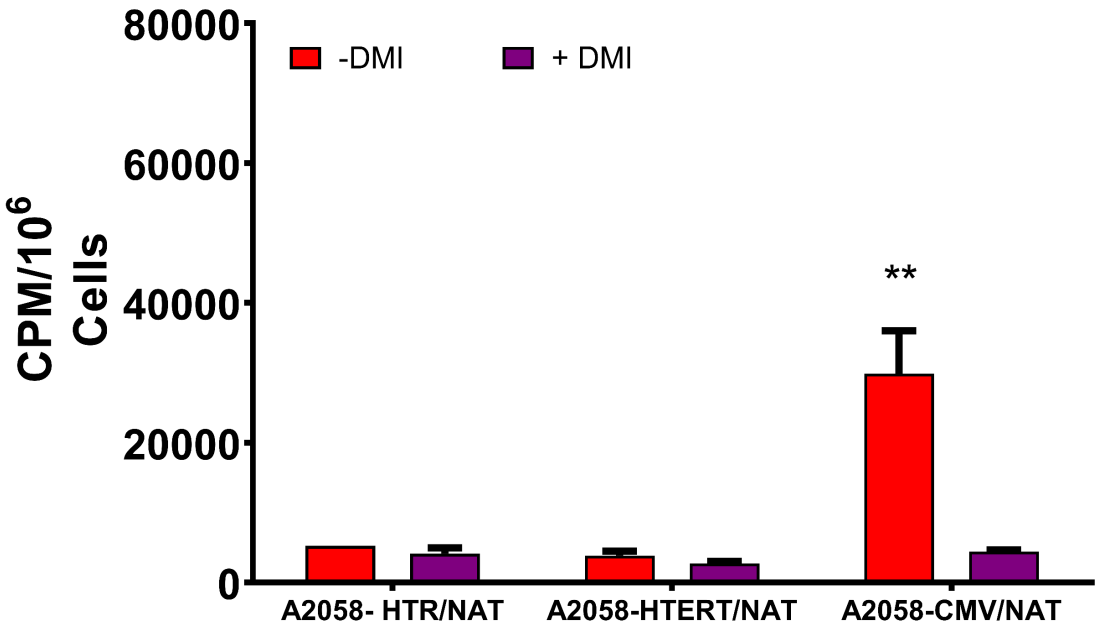



Figure 3-2 Uptake of [$^{131}$I]MIBG in the A2058-HTR/NAT, A2058-HTERT/NAT and A2058-CMV/NAT transfected cell lines.

Uptake is expressed as counts per minute (CPM) per 106 cells. Desmethylimipramine (DMI) inhibits the specific uptake of [$^{131}$I]MIBG by the noradrenaline transporter. Experimental data is representative of the mean of three experiments performed in triplicate, error bars are indicative of standard deviation (SD). The difference in uptake between the -DMI and +DMI treated samples only reached statistical significance in the A2058-CMV/NAT cell line, with no significant uptake in either the A2058-HTR/NAT or A2058-HTERT/NAT lines. **p≤0.01; paired t-test).

## 3.7 *Results of [$^{131}$I] MIBG uptake studies in NAT transfection B16-F10-Luc2 melanoma cell lines*

B16-F10-Luc2 cells were transfected with the NAT gene driven by the HTR, HTERT and CMV promoters respectively. However none of the cell lines demonstrated a statistically significant active accumulation of [$^{131}$I]MIBG (p≥0.9848, p≥0.9977 and p≥0.0676 respectively when analysed by 2-way ANOVA) . Figure 3-3 shows the [$^{131}$I] MIBG uptake capacity of transfectant B16 cell lines in the presence and absence of DMI.

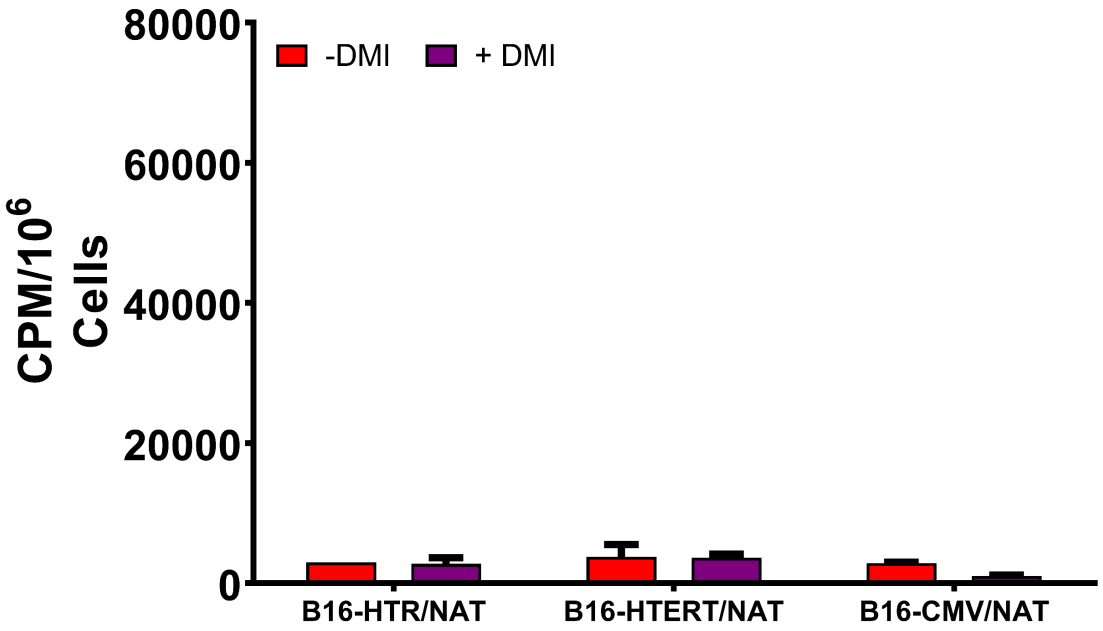



**Figure 3-3 Uptake of [$^{131}$I]MIBG in the B16-HTR/NAT, B16-HTERT/NAT and B16-CMV/NAT transfected cell lines.**

Uptake is expressed as counts per minute (CPM) per $10^6$ cells. Desmethylimipramine (DMI) inhibits the specific uptake of [$^{131}$I]MIBG by the noradrenaline transporter. Experimental data is representative of the mean of three experiments performed in triplicate, error bars are indicative of standard deviation (SD). The difference in uptake between the -DMI and +DMI treated samples was not found to be statistically significant ($p<0.05$; paired t-test)

These results demonstrated that the transfection of the Noradrenaline transporter transgene under the expression of the CMV, promoter resulted in uptake of [$^{131}$I]MIBG in the A375-CMV/NAT and A2058-CMV/NAT cell lines when compared to DMI treated controls. As the CMV driven A375/NAT cell line saw the most significant fold increase in [$^{131}$I]MIBG uptake of all cell lines and promoters tested. This cell line was taken forward to *in vitro* and *in vivo* [$^{131}$I]MIBG treatment studies.

## 3.8 *Clonogenic Survival assays.*

Clonogenic assays were performed in order to determine whether uptake of the radiopharmaceuticals [$^{131}$I]MIBG and [$^{131}$I]MIP1145 as well as exposure to external beam X-Ray irradiation translated to dose dependant cell kill *in vitro*.

### *3.8.1 Dose dependent toxicity of X-ray External Beam irradiation.*

In order to determine the inherent levels of radio sensitivity within each melanoma cell line the A375, A375/NAT, A2058 and B16-F10-Luc2 cell lines were exposed to X-ray irradiation at 0.5, 1, 2, 4, 6, 8 and 10 Gy at a dose rate of 2 Gy/min and plated for clonogenic assay, presented in Figure 3-4. Cell survival fractions at 2 Gy ($SF_2$) for the B16-F10-Luc2 cell line, was 0.64 (± 0.06), with the A2058 cell line demonstrating an SF2 of 0.63 (±0.04). The A375 and A375/NAT cell lines have SF2 values of 0.45 (±0.03) and 0.50 (±0.06) respectively, the SF2 values for both A2058 and A375 lines are similar to previously reported results (Leonard, Ramsay et al. 1995, Bujor, TrovV≤ et al. 1997) and establish an order of radio-resistance amongst the cell lines in the order of least to most resistant, as A375 and A375/NAT, followed by A2058 and B16-F10-LUC2 respectively.

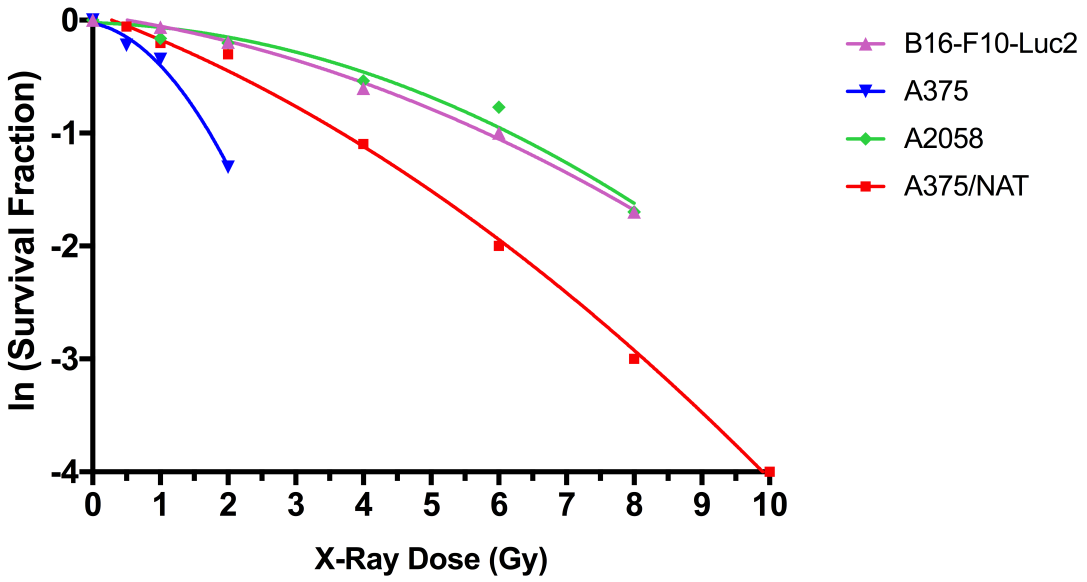



**Figure 3-4 Clonogenic survival following X-ray irradiation of A375, A2058, B16-F10-Luc2 and A375/NAT.**

The values are mean surviving fractions on a logarithmic scale, fitted to a linear quadratic curve. The Progressively steep shoulders of each cell line indicate the spectrum of radio resistance ranging from most resistant (B16-F10-LUC2 and A2058) to least resistant (A375) three independent experiments were performed in triplicate with error bars showing standard deviations (n=3). The difference in mean between untreated and X-irradiated cell line at all doses was found to be statistically significant ($p<0.005$).

### *3.8.2 Dose dependent toxicity following treatment with [$^{131}$I]MIBG in A375/NAT transfectant under the control of the CMV promoter and A375 Parental cells.*

In order to evaluate whether [$^{131}$I]MIBG uptake by the A375/NAT cell line translated into dose dependant toxicity, clonogenic assays were undertaken, the results of which are shown in Figure 3-5. Cell survival fractions in the A375/NAT line reached a minimum value of 0.62 (±0.02) after incubation with 2 Mbq/ml [$^{131}$I]MIBG. By comparison the A375 parental line showed minimal response to $^{131}$IMIBG treatment even at the highest administered dose of 4 Mbq/ml where the sf was 0.97 (±0.004) suggesting no sterilisation of clonogens even at this high administered [$^{131}$I]MIBG dose. A dose response was seen up to 2 Mbq/ml in the A375/NAT cell line with no additional benefit beyond that up to 4 Mbq/ml, which may be indicative of intracellular saturation of [$^{131}$I]MIBG beyond 2 Mbq/ml (Streby, Shah et al. 2015).

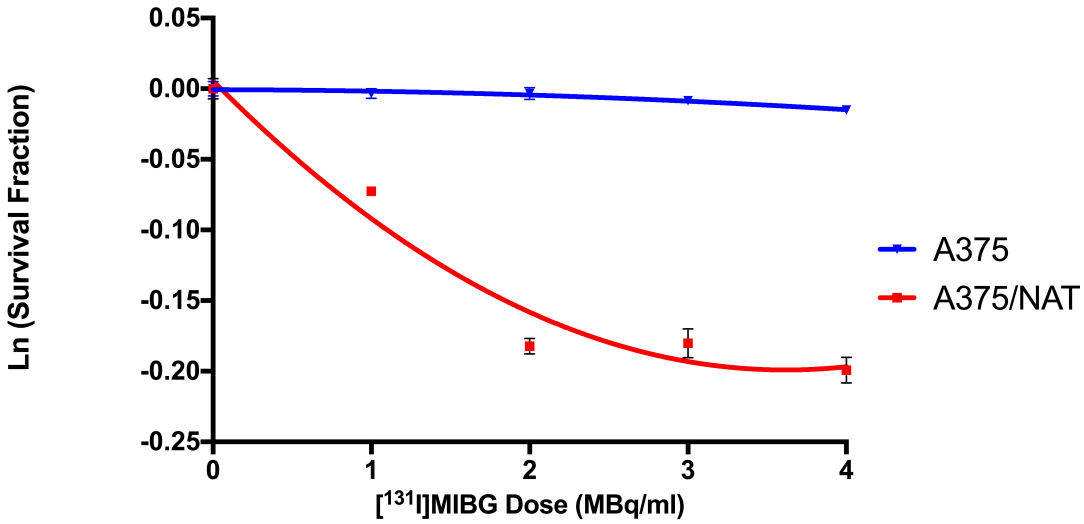


Figure 3-5 Clonogenic survival curves following exposure to escalating doses of [$^{131}$I]MIBG in A375/NAT transfected cell lines under the control of the CMV promotor and A375 parental cell line.

The values are mean surviving fractions on a logarithmic scale, fitted to a linear quadratic curve. The steep curve of the A375/NAT line is indicative of radiosensitivity and thus, uptake of [$^{131}$I]MIBG. A statistically significant minimum survival fraction of 0.62 (±0.02) was reached following administration of 2 MBq/ml $^{131}$IMIBG in the A375/NAT cell line. No significant difference in response was seen in the A375 parental line even at the highest dose of 4 Mbq/ml where survival fraction was 0.97 (±0.004). The values are mean surviving fractions of three independent experiments performed in triplicate with error bars showing standard deviations (n=3). The difference in mean between untreated and X-irradiated cell line at all doses was found to be statistically significant ($p<0.005$).

## 3.9 *Treatment of A375/NAT tumour xenografts with [$^{131}$I]MIBG in female nude mice.*

In order to assess the effectiveness of [$^{131}$I]MIBG treatment on NAT transfected cells *in vivo* tumour xenografts of the A375/NAT cell line were grown in female athymic nude mice as described in Sorensen, Mairs et al. 2012. Mice received 10 Mbq of [$^{131}$I]MIBG via intraperitoneal injection once tumours had reached approximately 100 mm$^3$. Tumours were measured twice weekly and the effect on tumour growth is shown in Figure 3-6. After administration of a PBS vehicle control tumours reached a maximum of 15 times the starting volume at day 25. By contrast the A375/NAT xenografts treated with 10 MBq [$^{131}$I]MIBG showing a statistically significant tumour

growth delay when compared to the PBS control ($p<0.0001$) with the time required to reach 15 times the tumour volume by day.

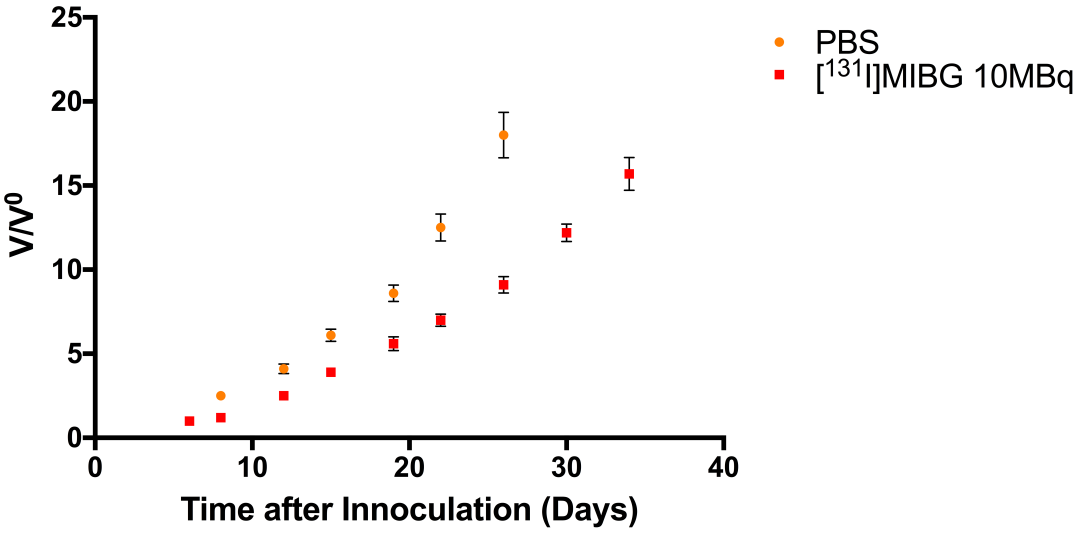



**Figure 3-6** **$^{131}$IMIBG significantly reduces NAT transfected melanoma tumour volume.**

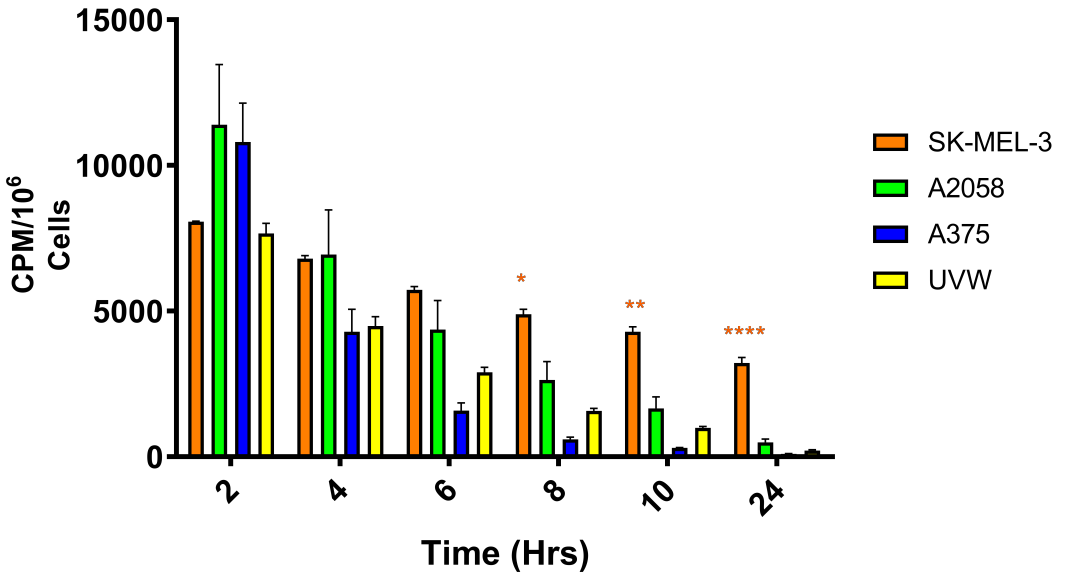
Growth of tumour xenografts in mice exposed to PBS or [$^{131}$I]MIBG (10 MBq). Xenografts were derived from the A375/NAT transfected melanoma cell line. Results are expressed as means of 6 mice per treatment group ± SD. V/V0 is representative of the volume at a given time point during the study over the volume at day 0 which is the time of MIBG treatment.

## 3.10 *[$^{131}$I]MIP1145 uptake and retention in vitro.*

The melanoma cell lines SK-MEL-3 (eumelanotic), A2058 (pheomelanotic) and A375 (amelanotic) along with the Glioma cell line UVW were treated with [$^{131}$I]MIP1145 pelleted and washed with PBS. The radioactivity of the cells (expressed as counter per minute per 105 cells) was determined by gamma counter at 2, 4, 6, 8, 10 and 24 hrs following removal of [$^{131}$I]MIP1145. All cell lines demonstrated [$^{131}$I]MIP1145 uptake at 2 hrs with notable [$^{131}$I]MIP1145 retention 84%, 61%, 40% and 59% in the SK-MEL-3, A2058, A375 and UVW cell lines compared to 2 hrs respectively. At 6 hrs

retention of radiopharmaceutical was seen to be 71%, 38%, 15% and 38% respectively. By 8 hrs retention was 61%, 23%, 6% and 20% of the initial 2 hr readings respectively. By the 10 hrs time point the SK-MEL-3 cell line had retained significantly more than other melanoma and UVW control cell lines 53% vs. 15%, 3% and 13% in the A2058, A375 and UVW lines respectively ($p<0.05$). By the final 24 hrs time point the SK-MEL-3 cell line had retained 40% of the initial radioactive dose of [$^{131}$I]MIP1145. Whilst the A2058, A375 and UVW cell lines retained 4%, 1%, and 3% radioactivity respectively, Dunnett's multiple comparison tests demonstrated no significant difference over the total duration of the 24 hrs time course between the A375 amelanotic and UVW non-melanoma control cells ($p = 0.9999$, Figure 3-7).

This result confirmed that [$^{131}$I]MIP1145 was selectively retained in melanotic cells as the darkly pigmented eumelanin containing melanoma cell line SK-MEL-3 over 24 hrs (40% retention) and moderately retained in the A2058 cell line over 10 hrs (15% retention), whilst egressing from amelanotic cells over time. This high specificity, particularly to eumelanin containing cells demonstrates that that [$^{131}$I]MIP1145 can possibly be applied as a targeted radiotherapy in malignant melanoma.




| | SK-MEL-3 | | A2058 | | A375 | | UVW | |
|---|---|---|---|---|---|---|---|---|
| Time Point | Significance | P Value | Significance | P Value | Significance | P Value | Significance | P Value |
| 2hrs vs. 4hrs | ns | 0.7682 | *** | 0.0003 | **** | <0.0001 | * | 0.0204 |
| 2hrs vs. 6hrs | ns | 0.161 | **** | <0.0001 | **** | <0.0001 | *** | 0.0001 |
| 2hrs vs. 8hrs | * | 0.0199 | **** | <0.0001 | **** | <0.0001 | **** | <0.0001 |
| 2hrs vs. 10hrs | ** | 0.0033 | **** | <0.0001 | **** | <0.0001 | **** | <0.0001 |
| 2hrs vs. 24hrs | **** | <0.0001 | **** | <0.0001 | **** | <0.0001 | **** | <0.0001 |

Figure 3-7 [$^{131}$I]MIP1145 uptake and retention *in vitro*.

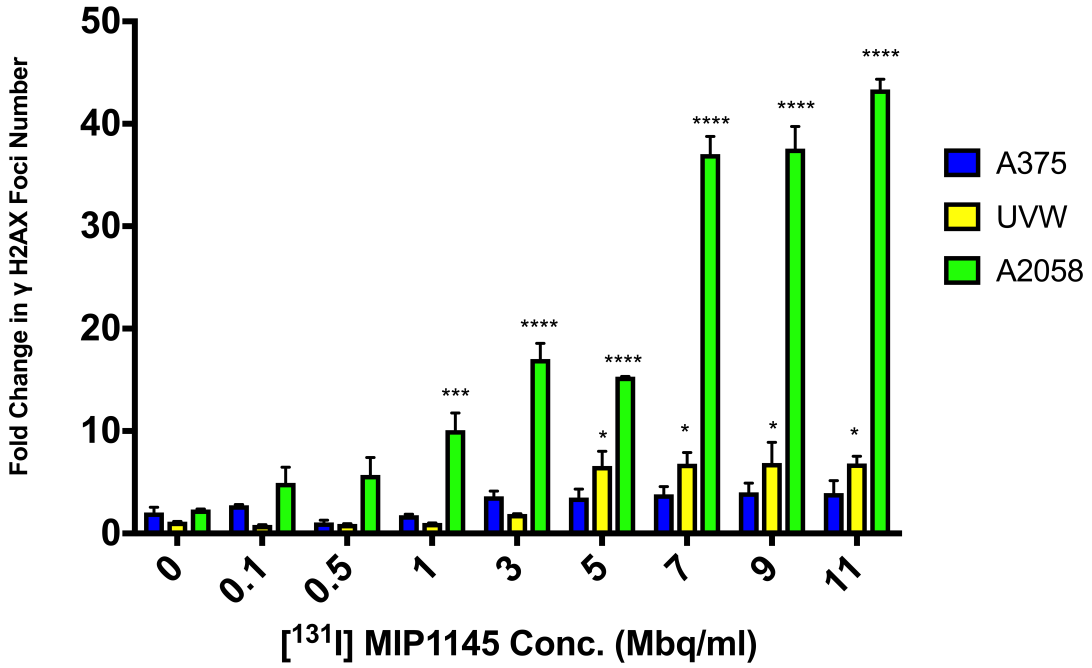
SK-MEL-3 (eumelanotic), A2058 (pheomelanotic) and A375 (amelanotic) along with the Glioma cell line UVW (amelanotic) were treated with 7 Kbq [$^{131}$I]MIP1145, pelleted and washed with PBS before determination of radioactivity via gamma counter at 2, 4, 6, 8, 10 and 24 hrs post treatment. The melanotic SK-MEL-3 cell line was the only cell line to retain significant levels of [$^{131}$I]MIP1145 over 24 hrs ($p < 0.0001$). The table presents the statistical significance and corresponding P values following 2-way ANOVA analysis. The values representative of three independent experiments performed in triplicate with error bars showing standard deviations (n=3).

## 3.11 *Expression of γH2AX foci in response to [$^{131}$I] MIP1145 treatment.*

In order to characterise the specificity of [$^{131}$I]MIP1145 induced damage, levels of genomic damage were assessed in melanotic *vs* amelanotic cell populations using FACS analysis was used to determine out to determine the γH2AX foci expression, a well-established marker of DNA damage (Mah, El-Osta et al. 2010, Sharma, Singh K Fau - Almasan et al. 2012, Mariotti, Pirovano et al. 2013).

A2058, A375, SK-MEL-3 and the UVW control cell lines were treated with escalating doses of [$^{131}$I]MIP1145 for 2 hrs, after which the cells were washed twice with PBS and incubated for a further 24 hrs at 37°C and 5% $CO_2$ prior to being suspended in cold 70% ethanol and subsequently analysed using the Millipore H2AX antibody quantified by FACS analysis. Following [$^{131}$I]MIP1145 treatment a dose dependant increase in the number of cells expressing H2AX foci was seen in the A2058 cell line, rising to 48% (± 1.78) of the population expressing γH2AX foci after the 2 hrs treatment of 11MbQ [$^{131}$I]MIP1145. In contrast 3.95% (± 2.11) of the A375 and 6.84% (± 1.20) of the UVW populations expressed γH2AX foci at the same dose, no survival was seen in any of the SK-MEL-3 treated groups. No statistically significant difference was found between the A375 and UVW lines following ANOVA analysis ($p = 0.2818$). Crucially, the levels of H2AX expression demonstrated by the A2058 lines was highly significant when compared to either A275 or UVW lines ($p < 0.0001$) which serves to clearly demonstrate, when combined with uptake data (Figure 3-8) that [$^{131}$I]MIP1145 is selectively retained by melanotic cells over time, whilst sparing

amelanotic cells significant levels of genomic damage over short term exposure (< 2 hrs). It should be pointed out however that ideally H2AX levels should have been assayed as a time course mirroring the uptake experiments at 2, 4, 6, 8, 10 and 24 hrs. In order to ascertain whether treated cells are able to repair [$^{131}$I]MIP1145 induced damage over time.




| | A375 | | UVW | | A2058 | |
|---|---|---|---|---|---|---|
| Radiation Dose (MBq) | Significance | P value | Significance | P value | Significance | P value |
| Untreated vs. 0.1 | ns | >0.9999 | ns | >0.9999 | ns | 0.7284 |
| Untreated vs. 0.5 | ns | 0.9992 | ns | >0.9999 | ns | 0.3991 |
| Untreated vs. 1 | ns | >0.9999 | ns | >0.9999 | *** | 0.0001 |
| Untreated vs. 3 | ns | 0.979 | ns | >0.9999 | **** | <0.0001 |
| Untreated vs. 5 | ns | 0.9872 | * | 0.0191 | **** | <0.0001 |
| Untreated vs. 7 | ns | 0.96 | * | 0.0128 | **** | <0.0001 |
| Untreated vs. 9 | ns | 0.9293 | * | 0.011 | **** | <0.0001 |
| Untreated vs. 11 | ns | 0.9403 | * | 0.0119 | **** | <0.0001 |

Figure 3-8 Expression of γH2AX foci in response to [$^{131}$I] MIP1145 treatment.

A2508, A375 and UVW cell lines were treated with 0.1, 0.5, 1, 3, 5, 7 and 11Mbq [$^{131}$I] MIP1145. A2058 cells saw a 48% (± 1.78) increase in cell expression of γH2AX foci whilst the A375 and UVW cell lines saw 3.95% (± 2.11) and 6.84 (± 1.20) increase respectively. The table presents the statistical significance and corresponding P values following 2-way ANOVA analysis. The values are mean surviving fractions

of three independent experiments performed in triplicate with error bars showing standard deviations (n=3).

## 3.12 *Clonogenic Survival of Melanotic and Amelanotic cell lines following [$^{131}$I] MIP1145 Treatment.*

Following on from [$^{131}$I]MIP1145 uptake studies that determined that only melanotic cells were able to retain [$^{131}$I]MIP1145 over clonogenic assays were carried out in order to ascertain whether this translated to selective cytotoxicity in melanotic melanoma cell lines. The A375 amelanotic and A2058 pheomelanotic cell lines were treated with escalating doses of [$^{131}$I] MIP1145 (Figure 3-9). [$^{131}$I] MIP1145 was found to induce significant levels of cytotoxicity in both melanoma cell lines over progressively higher concentrations as the survival fraction of the A2058 population dropping dramatically to 0.28 ($\pm$ 0.01) following 0.1 MBq/ml of the radiopharmaceutical. The A375 cell line unexpectedly responded to [$^{131}$I]MIP1145 treatment, albeit to a much lesser degree than the A2058 line, with 0.1 Mbq/ml reducing the survival fraction to 0.69 ($\pm$ 0.34) complete kill being observed following 5 Mbq/ml of radiopharmaceutical in the A2058 cell line and 11Mbq/ml [$^{131}$I ]MIP1145 in the A375 cell line indicating a greater susceptibility to [$^{131}$I ]MIP1145 induced damage in melanotic cell types. It should be noted that the SK-MEL-3 cell line was also assessed for clonogenic survival, however no survival was noted in any of the treatment groups.

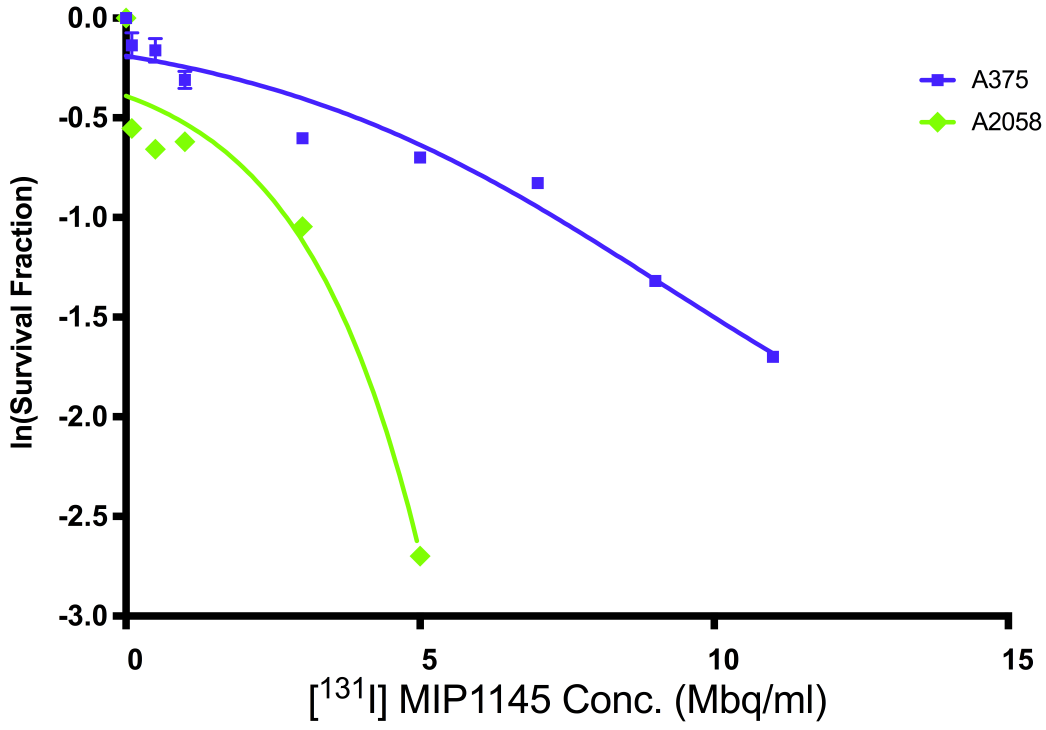



**Figure 3-9 Clonogenic survival of malignant melanoma cells following [$^{131}$I]MIP1145 treatment.**

A dose response was noted in both the amelanotic A375 cell line and pheomelanotic A2058 line. The values are mean surviving fractions on a logarithmic scale, fitted to a linear quadratic curve. The steep curve of the A2058 pheomelanotic line demonstrates a greater susceptibility to treatments requiring 0.1 Mbq/ml of compound to reduce the survival fraction to 0.28 (± 0.01), at the same dose levels A375 survival was reduced to 0.69 (± 0.34). The values are mean surviving fractions of three independent experiments performed in triplicate with error bars showing standard deviations (n=3).

## 3.13 *[$^{131}$I]MIP1145 uptake, retention and bio distribution in vivo.*

Preclinical evaluation of the uptake of [$^{131}$I]MIP1145 to melanin containing cells must be undertaken in order to rule out off site targeting of the compound. Prior to the uptake and retention data of [$^{131}$I]MIP1145 in figure 3-10. Bio distribution of the compound has also been investigated *in vivo* as part of pilot data generated by the manufacturer of the compound, Molecular Insight Pharmaceuticals (MIP) (figure 3-10). This pilot data from MIP formed the basis of the [$^{131}$I]MIP1145 *in vitro* and *in vivo* work throughout this investigation which was reported by Joyal, Barrett et al. 2010.

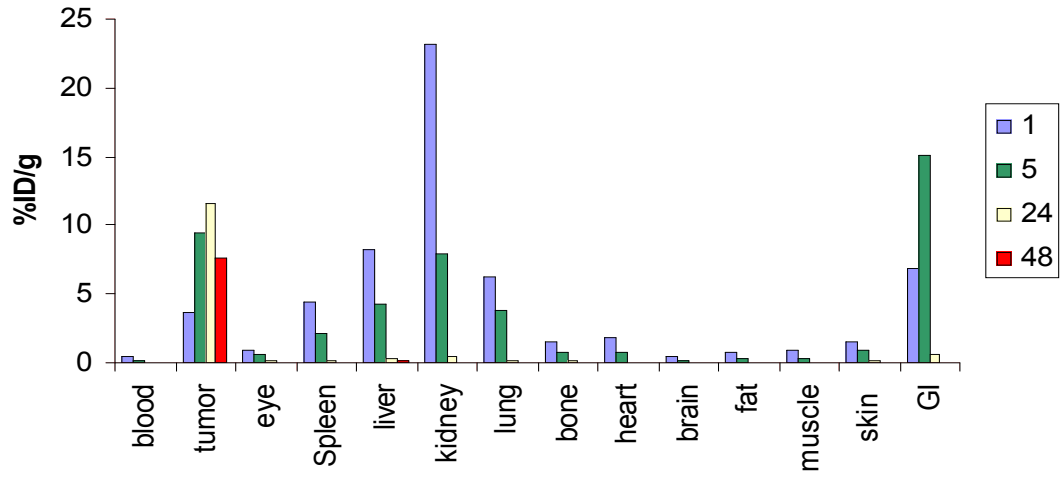



Figure 3-10 Tissue Distribution of [$^{131}$I]MIP1145 in SK-MEL-3 Nude Mice.

SK-MEL-3 tumour bearing nude mice were inoculated with 74Kbq of 131IMIP1145, mice (n=3) were euthanized at 1, 5, 24 and 48 hr post injection, tissues were removed and weighed with radioactivity being determined by use of a gamma counter. This figure was amended from Joyal et al 2010.

## 3.14 *Multiple administrations of [$^{131}$I] MIP1145 elicits a tumour growth delay in vivo.*

SK-MEL-3 tumour xenograft bearing athymic nude mice were administered a dose of 1.25 GBq/kg [$^{131}$I] MIP1145 as individual treatments either as a single dose on day 7 (once tumours had reached 5x5cm$^2$) or as a double or triple dose treatment on day 14 and 21 respectively. A combination of [$^{131}$I] MIP1145 and Dacarbazine administered as 3 separate doses at 1.25 Gb/Kg and 80 mg/kg for [$^{131}$I]MIP1145 and Dacarbazine respectively was also administered on the 3 dose schedule, along with control groups of PBS or 80 mg/kg of Dacarbazine alone.

Data shown in figure 3-11 shows that [$^{131}$I] MIP1145 administration produces a cumulative inhibitory effect on tumour growth. A combination of [$^{131}$I]MIP1145and Dacarbazine administered in multiple doses (3 administrations) was seen to be more effective than a single or double dose of [$^{131}$I] MIP1145 and a single dose of Dacarbazine administered alone. However, the combination treatment schedule proved less effective than three doses of [$^{131}$I] MIP1145 alone.

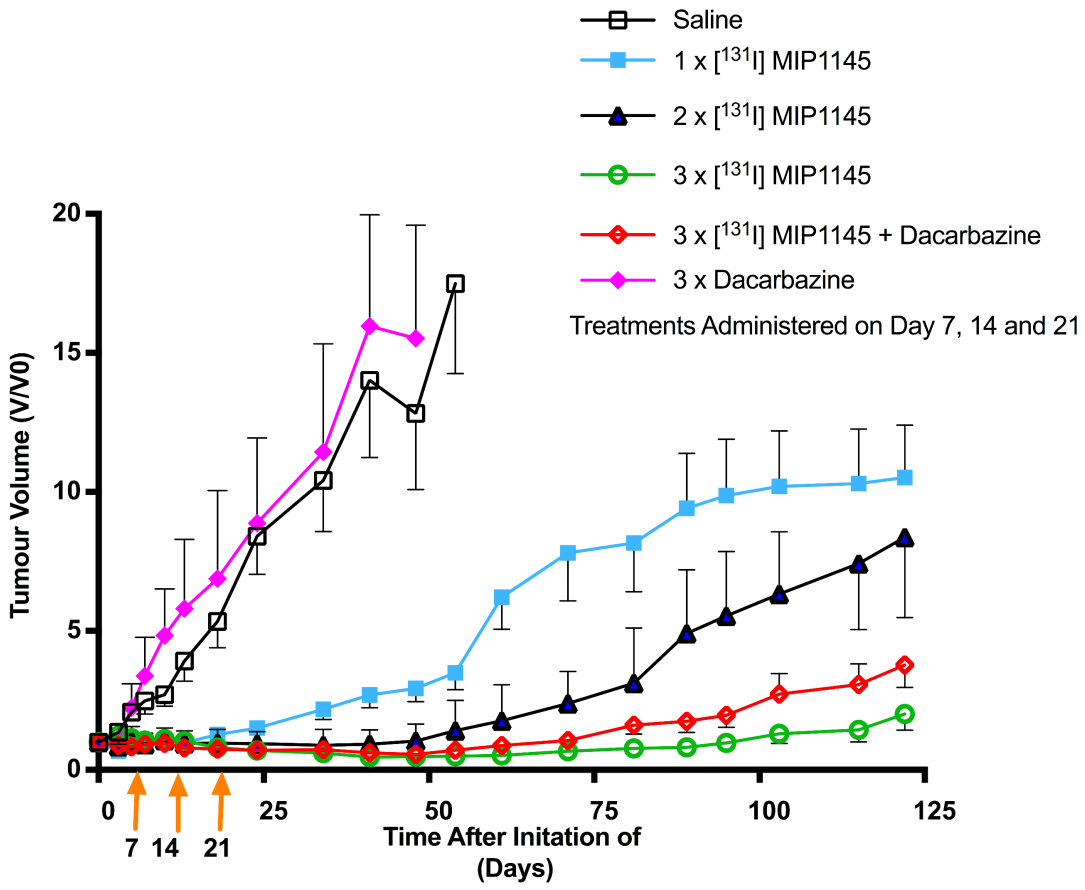



**Figure 3-11 Effect of [$^{131}$I] MIP1145 (1.25 GBq/kg) ± Dacarbazine (80 mg/kg)**

On the growth of SK-MEL-3 tumours in nude mice. Treatments were administered on day 7, 14 and 21. The graph demonstrates that [$^{131}$I]MIP1145 produces a cumulative inhibitory effect on tumour growth, with the combination of Dacarbazine and [$^{131}$I]MIP1145 proving more effective than two doses of [$^{131}$I]MIP1145 alone but less effective than three single doses of [$^{131}$I]MIP1145 when compared to Dacarbazine alone.

## 3.15 ***Discussion.***

The aim of this chapter was to characterise melanoma cell lines of differing melanin status, assessing the efficacy of [$^{131}$I] MIBG treatment following transfection with the noradrenaline transporter as well as to determine the susceptibility of melanoma cells of differing melanin status to the novel melanin targeting compound [$^{131}$I]MIP1145. Both [$^{131}$I] MIBG and [$^{131}$I]MIP1145 are radiopharmaceutical molecules produced from the conjugation of the beta emitter $^{131}$I to the noradrenaline analogue MIBG or the melanin binding benzamide MIP1145 respectively. By investigating two distinct radiolabelled compounds and exploring exogenous gene delivery dependant compounds, the present study addresses the heterogeneity of melanoma tumours, which do not universally contain melanin and often consist of a mixed population of melanotic and amelanotic cells (Miller, Emley et al. 2012, Brozyna, Jozwicki et al. 2016). This approach offers potential routes for treating patients stratified based upon the melanin status of their disease.

In order to assess both targeting methods, cell lines were chosen based on documented melanin expression, with lines either expressing Eumelanin (SK-MEL-3 and B16-F10-Luc2) (Choi, Sohn et al. 2010), Pheomelanin (A2058) (Matthews, Wilson et al. 2011) or reportedly lacking overall melanin expression (A375) (Niles, McFarland et al. 2003). The UVW line was used as a non-melanoma control for [$^{131}$I]MIP1145 studies Transfection experiments have led to the generation of the A375-CMV/NAT transfected cell line which demonstrating a 17.54 fold increase in [$^{131}$I] MIBG uptake

beyond those treated with DMI (desmethylimipramine), a selective inhibitor of NAT mediated MIBG uptake. Uptake experiments with the A2058-CMV/NAT and B16-F10-Luc2-CMV/NAT cell lines did not result in statistically significant [$^{131}$I]MIBG uptake. This may have been due to low transfection efficiency or post transfection epigenetic silencing of the CMV promoter by elevated HDAC activity and CPG island capping, both possibilities could be assessed via PCR of the NAT gene to confirm presence of the plasmid and visualisation of GFP - also present on the plasmid - to confirm active gene expression.

Transfection experiments with either the HTR or HTERT promoters did not result in uptake of [$^{131}$I] MIBG in any of the melanoma cell lines. This may be due to the aforementioned observations of epigenetic silencing and low transfection efficiency, but may also be attributed to the P53 status of the cells lines investigated, both the A375 andB16-F10-Luc2 cell lines and the A2058 cell line contains a point mutation of P53 which is not reported to effect the antiapoptotic and trans activating functions of the gene. (Sook YU 2009) Expression of factors related to P53, MDM2 and Menin are known negative regulators of HTR and HTERT activity (Zhao, Bilsland et al. 2005) and may very well have been responsible for suppressing HTR and HTERT activity in the transfected cell lines.

When characterizing cells for radiation experiments plating efficiencies were found to be satisfactory for conducting clonogenic assays in all cell lines when seeded at 250 cells per 60mm plate. Cells were exposed to an escalating dose range of ionizing radiation (Figure 3-4) and interestingly a biphasic dose response was observed with a slight increase in survival between 0.5 and 1Gy in the A2508 and B16-F10-Luc2 cell

lines. This may be as a result of the induction of pro survival pathways such as those associated with NFκ β, which is shown to be elevated in B16 melanoma cells at doses of 0.5gy and above (Prasad, Mohan et al. 1994). Boothman, Meyers et al. 1993 have also shown a panel of genes involved in DNA repair, apoptosis and mutagenesis that are induced by low dose exposure to X-rays in melanoma cell lines and there is evidence to suggest that integrin expression, considered a potential marker for metastatic growth (Albelda, Mette et al. 1990) are also induced following low dose X-Ray irradiation in melanoma cells (Onoda, Piechocki et al. 1992) All of which may account for the noted increase in survival at sub lethal ionizing radiation doses. The differences seen between the A375 parental line and A375-CMV/NAT plating efficiencies and survival at low dose X-ray exposure may be attributed to the presence of GFP and the CMV promoter. GFP is widely noted to elevate the free radical status of cells over time, although this would have a negative impact on conventional cells, elevated free radicals are potent growth stimulus for melanoma cells (Farmer, Gidanian et al. 2003).This, alongside the CMV promoter which exhibits several CREB binding sites and may positively influence antiapoptotic signalling (Wang, Qian et al. 2014) may have a marginal impact on cell survival beyond the parental cell lines.

The A375/NAT and A375 parental cell lines were investigated for [$^{131}$I] MIBG induced toxicity by *in vitro* clonogenic assay and *in vivo* xenograft inoculation. Both experiments have shown that the transfected A375/NAT transgenic cell line expresses stable and functional Noradrenaline transporters, which facilitated the uptake of [$^{131}$I] MIBG, which upon treatment results in dose dependant toxicity

specifically to transgenic cells. This is of considerable significance as these data demonstrate a potential application for $^{131}$IMIBG therapy in the treatment of malignant melanoma via the introduction of a non-native transgene.

The data obtained from the [$^{131}$I]MIP1145 studies provides encouraging evidence for the use of a native target for radiotherapy to melanoma. results demonstrated the efficacy of [$^{131}$I]MIP1145 In vitro with clonogenic toxicity in a dose responsive manner in both the A2058 pheomelanotic line and A375 amelanotic lines. The toxicity observed in the A375 line was unexpected as retention of [$^{131}$I]MIP1145 is only observed in melanotic tumours (Joyal, Barrett et al. 2010). However it has been noted previously that benzamide compounds can uptake in non-melanotic cells *in vitro* with retention of the compound only being observed in melanotic lines over time (Mansard, Papon et al. 2005). This was demonstrated during uptake and retention experiments whereby [$^{131}$I]MIP1145 retention was only observed in the melanotic SK-MEL-3 and A2058 cell lines over a 24hr period. The A375 cell lines showed a gradual efflux over 10hrs which may account for the unexpected cell kill observed in the amelanotic line as well as the limited increase in H2AX expression in the A375 and UVW lines, which also comparatively low when compared to the A2058 melanotic equivalent.

These observations suggest that further in vitro clonogenic studies utilising a modified experimental protocol involving more frequent PBS washes would facilitate more efficient efflux of the unbound compound and yield a more accurate representation of the potential cytotoxicity of the compound in non-target cells

[$^{131}$I]MIP1145 elicits significant tumour growth delay in the SK-MEL-3 nude mouse model, with rapid accumulation of [$^{131}$I]MIP1145 at the tumour site and all unbound compound being excreted 24hrs, treatment resulted in a cumulative inhibitory response, with the triple dose schedule producing significant growth arrest throughout the course of treatment.

Observation of tumour volume post treatment demonstrated an elevation in the rate of tumour growth, suggesting that the treatment did not result in the complete sterilisation of the tumour burden. A possible reason for this recurrence in tumour growth is the presence of so called 'cancer stem cell' populations within melanoma tumours.

Such cells have been shown to populate hypoxic regions of tumours. The effects of ionising radiation are reduced in these regions due to the lack of oxygen, providing radioresistant niche for these cells. 'cancer stem cells also exhibit lower amounts of double strand DNA damage upon assault with ionising radiation, as well as an ability to rapidly repopulate tumours due to an overexpression of developmental pathways such as notch and wnt (Pajonk, Vlashi et al. 2010).

Further studies using [$^{131}$I]MIP1145 by Joyal etal 2010 noted using Monte Carlo simulations in cymologous monkeys forecast the potential exposure and uptake of [$^{131}$I]MIP1145 in the human eye. It has been determined that retinopathy and

cataracts develop at 45Gy and 10Gy respectively, which would equate to a 20.8 Gbq dose of [$^{131}$I]MIP1145 reaching the retina to induce retinopathy and a dose of 7.7 GBq of [$^{131}$I]MIP1145 reaching the lens of the eye to induce cataracts. Based upon dosimetry and scintillation data it has been extrapolated a maximum safe administration of 16.2 GBq to humans and that the dose limiting organ is the large intestine. A therapeutically efficacious dose of [$^{131}$I]MIP1145 is estimated to be 2.5 Gbq/m$^2$ which would result a dose to the large intestine 3.3 times less than the maximum tolerable limit for that organ. Eye exposure is estimated to be 6.1 Gy, 39.9% lower than the 10 Gy toxicity limit (Joyal, Barrett et al. 2010). The evidence presented in this study and the further study by demonstrates the potential of $^{131}$I labelled MIP1145 for therapy in late stage melanoma patients.

# Chapter 4

# Investigating the use of the oncolytic HSV1716 viral vector for the delivery of the noradrenaline transporter to facilitate the uptake of [$^{131}$I] MIBG in an in vivo melanoma model.

## 4.1 *Introduction.*

In order to progress investigations using [$^{131}$I ]MIBG in an *in vivo* context a suitable delivery vector must be found. The Herpes simplex type 1 virus (HSV-1) presents itself as an attractive option for cancer therapy and the delivery of transgenic material. The virus has a relatively pliable genome, allowing for the alteration of up to 30 kb of material. HSV-1 has a wide a tropism and has shown the capacity to infect both dividing and non-dividing cells without integration into the host genome (Shen and Nemunaitis 2006). The ease of manipulation of the HSV-1 genome has enabled the development of selectively replication-competent viruses via alterations to the expression of the ICP34.5 virulence factor a key protein in the initiation of viral replication and evasion of the host immune response (Li Y Fau - Zhang, Zhang C Fau - Chen et al. , Shen and Nemunaitis 2006, Grandi, Peruzzi et al. 2009). This study utilizes such a virus, HSV1716 a variant of the Glasgow strain 171, which has deletions in both copies of the RL1 gene encoding the ICP34.5 factor (MacLean, ul-Fareed et al. 1991),

resulting in the selective replication of the virus in non-differentiated dividing cells (Brown, Harland et al. 1994). *In vivo* studies using the HSV1716 strain have demonstrated significantly improved survival in human tumour baring xenograft murine models, and the virus has shown itself to be safe on exposure to normal tissue following inoculation into a normal animal brain (Randazzo, Bhat et al. 1997, McKie, Brown et al. 1998, Toyoizumi, Mick et al. 1999, Coukos, Makrigiannakis et al. 2000, Thomas and Fraser 2003). The safety and clinical efficacy of the HSV1716 strain has been further proven in the clinical context following Phase I clinical trials with the virus administered as a monotherapy in patients suffering from Glioma (Rampling, Cruickshank et al. 2000, Papanastassiou, Rampling et al. 2002, Harrow, Papanastassiou et al. 2004), squamous cell carcinoma of the head and neck (Mace, Ganly et al. 2008) and most prominently for this study, refractory malignant melanoma (MacKie, Stewart et al. 2001).

The HSV1716 virus utilized in this study is a second-generation variant of the original strain, modified to express the Noradrenaline Transporter. This virus, HSV1716/NAT was constructed to facilitate the uptake of [$^{131}$I] MIBG in non-neural crest-derived tumours and has previously demonstrated efficacy *in vitro* following infection of a glioma cell line, achieving expression of the NAT gene and facilitating uptake of [$^{131}$I] MIBG (Quigg, Mairs et al. 2005).

The success of NAT gene transfer invitro has large implications for in vivo cancer gene therapy, as the delivery of a gene facilitating the uptake of radionuclides will not only

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deliver therapy to target cells but potentially expose cells to the cross-fire effect on on-targeted cells in the vicinity of targeted and radiopharmaceutical containing counter parts. This is further compounded by the radiation induced bystander biological bystander effects (RIBBE), which have particular significance with targeted radiotherapies that typically deliver a low dose and low dose rate dose of radioactivity (Mothersill and Seymour 2001, Boyd, Mairs et al. 2002) where by irradiated cells generate toxic factors, which impinge on the functions of the surrounding cellular material (Boyd, Ross et al. 2006). The data presented in this chapter serves to demonstrate the potential efficacy of the HSV1716/NAT virus in combination with [$^{131}$I]MIBG treatments in *in vivo* Melanoma and Glioma models.

## 4.2 ***Aims***

- Determine the bio distribution of HSV1716/NAT and [$^{131}$I] MIBG in UVW and SK-MEL-3 bearing nude mouse xenografts.
- Investigate the effects of intratumoural and intravenous administration of HSV1716/NAT and [$^{131}$I] MIBG on tumour growth delay.
- Consider the effects of HSV1716/NAT and [$^{131}$I] MIBG scheduling on tumour growth delay following intratumoural and intravenous administration.

## 4.3 *Biodistribution of [$^{131}$I]-MIBG following virus administration.*

A functional [$^{131}$I] MIBG-uptake assay was used to determine NAT gene expression in UVW and SK-MEL-3 xenografts and normal organs following infection with HSV1716/NAT.

Initially, nude mice bearing tumours of both UVW and SK-MEL-3 were treated with [$^{131}$I] MIBG radiopharmaceutical, HSV1716 and HSV1716/NAT virus in two schedules. Simultaneous administration of [$^{131}$I] MIBG with 1x10$^{6}$ PFU of HSV1716, HSV1716/NAT or PBS and/or PBS and virus inoculated 24 hrs prior to [$^{131}$I] MIBG administrations was compared (Figure 4-1A,B). In order to determine the bio distribution of [$^{131}$I] MIBG in the absence of the NAT transgene, control cohorts received PBS/HSV1716 viral inoculation.

Results from one-way ANOVA testing determined no significant difference in [$^{131}$I] MIBG concentrations between PBS and HSV1716 control cohorts, regardless of schedule. This is in contrast with comparisons between the transgenic and wild type virus/PBS, where by a significant difference in [$^{131}$I] MIBG concentrations were seen between HSV1716/NAT and the HSV1716/PBS groups respectively ($p < 0.001$) regardless of administration schedule. Mann-Whitney U testing post hoc analysis revealed that tumour uptake of [$^{131}$I] MIBG in both xenograft models was significantly enhanced by administration of HSV1716/NAT 24 hrs prior to [$^{131}$I] MIBG treatments

when considered against simultaneous treatments ($p < 0.001$). Further, these analyses show no significant difference between the retention levels of [$^{131}$I] MIBG at 24 and 48 hrs, indicating that [$^{131}$I] MIBG was retained within treated tumours.

The uptake and distribution of [$^{131}$I]-MIBG in other organ systems following intratumoural (IT) administration was assessed by comparison of uptake between tumour, liver, lung, heart, spleen, kidneys and adrenals. Both the UVW and SK-MEL-3 xenografts were studied 24 and 48 hrs post [$^{131}$I] MIBG administrations (Figure 4-2). Results show similar levels of distribution between both tumor types and no significant difference in uptake between PBS controls and HSV1716/NAT in any of the organ systems examined ($p > 0.05$) and this was seen to be uniform across the results obtained, regardless of administration schedule.

It has been previously reported that a similar modified Glasgow strain 171 simplex viruses, HSV1790, (expressing the nitroreductase transgene) administered intravenously at 1 x $10^7$ PFU results in significant accumulation of virus and thus $^{131}$I-MIBG upon administration within UVW tumour xenografts (Braidwood, Dunn et al. 2009). In order to assess whether similar cumulative effects are witnessed using HSV1716/NAT, 1 x $10^7$ PFU of virus was administered via tail vein injection 24 hrs prior to administration of [$^{131}$I] MIBG. The data shows significant uptake of [$^{131}$I] MIBG in virus treated tumours *vs.* PBS following intravenous administration ($p < 0.01$, Figure 4-2).

IT administration did not result in toxicity in members of any cohort treated, regardless of virus type, titre or radiopharmaceutical administration.

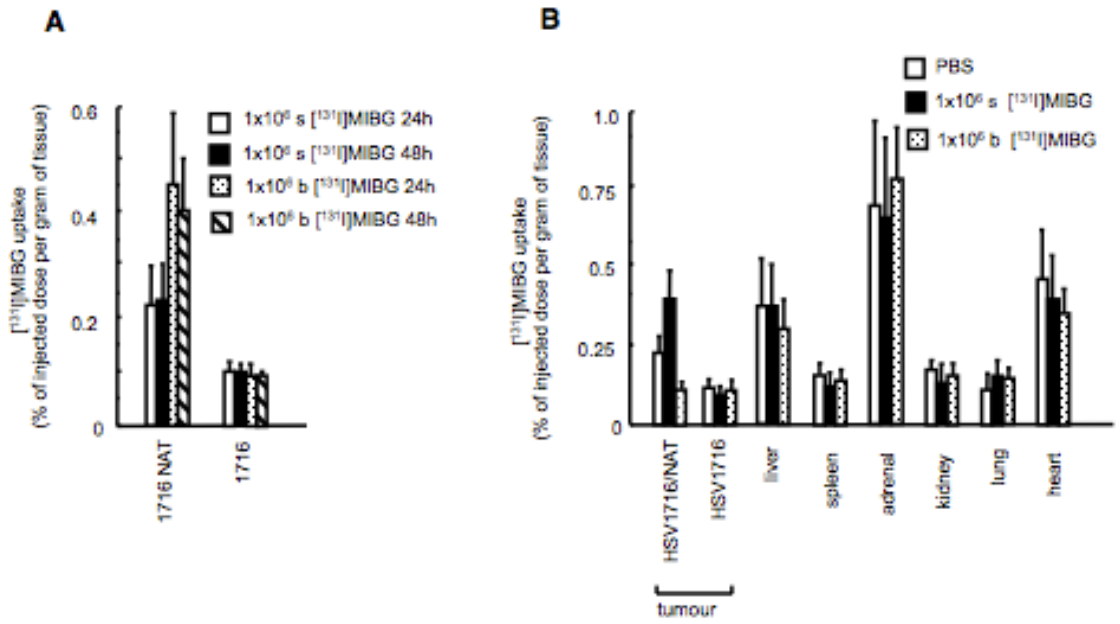



**Figure 4-1 The effect of scheduling of viral delivery on the accumulation of [$^{131}$I]-MIBG.**

This figure shows the effect of scheduling of viral delivery on the accumulation of [$^{131}$I]-MIBG by human UVW glioma (A) and normal organs (B). 1x10$^{6}$ PFU of HSV1716/NAT or HSV1716 were injected intra-tumorally 24 hrs before (b) or simultaneously (s) with intra-peritoneal administration of 2 MBq of [$^{131}$I]-MIBG. Radiation accrual was measured 24 or 48 hrs after radiopharmaceutical administration in excised tumours and 48 hrs after in normal organs. Data are means and s.d. of twelve determinations.

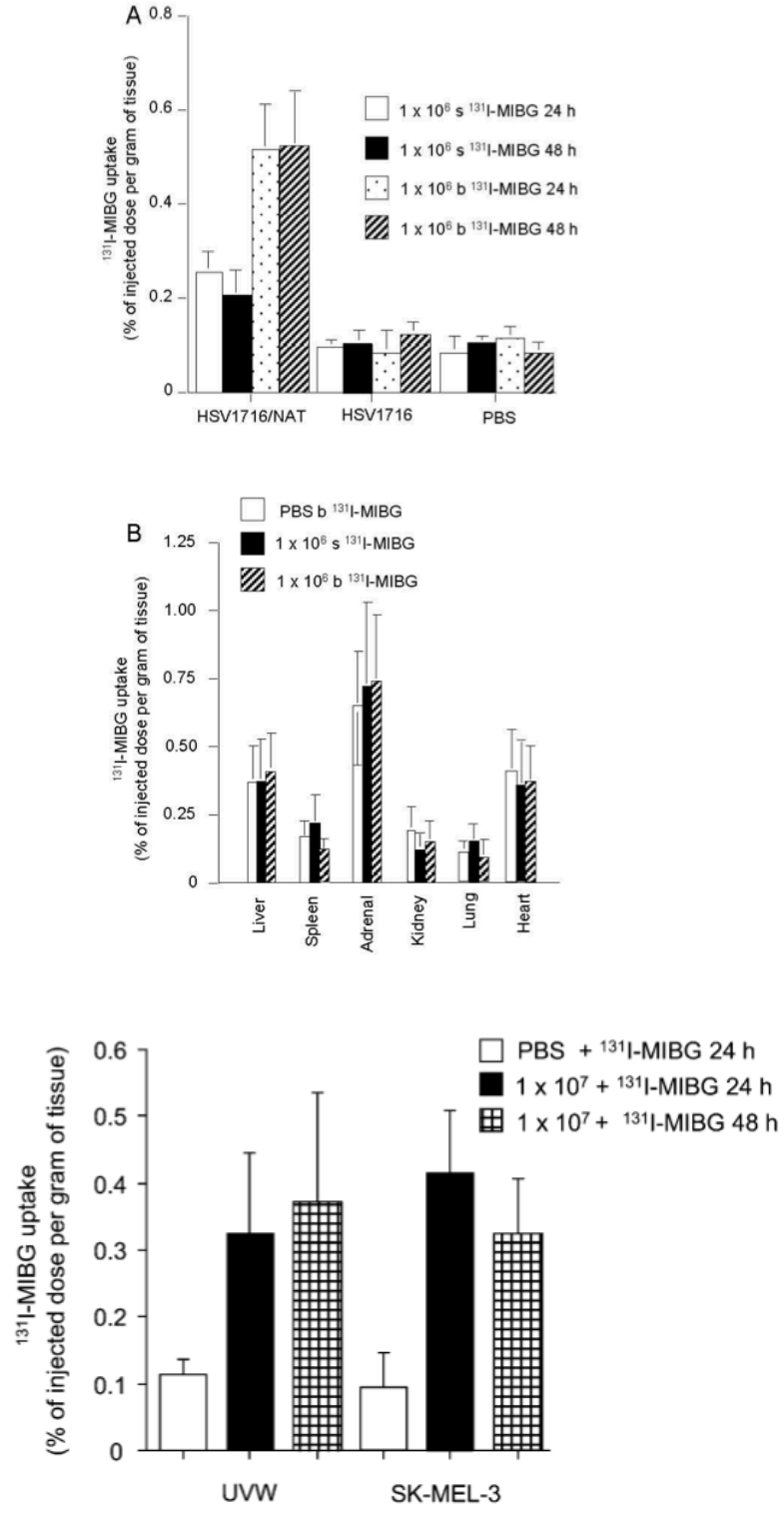



Figure 4-2 The effect of intra-venous injection of HSV1716/NAT on the accumulation of [$^{131}$I]MIBG in human UVW glioma and SK-MEL-3 tumour xenografts.

Chapter 4 - Investigating the use of the oncolytic HSV1716 viral vector for the delivery of the noradrenaline transporter to facilitate the uptake of [131I] MIBG in an in vivo melanoma model.

$1x10^7$ PFU of HSV1716/NAT was administered 24 hrs prior to intra-peritoneal administration of 2 MBq $^{131}$I-MIBG. The radioactivity in excised tumors was measured 24 or 48 hrs after radiopharmaceutical administration. Data are means and s.d. of twelve determinations.

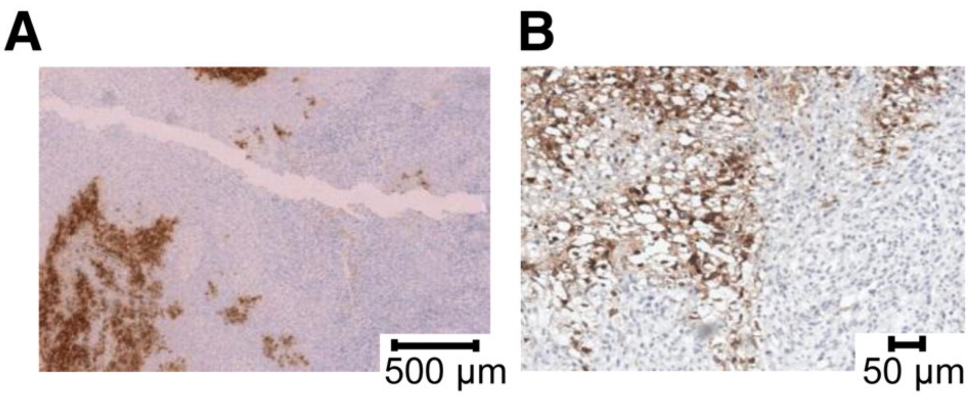
## 4.4 *Biodistribution of HSV1716/NAT.*

Immunohistochemistry techniques were utilised to ascertain the biodistribution of the HSV1716/NAT virus within UVW and SK-MEL-3 xenograft tumour tissue and organ systems.

Assessment was carried out 24 hrs post administration of virus by either intravenous and intratumoural injection. HSV positive cells (brown) were present in UVW tumour samples (fig 4-3A-C). Figure 3B demonstrates a typical outcome of infection; large multinucleated cells are present surrounded by visible holes in the tissue. This is the result of viral replication and subsequent cell lysis. Observation of samples obtained from subjects who received an intratumoural injection show evidence of HSV1716/NAT infection limited to the tumour site, with no sign of viral infection in any of the organs analysed.

Tissue samples from subjects receiving intravenous injection did not show any evidence of HSV1716 infection following immunohistochemical analysis (data not shown). This may have been as a result of the route of administration (intravenous) whereby virus did not accumulate within the tumour. Due to this, samples were taken at day 3 and day 7 for both intratumoral and intravenous administrations schedules in SK-MEL-3 xenograft bearing mice. Virus was titrated from the tissue samples onto BHK21/13C hamster kidney cells and the number of plaque forming units (PFU) was assessed.

Results demonstrate infectious virus particles in both intratumoral and intravenous samples from 3 days post administration of HSV1716/NAT, (fig 3C.). Of these, intratumorally injected samples were found to contain higher viral titres than the initial injection at day 3 and day 7, indicating viral replication within the tumour. The data shows no other accumulation of virus in any of the organs examined, either by intratumoural or intravenous injection.




C

| Tissue | Titer day 3 IV | Titer day3 IT | Titer day 7 IV | Titer day 7 IT |
|---|---|---|---|---|
| SK-MEL-3 tumor | $5.7 \times 10^4 \pm 1.2 \times 10^4$ | $2.3 \times 10^8 \pm 2.2 \times 10^8$ | 0 | $3.2 \times 10^7 \pm 4.0 \times 10^6$ |
| Liver | 0 | 0 | 0 | 0 |
| Spleen | 0 | 0 | 0 | 0 |
| Adrenal glands | 0 | 0 | 0 | 0 |
| Kidney | 0 | 0 | 0 | 0 |
| Lung | 0 | 0 | 0 | 0 |
| Heart | 0 | 0 | 0 | 0 |

Figure 4-3 Biodistribution of HSV1716/NAT.

Representative images of immunohistochemical staining of HSV (brown staining) in UVW xenograft tumors 24 hrs after intra- tumor (IT) administration of $1x10^6$ PFU of HSV1716/NAT (n=3) (C) Titer of infectious virus (PFU) present 3 and 7 days post administration of HSV1716/NAT (intra-tumor (IT) $1x10^6$ PFU, intra-venous (IV) $1x10^7$ PFU) in tumor samples from mice bearing SK-MEL-3 melanoma tumors. Data are means and s.d. of three determinations.

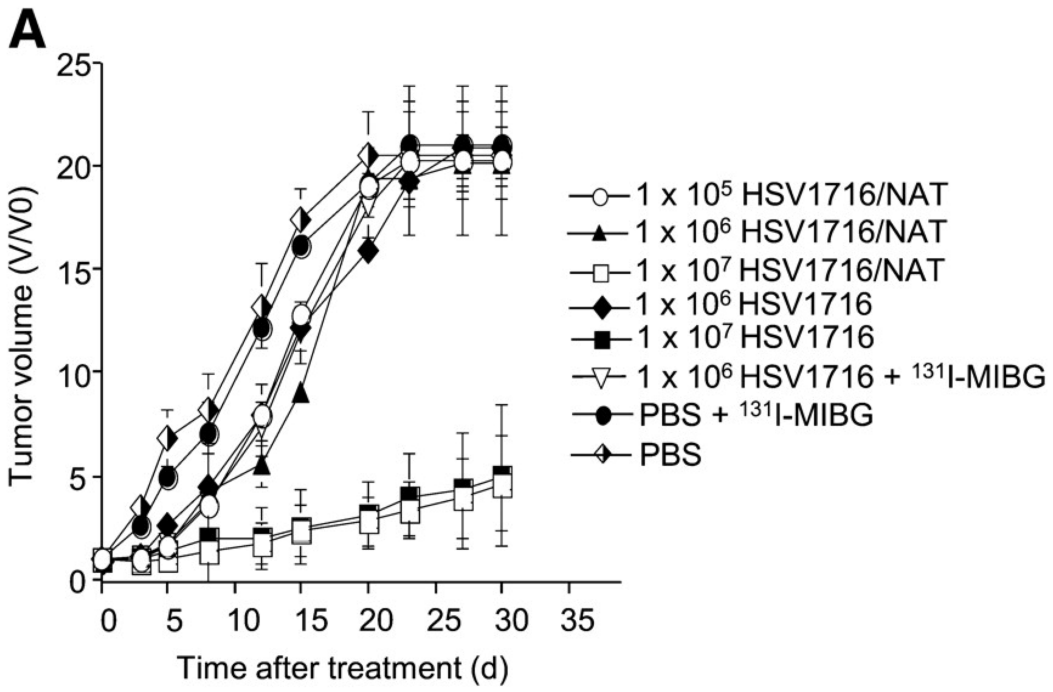
## 4.5 *Tumor Growth Delay After Intratumoral Administration of HSV1716/NAT and $^{131}$I-MIBG*

In order to determine the effects of intratumoral administration of HSV1716/NAT as a single agent, as well as ascertain any effects of the NAT transgene or wild type virus in the absence of and in combination with [$^{131}$I]MIBG. HSV1716 or HSV1716/NAT virus was administered intra tumorally alone at $1x10^5$, $1x10^6$ and $1x10^7$ PFU. One group received a control of PBS alone. 10 MBq of [$^{131}$I]-MIBG was administered 24 hrs post-viral injection to HSV1716 $1x10^6$ wild type and PBS groups.

When compared by the Kruskal-Wallis test significant differences were seen between all groups ($p < 0.001$, Figure 4-4 ). However, on comparison between pairs of individual groups it was seen that HSV1716/NAT treatment resulted in significantly greater tumour growth delay than PBS alone ($p < 0.05$). Further, administration of $1x10^6$ PFU of HSV1716/NAT resulted in significant tumour growth delay when compared to the $1x10^5$ treatment and $1x10^7$ treatment resulted in significantly slower growth than the $1x10^6$ treated group ($P<0.02$). Groups treated with either wild type HSV1716 or HSV1716/NAT showed no statistical difference between treatments with each virus ($p = 0.3$). Upon administration of 10 MBq of [$^{131}$I]MIBG 24 hrs following HSV1716 wild type virus no significant growth delay beyond observations of HSV1716 wild type alone. Similarly, [$^{131}$I]MIBG treatment administered to PBS control did not yield significantly tumour growth delay beyond PBS alone ($p < 0.05$).

These results demonstrate that [$^{131}$I]MIBG administrations in the absence of the HSV1716/NAT transgene vector do not affect the rate of tumour growth the UVW xenograft model. Cures (fig 4-4 B) were only seen in groups that received 1x10$^{7}$ PFU of either wild type or transgenic virus, indicating that the lower titres of 1x10$^{5}$ and 1x10$^{6}$ should be considered for combination therapy studies.

**A**




**B**

| Treatment | Cure rate (%) |
|---|---|
| 1 x $10^5$ PFU HSV1716/NAT | 0 |
| 1 x $10^6$ PFU HSV1716/NAT | 0 |
| 1 x $10^7$ PFU HSV1716/NAT | 58 |
| 1 x $10^6$ PFU HSV1716 | 0 |
| 1 x $10^7$ PFU HSV1716 | 50 |
| 1 x $10^6$ PFU HSV1716 + $^{131}$I-MIBG | 0 |
| PBS +$^{131}$I-MIBG | 0 |
| PBS | 0 |

Figure 4-4 Determination of an appropriate dose of virus to utilise in combination therapies.

$1x10^5$, $1x10^6$ and $1x10^7$ PFU of HSV1716/NAT and $1x10^6$ and $1x10^7$ PFU of HSV1716 was administered and assessed against the growth (A) and cure (B) of human glioma xenografts. As a control for the absence of NAT transgene delivery, [$^{131}$I]MIBG (10 MBq) treatment was preceded by intra-tumoral delivery of HSV1716 or PBS. Cure was defined as the failure of xenografts to grow over the experimental period (day 0-30). Data are means and s.d. of twelve determinations.

## 4.6 *Comparison of treatment schedule tumour growth delay following intratumoral injection of HSV1716/NAT and [$^{131}$I]-MIBG.*

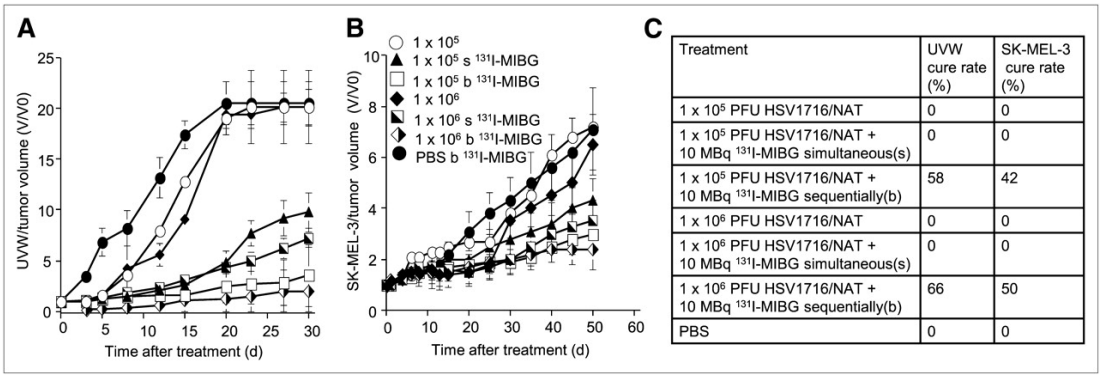
The HSV1716/NAT virus was assessed in combination with [$^{131}$I]MIBG administration via either simultaneous intratumoural injection or intratumoural [$^{131}$I]MIBG administration 24 hrs following treatment with the virus in both UVW and SK-MEL-3 xenografts (Fig 4-5 A+B) . Control groups were administered PBS 24 hrs prior to [$^{131}$I]MIBG treatments.

All groups treated with combinations of virus and radiopharmaceutical demonstrated significant differences over those treated with virus alone, regardless of treatment schedule ($p < 0.001$) which is an indication that the addition of [$^{131}$I]MIBG results in greater tumour growth delay than virus alone.

It was observed that on paired comparison, [$^{131}$I]MIBG treatments significantly influenced tumour sterilization regardless of schedule (simultaneous or 24hrs post) or quantity of virus ($1x10^5$ or $1x10^6$ PFU) ($p < 0.001$). There were significant

differences observed between the $1x10^6$ and $1x10^5$ PFU treatment groups regardless of [$^{131}$I]MIBG administrations or treatment schedule ($p < 0.001$). This effect was seen in both tumour types.

The administration of virus 24hrs prior to [$^{131}$I]MIBG treatment yielded a significant enhancement over the simultaneous schedule, demonstrating a highly effective schedule for HSV1716/NAT – [$^{131}$I]MIBG combination therapies as tumour cure was only achieved in groups receiving virus 24 hrs prior to radiopharmaceutical (figure 4-5C).




| Treatment | UVW cure rate (%) | SK-MEL-3 cure rate (%) |
|---|---|---|
| 1 x $10^5$ PFU HSV1716/NAT | 0 | 0 |
| 1 x $10^5$ PFU HSV1716/NAT + 10 MBq $^{131}$I-MIBG simultaneous(s) | 0 | 0 |
| 1 x $10^5$ PFU HSV1716/NAT + 10 MBq $^{131}$I-MIBG sequentially(b) | 58 | 42 |
| 1 x $10^6$ PFU HSV1716/NAT | 0 | 0 |
| 1 x $10^6$ PFU HSV1716/NAT + 10 MBq $^{131}$I-MIBG simultaneous(s) | 0 | 0 |
| 1 x $10^6$ PFU HSV1716/NAT + 10 MBq $^{131}$I-MIBG sequentially(b) | 66 | 50 |
| PBS | 0 | 0 |

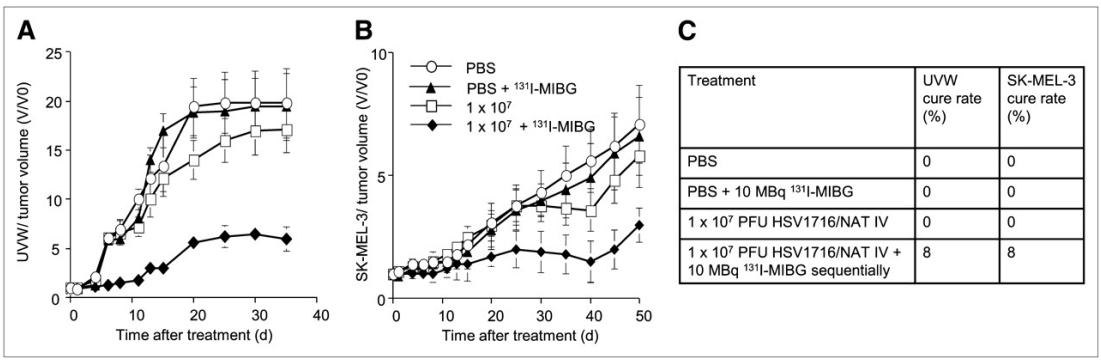
Figure 4-5 The effect of intra-tumoral administration of various titers.

HSV1716/NAT alone or in combination with [$^{131}$I]-MIBG treatment (10 MBq) on the growth of UVW (A) and SK-MEL-3 (B) xenografts. The radiopharmaceutical was administered either simultaneously with (s) or 24 h after (b) virus injection. (C) Cure of UVW and SK-MEL-3 xenografts following treatment schedules was defined as the failure of xenografts to grow over the experimental period. Data are means and s.d. of twelve determinations.

## 4.7 *Comparison of treatment schedule tumour growth delay following intravenous injection of HSV1716/NAT and $^{131}$I-MIBG.*

In order to assess whether HSV1716/NAT – [$^{131}$I]MIBG is effective following intravenous administration, 1x10$^7$ PFU HSV1716/NAT was injected as a single agent and in combination with $^{131}$I-MIBG. A schedule of viral injection 24 hrs prior to [$^{131}$I]MIBG treatments was followed, based upon previously detailed intratumoural experiments demonstrating the higher effectiveness of this schedule (section 3.3.5). As with the intratumoural study the addition of 10 MBq [$^{131}$I]MIBG to the treatment schedules resulted in significantly greater tumour sterilization than virus alone in both xenograft models ($p < 0.01$, figure 4-6 A and B).

The intravenous administration of 1x10$^7$ PFU HSV1716/NAT and 10 MBq [$^{131}$I]MIBG in combination resulted in tumour cures in both xenograft models (figure 4-6 C). This effect was not seen the in single treatment groups, demonstrating the importance and potential for use of HSV1716/NAT and [$^{131}$I]MIBG as a combination therapy.




| Treatment | UVW cure rate (%) | SK-MEL-3 cure rate (%) |
|---|---|---|
| PBS | 0 | 0 |
| PBS + 10 MBq $^{131}$I-MIBG | 0 | 0 |
| 1 x 10$^7$ PFU HSV1716/NAT IV | 0 | 0 |
| 1 x 10$^7$ PFU HSV1716/NAT IV + 10 MBq $^{131}$I-MIBG sequentially | 8 | 8 |

Figure 4-6 The effect of intra-venous (IV) administration of HSV1716/NAT (1x107 PFU).

This figure shows the effect of intra-venous (IV) administration of HSV1716/NAT ($1x10^7$ PFU) or PBS alone or in combination with $^{131}$I-MIBG treatment (10 MBq) on the growth of xenografts derived from (A) UVW glioma and (B) SK-MEL-3 melanoma cells. Radionuclide was administered 24 h after injection of virus. (C) Cure of UVW and SK-MEL-3 xenografts following treatment was defined as the failure of xenografts to grow over the experimental period. Data are means and s.d. of twelve determinations.

## 4.8 *Discussion.*

The construction of the HSV1716/NAT virus presents an opportunity to deliver the [$^{131}$I]MIBG radiopharmaceutical to a much wider variety of tumour types than those derived from the neural crest, through delivery of the Noradrenaline Transporter (Mairs and Boyd 2008).

The results presented herein demonstrate that the HSV1716/NAT virus successfully endows a human Melanoma and human Glioma cell line with the capacity to uptake [$^{131}$I]MIBG in athymic nude mouse xenografts. There was significant anti-tumour activity upon inoculation with the HSV1716 parental strain, which was further enhanced when utilizing the HSV1716/NAT recombinant strain followed by the addition of [$^{131}$I]MIBG.

Investigations into the use of the HSV1716/NAT virus and [$^{131}$I]MIBG have shown that therapeutic efficacy of the combination of treatment modalities is schedule

dependent, whereby the application of the therapy modality was found to be curative.

We show that inoculation with the HSV1716/NAT through either Intra-tumoural (IT) or Intravenous (IV) injection is non-toxic to normal tissue whilst enabling the accumulation of [$^{131}$I]MIBG in xenograft tissue with no uptake observed in normal organ systems. Notably, [$^{131}$I]MIBG uptake was negligible in tumours receiving either PBS or the HSV1716 parental strain, showing that [$^{131}$I]MIBG uptake is dependant on successful NAT gene transfection by the virus in xenografts which lack native noradrenaline transporter expression.

Scheduling of HSV1716/NAT inoculation and [$^{131}$I]MIBG administrations is a highly important factor when utilizing this therapy method. Intratumoural inoculations are the preferred clinical mode of administration for the virus, with a proven record of safety and efficacy (Rampling, Cruickshank et al. 2000, MacKie, Stewart et al. 2001, Papanastassiou, Rampling et al. 2002, Harrow, Papanastassiou et al. 2004, Mace, Ganly et al. 2008). HSV1716/NAT virus was administered via IT injection 24 hrs prior to $^{131}$I-MIBG treatment resulting in the accumulation of approximately double the concentration of $^{131}$I-MIBG when compared to levels of the radiopharmaceutical following simultaneous injection. This viral pre-treatment schedule resulted in greater inhibition of tumour growth and proved cure rates. The enhanced therapeutic effect of pre-treatment may be as a result of the pause between each

treatment mode allowing for replication of the virus in target cells, resulting not only in viral oncolysis but also simultaneous expression of the Noradrenaline transporter by cells that are undergoing viral replication or those that are infected but unable to maintain a complete viral life cycle, either due to cell cycle or metabolically unfavourable conditions.

IV administrations of the HSV1716/NAT virus were undertaken in order to provided evidence for the efficacy of the virus in patients with metastastic disease, whereby IT administration may prove to be ineffective. Here we show that, as with IT schedules, HSV1716/NAT infection facilitated [$^{131}$I]MIBG uptake in Xenograft models whilst sparing normal tissue from both virus and radiation related toxicity.

These results are in agreement with previous studies, which have demonstrated that radiation utilized in combination with HSV-1 mutants enhances cell kill in tumour models (Advani, Sibley et al. 1998, Adusumilli, Stiles et al. 2005, Jarnagin, Zager et al. 2006, Dai, Zamarin et al. 2010). One of the advantages of using targeted radiotherapy in this manner is the impact of the radiation induced biological bystander effect (RIBBE), whereby cells not directly targeted by therapy are exposed to toxic factors induced in the target population (Boyd, Ross et al. 2006). This phenomenon may act in complement with the direct cell lysis, and radiation induced toxicity in NAT expressing cells to enhance overall cell kill.

# Chapter 5

# Investigating the small molecule Mirin as a radio sensitizer in malignant melanoma.

## 5.1 *Introduction*

A multitude of internal and environmental factors contribute to continual DNA damage and cellular stress, including but not limited to; radioactive assault, reactive oxygen stress, mutagens and alkylating agents. The ability of mammalian cells to sense and efficiently repair this genetic damage is key to the long-term survival and genetic stability of an organism. To this end multiple self -checking, damage sensing and repair pathways have evolved to address the variety of damage experienced by an organism.

Malignant Melanoma tumours are typically regarded as highly radio and chemo resistant, which is in part due to the significantly elevated activation of both small and large scale DNA repair pathways, such as nucleotide excision repair and homologous recombination repair which can have a drastically negative effect on the outcome of treatment with conventional therapeutics (Bradbury and Middleton 2004). Although this poses a serious problem to treatment it also presents itself as a potential target for combination therapies. Inhibitors of DNA repair are increasingly employed as single agents and in conjunction with radiotherapy, our labs have previously reported the effectiveness of the Topoisomerase 1 inhibitors and Poly ADP ribose polymerase 1 inhibitors Topotecan and PJ34 in combination with [$^{131}$]IMIBG (McCluskey, Boyd et al. 2008, McCluskey, Mairs Rj Fau - Tesson et al. 2012).

## 5.2 *Mirin – A small molecule inhibitor of MRE11*

Mirin is a novel small molecule identified through high throughput screening targeting the MRE11 subunit of the MRE11-RAD50-NBS1 (MRN) complex, responsible for the activation of the double strand DNA repair pathways homologous recombination repair (HRR) and non-homologous end joining (NHEJ). Acting to induce the accumulation of phosphoATM, RPA and γ H2AX at DSB sites and abolishing the G2/M cell cycle checkpoint (Dupre, Boyer-Chatenet et al. 2008) , which are essential for repairing the extensive genomic damage produced by radiotherapy treatments such as external beam X irradiation and [$^{131}$]IMIBG. The DSB repair pathways are activated via phosphorylation of ATM and ATR by the exonuclease activity of MRE11, which presents compounds such as Mirin as an attractive option for combination therapies as a radio sensitizer resulting in melanoma cells being left highly vulnerable to radiotherapy treatments, a key focus of this study.

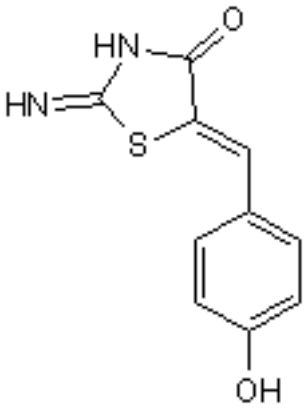



Figure 5-1 The Structure of Mirin, an inhibitor of MRE11 exonuclease activity.

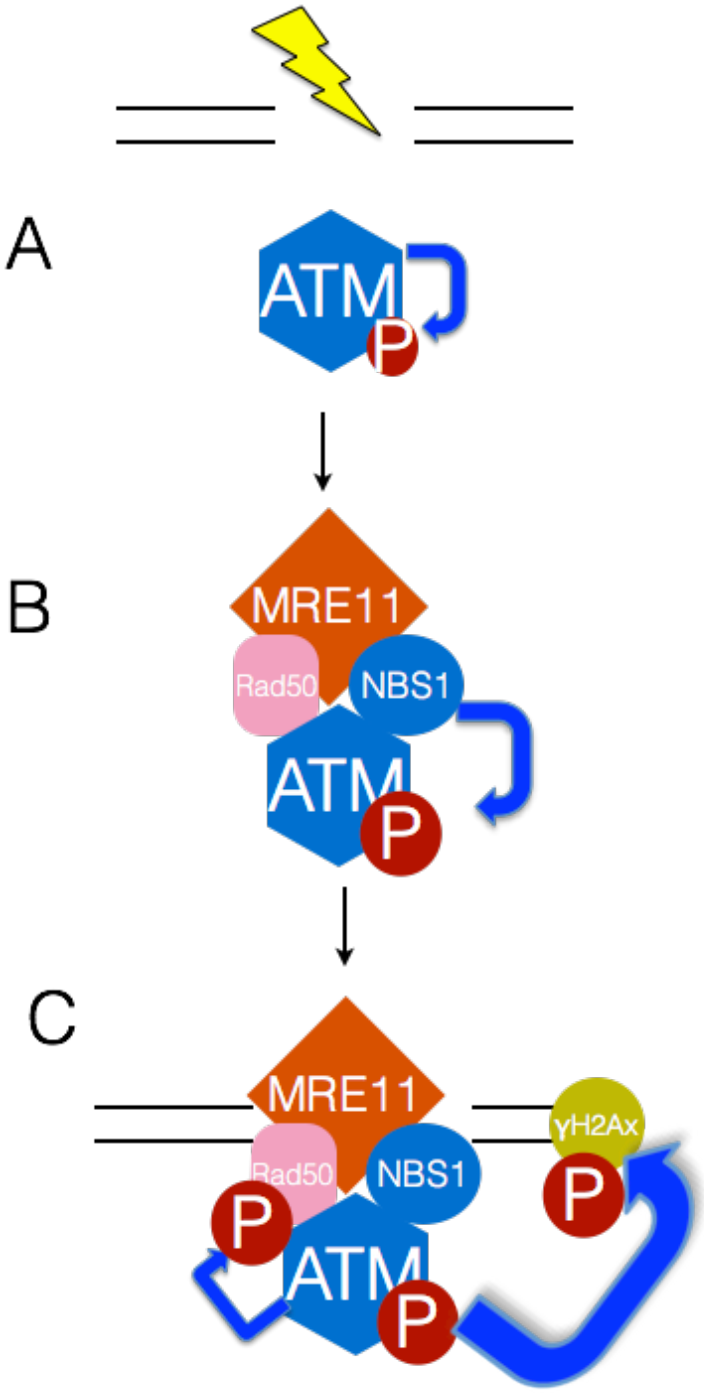



Figure 5-2 Outline of ATM Dependant Homologous recombination repair.

The MRN complex phosphorylates ATM (A and B). This in turn phosphorylates γ H2AX which accumulates at the site of DSB damage and leads to the initiation of DNA repair (C).

Chapter 5 - Investigating the small molecule Mirin as a radio sensitizer in malignant melanoma.

When considering the extensive double strand break damage produced by radiation therapies such as external beam and [$^{131}$I]MIBG treatments two pathways, the DNA double strand break repair and non-homologous end-joining pathways, are critical for the damage repair and survival of the exposed cells and tissue, requiring precise activation dependent on phosphorylation of ATM and ATR following genetic insult. These phosphorylation reactions are regulated by the Mre11-Rad50-NBS1 (MRN) complex. The exonuclease activity of Mre11, which acts to phosphorylate the PI3K-like protein ATM is of particular interest within this study as the phosphorylation is heavily involved in the activation of the DNA double strand break repair mechanisms, a key cellular defense to radioactive. This same exonuclease activity of the Mre11 phosphoesterase domain has recently been implicated in the regulation of deletional non-homologous end joining repair pathway also.

As activation of the DDSB repair pathway is dependent on ATM phosphorylation, it is theorized that inhibitors of proteins such as Mre11, may prove to be of use as radio-sensitizing agents. One such agent, 'Mirin', which has been identified from a recent for forward genetic screen, inhibits the exonuclease activity of Mre11 and thus prevents Mre11 phosphorylation of ATM. This, in theory would diminish the cells ability to repair double strand breaks induced by radioactive assault as well as abolish the ionizing radiation induced G2/M check point (Dupre, Boyer-Chatenet et al. 2008).

HRR is one of the two primary repair mechanisms (along with NHEJ) for large scale DNA damage. HRR is active during S and/or G2 phase during the cell cycle, stimulated

in response to double breaks in the DNA, which are commonly induced upon exposure to ionizing radiation (Viniegra, Martinez et al. 2005, Irarrazabal, Burg et al. 2006, Hakem 2008).

One of the initial steps in HRR activation is the phosphorylation of ATM by the Mre11-Rad50-NBS1 (MRN) complex. Once phosphorylated, ATM and the MRN complex accumulate at the DSB site, recruiting $\gamma$ H2AX. The accumulation of $\gamma$ H2AX leads to RPA attachment to the DNA and subsequent stabilization and repair of the DNA strands (Hakem 2008, Smith, Tho et al. 2010).

## 5.3 *Aims*

- Determine whether mirin effects $\gamma$ H2AX foci formation and clearance following X-ray exposure.
- Consider the effects of Mirin on Clonogenic Survival in melanoma cell lines treated with 2Gy X-rays.
- Explore whether Mirin elicits tumour growth delay *in vivo* as a single agent and in conjunction with 6Gy X-rays.

## 5.4 *Expression of ϒH2AX foci in response to treatment with Mirin.*

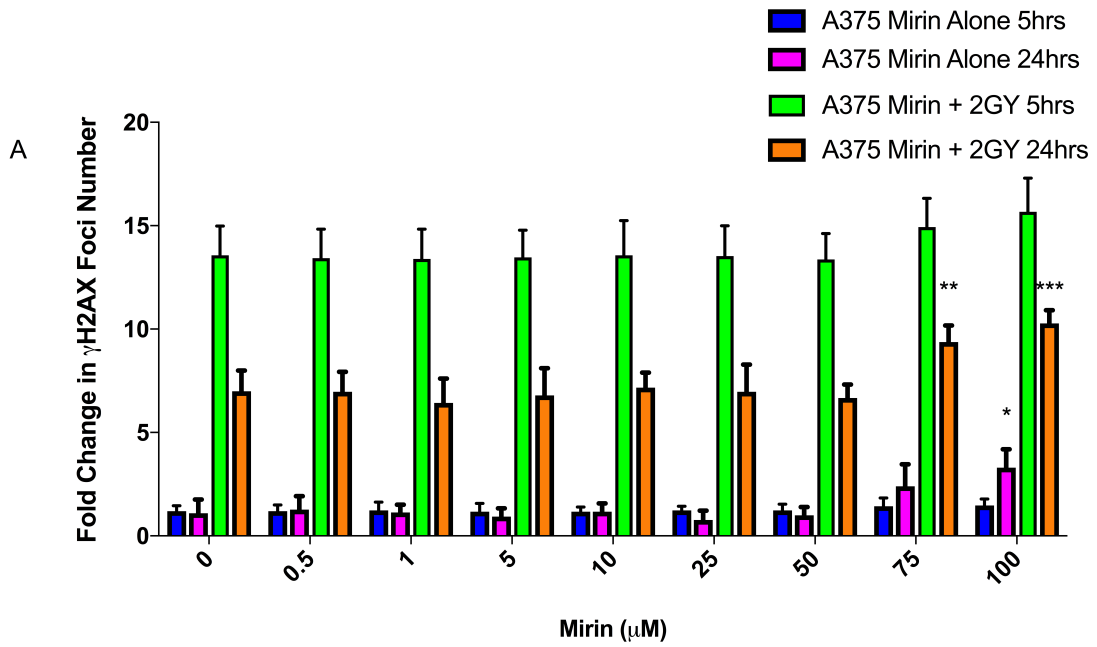
Dupre, Boyer-Chatenet et al. detailed the inhibitory effect of Mirin on the MRN subunit MRE11, resulting in failure to repair DNA double strand break damage. (Dupre, Boyer-Chatenet et al. 2008) In order to investigate whether this mechanism proves effective in the context of radiation induced damage, Mirin was administered as a pre-treatment 2 hrs prior to 2Gy X-ray radiation exposure, a level of radiation which is known to induce DNA double strand breaks (Lomax, Folkes et al. 2013).

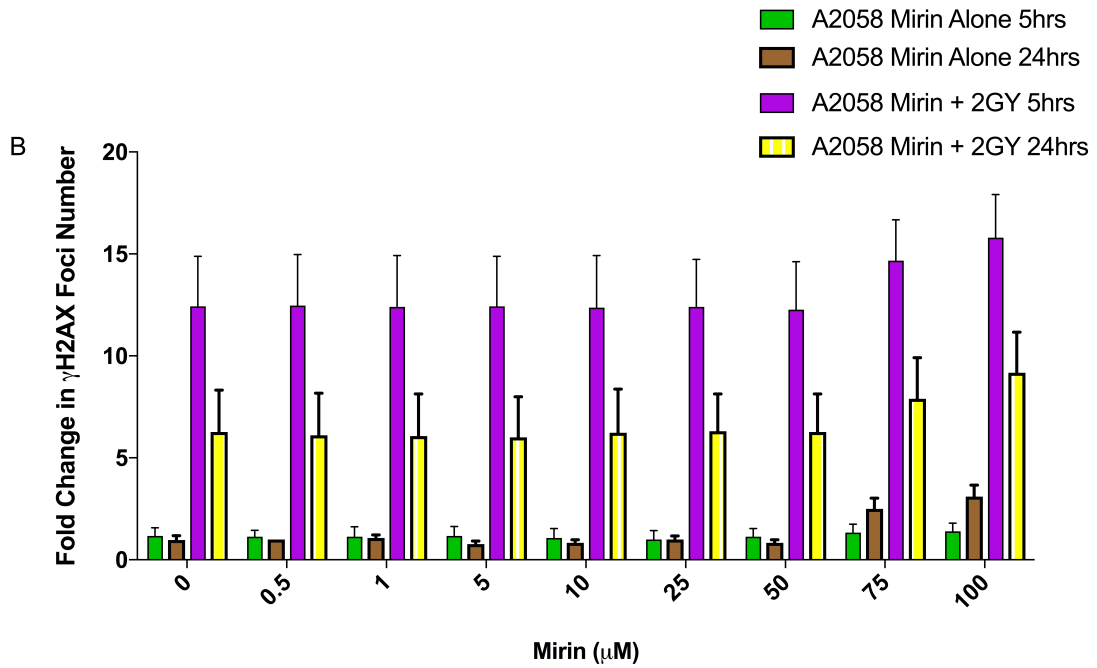
Assessment of DNA repair was carried out using Phospho**γ**H2AX, an early marker for DNA double strand break damage activated in response to phosphorylation by ATM and the MRN complex, triggered by exposure to ionising radiation. A reduction in phosphorylated**γ**H2AX levels following Mirin pre-treatment would for example indicate an inhibition of the MRN complex by Mirin and thus a reduced capacity to sense double strand DNA damage and activate repair. The**γ**H2AX assay, as analysed via fluorescence activated cell sorting (FACS) analysis, is a method of determining the level of phosphylated**γ**H2AX expression in a given cell sample. The levels of **γ**H2AX were recorded following Mirin treatment alone and as a pre-treatment to 2Gy X-Ray irradiation in an attempt to establish whether the expression of phosphorylated **γ**H2AX became altered in response to treatment with the compound.

Cell samples were collected at 5 hrs and 24hrs post irradiation and are shown in Figure 5-3 A-C. Treatment with Mirin as a single agent did not result in significant alteration in phospho $\gamma$ H2AX expression after 5 hrs in either the A375 (A), A2058 (B) and B16F10Luc2 (C) cell lines respectively ($p < 0.9999$) no significant reduction in $\gamma$ H2AX levels in Mirin groups compared to 2Gy alone ($p<0.99$) at the 5hr time point indicating that Mirin was not effective at inhibiting or altering the recruitment of phospho $\gamma$ H2AX to the site of DNA damage induced by 2Gy X-Irradiation in melanoma cell lines 5hrs after exposure.

Phospho $\gamma$ H2AX expression levels 24 hrs post irradiation showed a weakly significant accrual in phospho $\gamma$ H2AX expression at 75 µm in the B16-F10-LUC2 cell line (p 0.0317) as well as significant accruel treating with 100 µm Mirin alone in the B16-F10-LUC2 and A375 cell lines (p 0.0005) and (p 0.0160) respectively, with phospho $\gamma$ H2AX levels at lower doses remaining unchanged following Dunnett's multiple comparison tests. This was contrasted in the Mirin/2Gy combination groups which saw a 46% ($\pm$0.061), 49% ($\pm$0.03) and 60% ($\pm$0.023) reduction in phospho $\gamma$ H2AX expression in the A375, A2058 and B16-F10-LUC-2 lines respectively when compared to levels at 5 hrs ($p < 0.0001$), indicating that surviving cells were able to repair damage induced by 2Gy X-ray irradiation irrespective of Mirin pre-treatment. 2Gy X-ray in conjunction with 75µm Mirin resulted in moderate accrual of phospho $\gamma$ H2AX foci in A375 cells (p 0.0083) and a strong accrual of foci following 100 µm in conjunction with 2Gy X-ray which may be indicative of an impaired clearance of phospho $\gamma$ H2AX and

reduction but not abolishment, in the capacity to repair DNA double strand break damage.



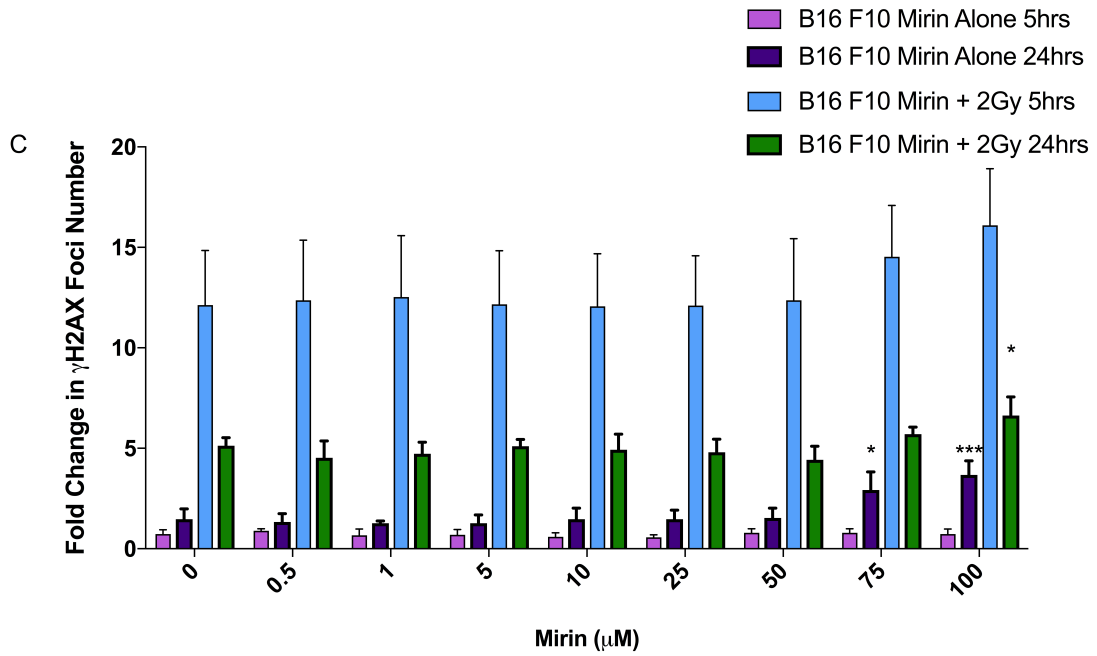


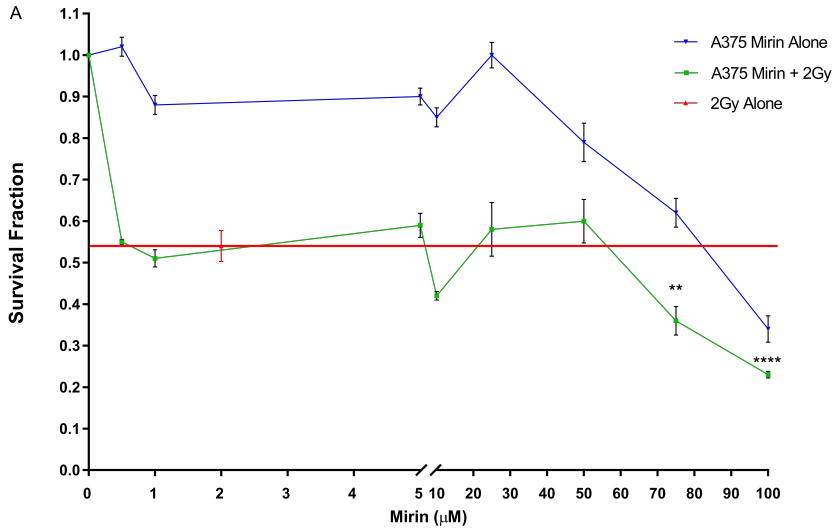

**Figure 5-3 Phospho γH2AX expression following 2hr incubation with Mirin.**

Samples were collected 5hrs post treatment and stored at -20$^0$C (see materials and methods) prior to FACS analysis. No significant difference was seen in A375, A2058 or B16-F10-Luc2 cell lines when treated with Mirin alone (P<0.9999). 2Gy treated groups demonstrated a 13-fold increase in phosphoγH2AX expression when compared to unirradiated controls ($p < 0.05$) following Bonferroni multiple comparisons. Significant reductions of 46% (±0.061), 49% (±0.03) and 60% (±0.023) were seen in the A375, A2058 and B16-F10-Luc2 cell lines respectively at 24hrs post treatment vs. 5hrs ($p < 0.0001$). Moderate accrual of phosphoγH2AX foci at 75μm and 100 μm appears to indicate impaired phosphor γH2AX clearance. Results are representative of the mean of 3 experiments seeded in triplicate plus standard deviations.

## 5.5 *Clonogenic survival of Melanotic and Amelanotic cell lines following treatment with Mirin.*

In order to assess whether the results from the Phospho **γ**H2AX expression assay were reflected in the clonogenicity of treated cells, clonogenic assays were carried out using Mirin as a single agent and in conjunction with 2Gy external beam X- ray Irradiation (figure 5-4). The A375 amelanotic (A), A2058 Pheomelanotic (B) and B16-F10-Luc-2 cell lines (C) were treated with Mirin either as a single agent for 24 hrs or as a pre-treatment 2 hrs prior to an exposure of 2Gy of X-ray radiation and plated for clonogenic assay.

Clonogenic survival following Mirin treatment alone did not induce toxicity until dosage reached 75 µm and 100 µm (0.62 $\pm$ 0.0082) (p0.0001) (0.34 $\pm$ 0.055) (p0.0001), (0.79 $\pm$ 0.055) (0.49 $\pm$ 0.062) (p0.0001), (0.68$\pm$0.068) (0.34 $\pm$ 0.052) for A375, A2058 and B16-F10-LUC2 respectively, which reflects the pattern of elevated Phospho **γ**H2AX expression noted in figure 5-3A-C and further noted in the Mirin/2Gy treated groups where treatment with Mirin was only found to be significantly different from treatment with 2Gy alone in the 75 µm and 100 µm concentrations in all cell lines ($p < 0.0001$,), which when coupled with the Phospho **γ**H2AX data indicate an abrogation of the DNA repair process and potentially DNA replication in melanoma cells at these concentrations.


A
A375 Mirin Alone
A375 Mirin + 2Gy
2Gy Alone
Survival Fraction
Mirin (μM)
**
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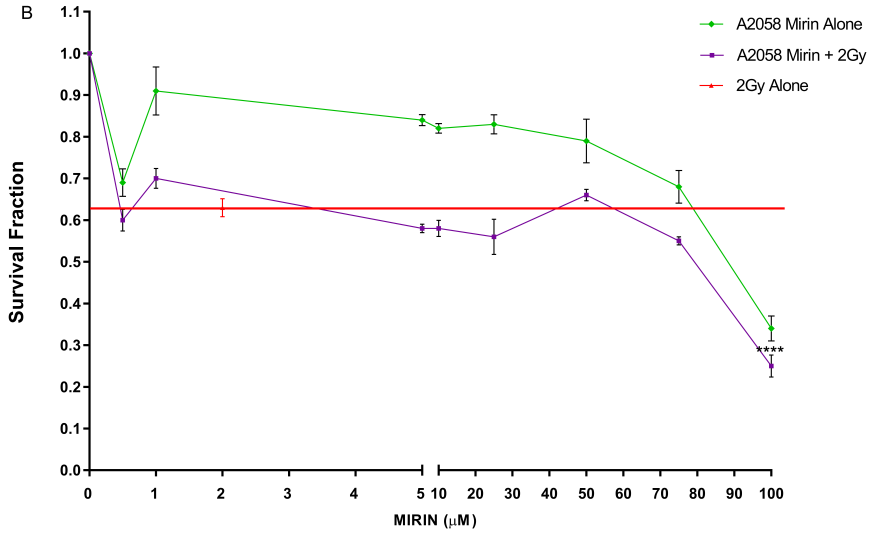

B
A2058 Mirin Alone
A2058 Mirin + 2Gy
2Gy Alone
Survival Fraction
MIRIN (μM)
****

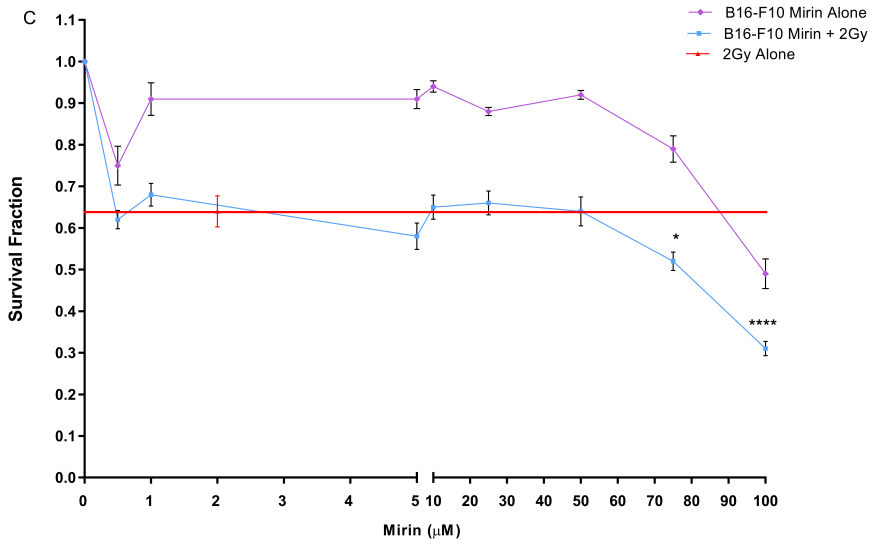

C
B16-F10 Mirin Alone
B16-F10 Mirin + 2Gy
2Gy Alone
Survival Fraction
Mirin (μM)
*
****

Figure 5-4 Clonogenic survival of melanoma cell lines following Mirin treatment.

Clonogenic survival of A375 (A), A2058 (B) and B16F10Luc2 (C). Following Mirin treatment as a single agent or in combination with 2Gy X-ray. Significant toxicity was seen in in both Mirin alone and Mirin/ 2Gy combination treatments in all cell lines treated ($p < 0.0001$). Results are representative of the mean of 3 experiments plus standards deviation, seeded in triplicate.

## 5.6 *In vivo results.*

In order to assess whether Mirin effects X ray induced tumour shrinkage *in vivo* (figure 5-5), athymic nude mice were inoculated *via* subcutaneous injection with $3x10^6$ A375 melanoma tumour cells. All groups, including controls, consisted of 6 mice. Mice received 50 mg/kg of Mirin either alone or 2 hrs pre 6Gy irradiation via oral gavage. Following the 2 hrs pre-treatment subjects were anesthetized and placed in an X-Rad 225 X-ray irradiation system. Subjects were shielded to ensure the radioactive dose was concentrated at the tumour site, receiving a 6 Gy irradiation of X-rays at a dose rate of 2.2 Gy/min. Tumour xenografts were measured with calipers every 2-3 days following treatment. 50 mg/kg Mirin alone produced a significant tumour growth delay at 20 days post treatment ($p < 0.0001$) Both 6 Gy X-ray radiation and 6 Gy/Mirin combinations produced significant tumour growth delay over the course of 20 days ($p < 0.0001$,). However there was no significant difference found between the combination and radiation alone treatment groups ($p > 0.5$,) at day 20 following Dunnett's multiple comparison tests.

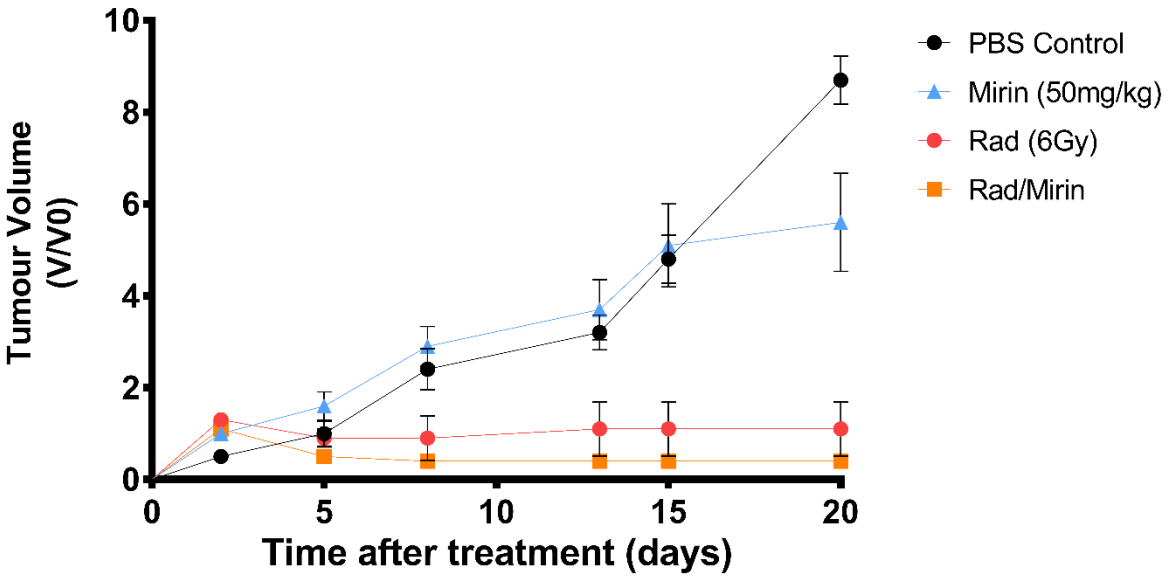



**Figure 5-5 Effect of 6Gy X-ray radiation ± 50mg/kg Mirin on the growth of A375/NAT tumours in nude mice.**

Mirin treatment was administered 2hrs prior to X-ray exposure on day 0. The graph demonstrates that the addition of Mirin as a pre-treatment to 6 Gy x-ray exposure does not produce a statistically significant effect on tumour growth when compared to 6 Gy alone. V/V0 is the volume at day x over the volume at day 0 which was the time of X-Ray treatment.

## 5.7 *Discussion*

Mirin is a small molecule inhibitor of MRE11, a key component of the MRN complex responsible for the activation of ATM and initiation of DNA double strand break repair. As such, Mirin has potential for development as a component of radiation combination therapies. The initial hypothesis during this investigation centred around the inhibition of MRE11 exonuclease activity - required for the phosphorylation of ATM - by Mirin, with the result of abolition of G2/M checkpoint arrest and disruption of radiation induced double strand break repair (Dupre, Boyer-Chatenet et al. 2008).

Consequently, our experiments were designed with Mirin treatments in conjunction with external beam irradiation ranging between 2-6 Gy with the intent of inducing a significant level of DNA damage which would either be repaired or persist dependent upon the presence of Mirin.

To investigate this, we performed both *in vivo* and *in vitro* experiments assaying for Phospho **γ**H2AX expression via FACS analysis as a surrogate for ATM mediated sensing and repair of double strand DNA damage following a 2 hrs treatment with Mirin prior to 2 Gy irradiation, the levels of Phospho **γ**H2AX expression were assessed at 5 hrs and 24 hrs post treatment. In contradiction to previous studies which cited a distinct inhibition of **γ**H2AX phosphorylation following Mirin treatment between 50 µm and 100 µm (Dupre, Boyer-Chatenet et al. 2008), baseline levels were found to

be slightly elevated in the Mirin alone groups at these concentrations, with a substantial Phospho-**γ**H2AX elevation in response to 2Gy X-Ray irradiation, which was accompanied by an impaired clearance Phospho **γ**H2AX over 24 hrs in the Mirin/2Gy radiation groups. Though unexpected, the observed rise in Phospho **γ**H2AX levels has been reported in the literature (Mishima, Mishima-Kaneko et al. 2014) . This effect may not be a failure of Mirin to inhibit Mre11 or ATM function but might be as a result of actions of DNA-dependent protein kinase (DNA-PK's) known to be elevated in malignant melanoma's (Kotula, Berthault et al. 2015) and able to phosphorylate γH2AX in the absence of functional ATM (Stiff, O'Driscoll et al. 2004) as well as function in the non-homologous end joining repair pathways (Davidson, Amrein et al. 2013) and cell cycle progression (An, Huang et al. 2010). However, in order to validate this hypothesis future studies should include cell cycle data and western blot assays for DNA PK and phospho-ATM to confirm the functional reduction in ATM activity and subsequent overlap of function with DNA-PK in the presence of Mirin, knocking down function of ATM and DNA-PK with inhibitors such as KU-55933, Wortmannin and NU7026 (Hashimoto, Rao S Fau - Tokuno et al. 2003, Willmore, de Caux S Fau - Sunter et al. 2004, Li and Yang 2010) may also help to confirm this interaction.

Clonogenic assays were undertaken to assess the levels of cytotoxicity for treatments of 2 Gy X ray radiation alone and in conjunction with escalating concentrations of Mirin, demonstrating significant toxicity at the 75-100μm levels. Taken in conjunction with the H2AX data, this may indicate that although DNA damage was detected and H2AX phosphorylated the subsequent repair of the damage in the absence of ATM,

possibly via ATR dependant repair (Cuadrado, Martinez-Pastor et al. 2006) was not efficient enough to ensure the survival of the majority of treated cells. Again, this can only be proven if future studies include assays for ATM function and HRR repair.

*In vivo* experiments involving Mirin have not been reported in the literature, a pilot experiment consisting of a 6Gy X-ray irradiation in combination with a 2hr Mirin pre-treatment in A375 nude mouse xenografts failed to show significant differences in lethality in the combination treatment groups when compared to 6Gy irradiation. However further detailed studies are required for *in-vitro* and *In-vivo* use of Mirin to determine effective therapeutic concentrations of the compound both as a single agent as well as in combination with external beam radiation.

Interestingly, due to the difference in dose rate between the two forms of radiation. The X irradiation treatment was carried out at high dose rate 2.2 Gy/min, whilst treatment with radionuclides is accepted to be 0.01-1.0 Gy/hr. High dose rate exposures have been found to result in the phosphorylation of ATM, activating double strand break repair, where-as low dose rates have been shown not to stimulate an ATM phosphorylation response (Carlsson, Stigbrand et al. 2008).

The hypothesize that although the presence of Mirin may inhibit this repair pathway through negated ATM phosphorylation, the acute nature of the damage allows for the up regulation of other repair pathways such as the nucleotide excision repair pathway and base excision repair pathway over time (Hakem 2008) whereas low dose

rate radionuclides, such as [$^{131}$I] have been shown not to induce ATM phosphorylation, negating the potential for the repair of the damage induced and allowing for a chronic accrual of DNA damage over time (Collis, Schwaninger et al. 2004). a possible hypothesis is that the enhanced cell kill noted within the Mirin treatments may be seen due to the inhibition of Mre11 activity within the Non Homologous End Joining pathways, further negating the potential for DNA repair over time (Zha, Boboila et al. 2009, Zhuang, Jiang et al. 2009). Investigating Mirin when used in conjunction with other compounds directly effecting specific points in the double strand break and NHEJ pathways such as the AKT, MAPK and ERK related pathways (Golding, Morgan et al. 2009) may prove fruitful for generating a radiotherapeutic combination.

# Chapter 6

# Investigation of Novel IKKβ Inhibitors as radio sensitizers in malignant melanoma

NF-κB transcription factors are a relatively ubiquitous family of proteins including RelA (P65), RelB, P50 and P52 - commonly activated in response to cellular stresses such as infection from pathogenic organisms, elevated levels of reactive oxygen species and radioactive assault (Orlowski and Baldwin 2002, Amiri and Richmond 2005, Ueda and Richmond 2006).

Constitutive activation of the NF-κB pathway has been reported in multiple cancers, including malignant melanoma (Ueda and Richmond 2006,Baldwin 2001) Where it is thought to stem from a combination of environmental stimuli (UV irradiation), was gene mutation and activation associated with melanoma development and progression (NRas/BRaf mutation, and PTEN loss) as well as over expression of growth factor receptors. (Ueda and Richmond 2006), (Gordon-Thomson, Jones et al. 2005)).

This permissive NF-κB activation has been associated with anti-apoptotic activity, angiogenesis, cellular proliferation and excessive inflammatory responses which contribute to tumour progression and metastasis (Karin and Lin 2002) And thus make components of the NF-κB pathways highly attractive targets for cancer therapies.

Chapter 6 - Investigation of Novel IKK Inhibitors as radio sensitizers in malignant melanoma

## 6.1 *IKK's – the regulators of NFKB*

NF-κB activation is regulated by the I kappa kinase Kinase (IKK) enzyme family, of which 3 of it members, IKKα, IKKβ, and IKKγ function as complexes in order to activate NF-κB signalling via two largely distinct signalling cascades referred to as the classical (canonical) and alternative (non-canonical) pathways. Each cascade is dependent upon different IKK complexes for activation, typically IKKγ is required as a scaffold protein for complexes consisting of an IKKα and IKKβ subunit for classical activation, whereas two IKKα subunits are needed to activate the alternative pathway.

Each cascade is triggered in response to external stimulus. Classical activation is typically activated in response to pro inflammatory signals mediated by receptors such as the tumour necrosis factor receptors, interleukin 1 receptor and toll like receptor 1 alongside molecules such as lipopolysaccharide (LPS), whereas alternative activation is triggered almost exclusively by the tumour necrosis factor receptors.

Classical activation relies on a complex of IKKβ / IKKα and is governed by enzymes such as ERK kinase kinase 3 (MEKK3) and mitogen-activating protein (MAP), these factors phosphorylate IKKβ, which in turn phosphorylates IκB. This phosphorylation event causes IκB to be ubiquitinated and degraded by the 26S proteasome, releasing the RelA/P50 homodimer which influences inflammatory and pro-survival pathways.

Alternative activation is IKKβ independent and relies solely on IKKα dimers triggered by tumour necrosis factor receptors. This pathway is governed by NF-κB inducing kinase (NIK), which phosphorylates IKKα leading to the phosphorylation of p100. p100 is subsequently degraded and results in RelB/p52 nuclear translocation (Chariot 2006, Hacker and Karin 2006) where it influences organogenesis and the adaptive immune response via B-cell maturation (Bonizzi and Karin , Claudio, Brown K Fau - Park et al.), and influence cell cycle progression via Aurora A phosphorylation to affect the classical pathway via direct histone phosphorylation.

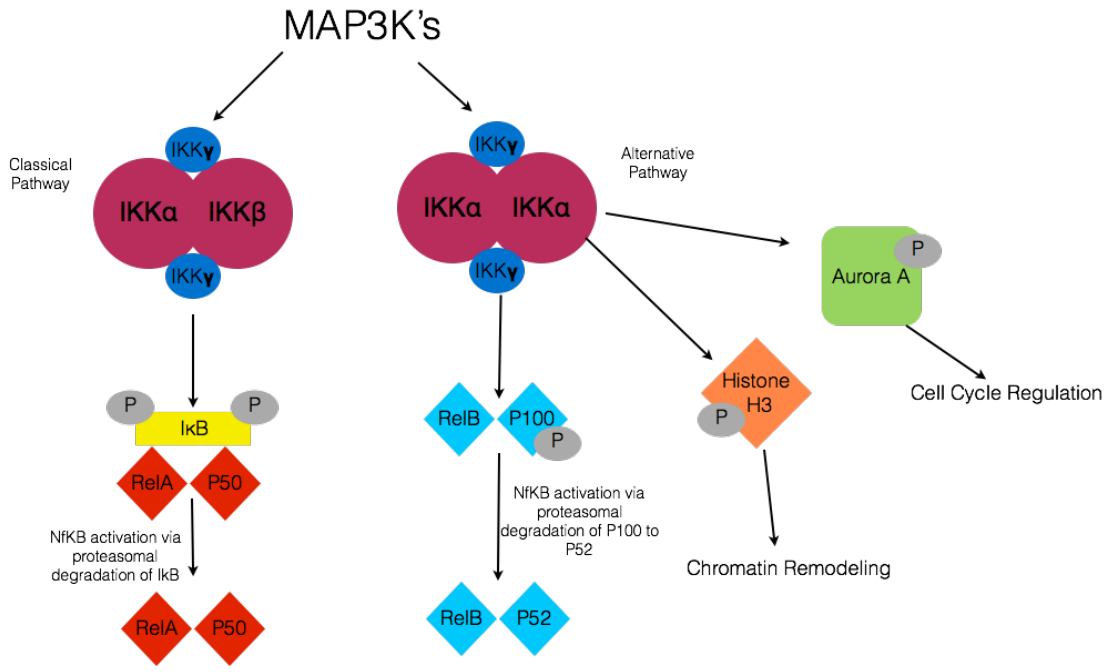



Figure 6-1 NF-κB activation cascades.

The IκB Kinase complexes govern NF-κB subunit activation via the classical IKKα/IKKβ pathway or alternative IKKα pathways.

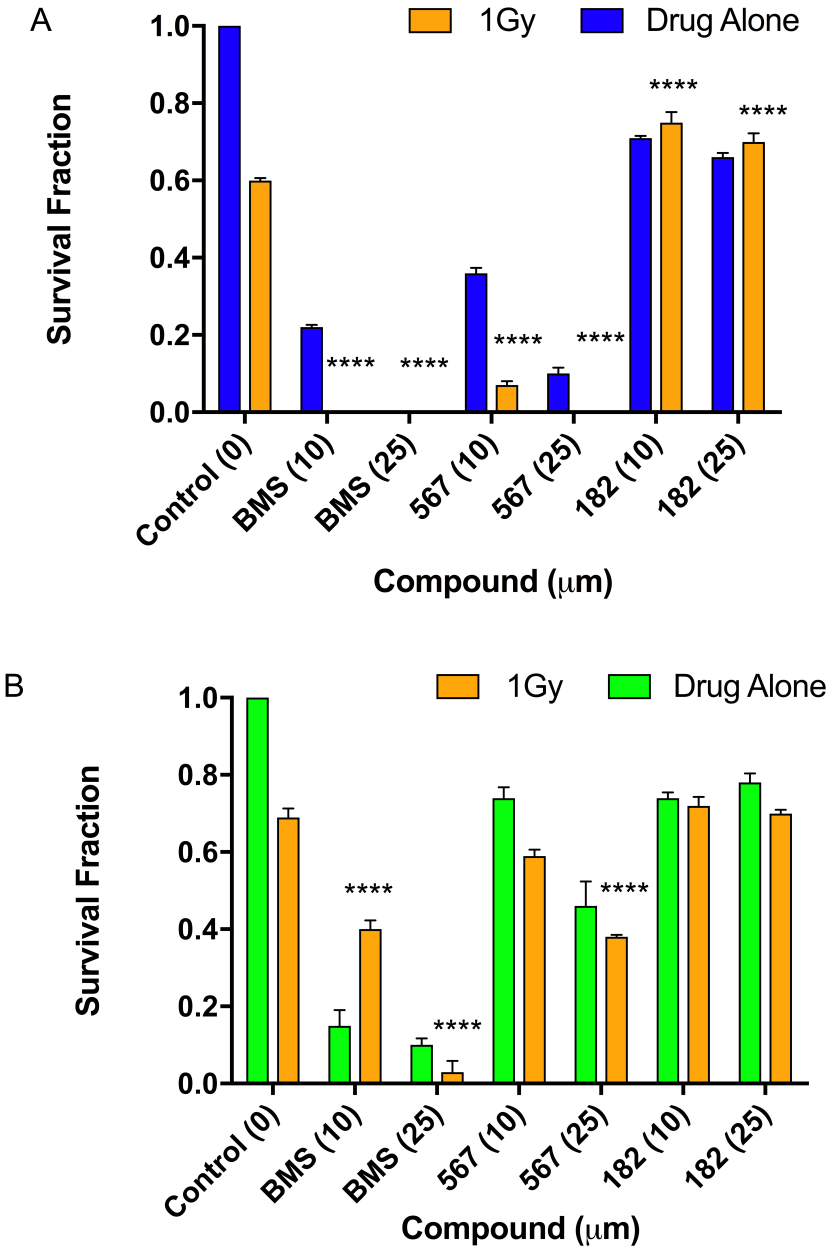
The IKK family is an attractive target when considering strategies for abrogating the NF-κB pathways. The essential role of the IKK complexes in NF-κB activation means that selective targeting of these individual and early components could potentially nullify entire NFKB signalling cascades. Considering this, there has been a drive to produce compounds that inhibit both the IKKα and IKKβ subunits, however it has proven difficult to produce compounds which bind to either IKKα or IKKβ exclusively (Liu, Xia et al. 2012). The small molecule drug discovery program at Strathclyde university has developed a range of compounds targeting either IKKα or IKKβ . This study investigates two of these compounds, SU182 and SU567. During development both have shown selectivity for IKKβ and this study aims to confirm IKK inhibition and potential therapeutic effectiveness in Melanoma cell lines, versus the known commercial compound BMS34511, a small molecule allosteric IKK Inhibitor strongly favouring IKKβ over IKKα (IC50 0.3µm/L vs 4µm/L)(Lee and Hung 2008).

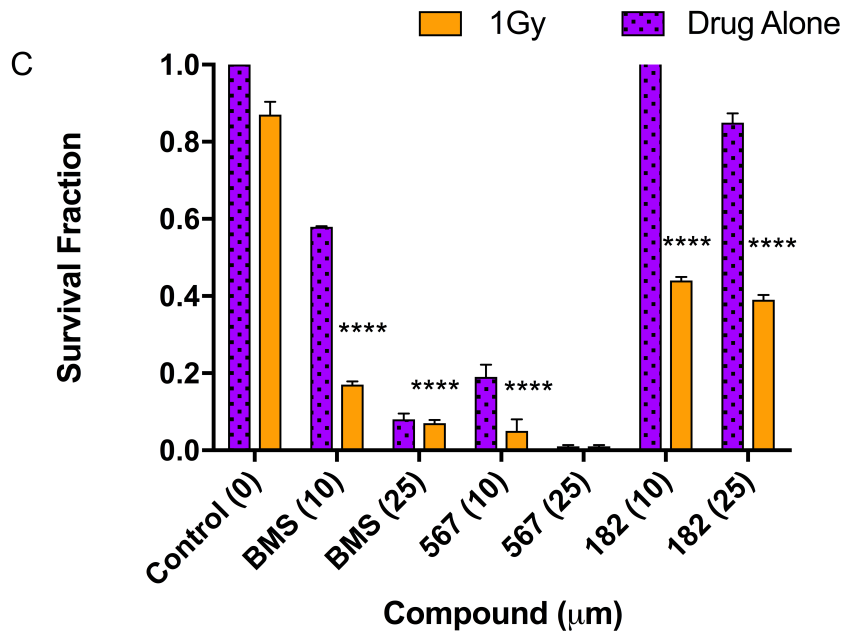
## 6.2 *Clonogenic survival of Melanoma cell lines treated with Novel IKKβ inhibitors as single agents and in combination with 1Gy of external beam radiation.*

Assessments within the drug discovery unit have indicated that SU567 and SU182 have high IKKβ inhibitory effects at a concentration of 25µm, here the compounds have been assessed at 10µm and 25µm In order to assess their potential in combination with radiotherapy. The A375, B16-F10 and A2058 cell lines were

pretreated with SU567 or SU182 at 10 µm and 25µm for 1hr prior to a 1Gy X-ray exposure. Cells were incubated for a further 24hrs before being plated via clonogenic assay and incubated for 7-10 days to allow for colony formation. Figure 6-2 shows the clonogenic kill induced by the SU compounds both as single agents and in combination with 1Gy of X-ray in the A375, A2058 and B16-F10-LUC2 melanoma cell lines.

Treatment with SU567 and BMS 345541 elicited considerable cytotoxicity in all cell lines as a single agent (p 0.0001) following 2-`way ANOVA and post hoc Sidaks multiple comparison and considerable enhancement in cytotoxicity beyond 1Gy alone (p 0.0001). SU182 failed to induce significant cytotoxicity in the A375 and A2058 cell lines but produced a marked response in B16-F10-LUC2 cells in combination with 1 Gy rays.


A
1Gy
Drug Alone
Survival Fraction
1.0
0.8
0.6
0.4
0.2
0.0
****
Control (0)
BMS (10)
BMS (25)
567 (10)
567 (25)
182 (10)
182 (25)
Compound (μm)
B
1Gy
Drug Alone
Survival Fraction
1.0
0.8
0.6
0.4
0.2
0.0
****
Control (0)
BMS (10)
BMS (25)
567 (10)
567 (25)
182 (10)
182 (25)
Compound (μm)




| | A375 (A) | | A2058 (B) | | B16-F10-LUC2 (C ) | |
|---|---|---|---|---|---|---|
| | Significance | P value | Significance | P value | Significance | P value |
| 1Gy vs. 1Gy + BMS (10) | **** | <0.0001 | **** | <0.0001 | **** | <0.0001 |
| 1Gy vs. 1GY + BMS (25) | **** | <0.0001 | **** | <0.0001 | **** | <0.0001 |
| 1Gy vs. 1Gy + 567 (10) | **** | <0.0001 | ns | 0.2657 | **** | <0.0001 |
| 1Gy vs. 1Gy + 567 (25) | **** | <0.0001 | **** | <0.0001 | **** | <0.0001 |
| 1GY vs. 1Gy + 182 (10) | **** | <0.0001 | ns | >0.9999 | **** | <0.0001 |
| 1Gy vs. 1Gy + 182 (25) | **** | <0.0001 | ns | >0.9999 | **** | <0.0001 |

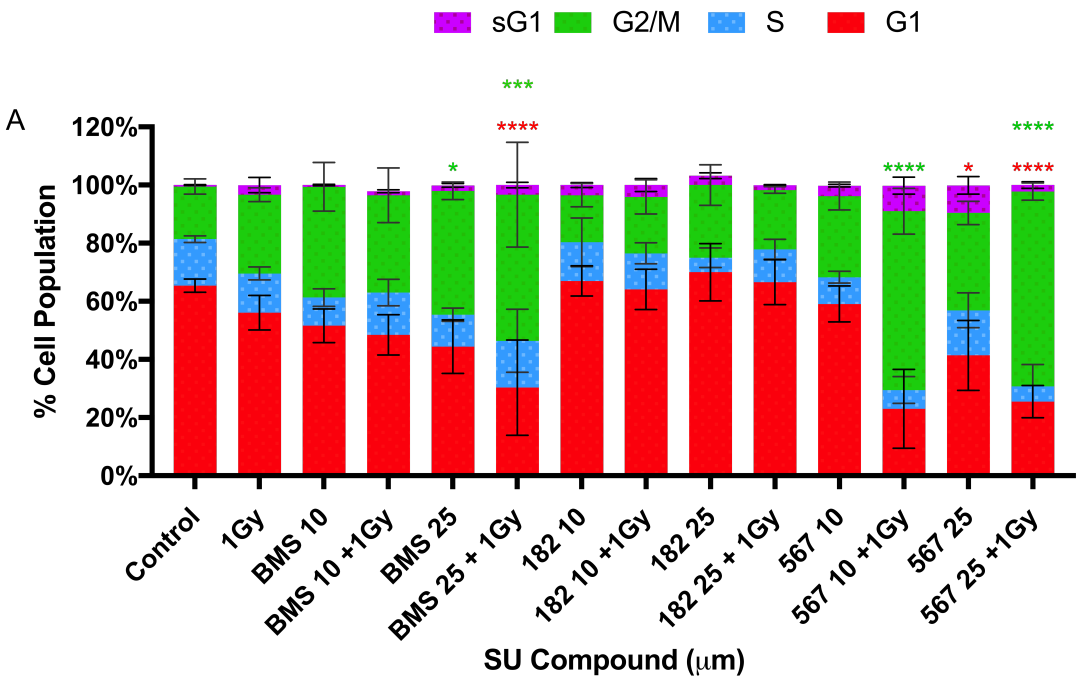
**Figure 6-2 Clonogenic survival of A375, A2058 and B16-F10-LUC2 cells following IKKβ inhibitor treatment.**

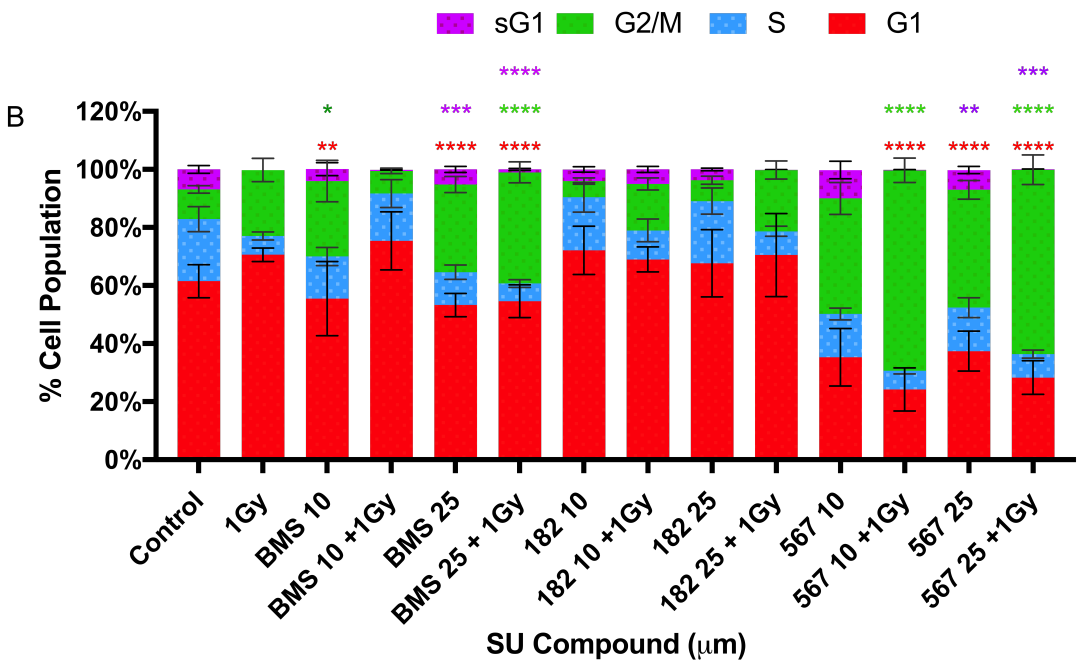
Clonogenic survival of A375 (A), A2058 (B) and B16-F10-Luc2 9 (C) cells respectively, treated with SU567, SU182 and BMS 345541 compounds alone and in combination with 1Gy X-rays. The table summarises the significant differences between X-ray alone and combination treatments following 2-way ANOVA and Sidaks multiple comparisons. Results represent the mean of 3 experiments plus standard deviation, seeded in triplicate.

## 6.3 *The effects of treatment with SU567 and SU182 as single agents and in combination with 1Gy external beam radiation on cell cycle.*

Cell cycle analysis of the three Melanoma lines was carried by propidium iodide treatment and analyzed by FACS analysis. The A375, A2058 and B16F10-LUC2 cell lines were treated with SU567, SU182 and BMS345541 as both single agents and in combination with 1Gy of external beam irradiation. Samples received the SU compounds 1hr prior to irradiation in the combination group; cells were collected 24hrs after administration in order to ascertain whether any inhibitions in NFKB expression would affect alterations in the cell cycle.

The data shows marked alterations in the cell cycle of treated groups versus 1Gy alone. Figure 6-3 demonstrates the alterations in the cell cycle induced by the SU compounds both as single agents and in combination with 1Gy of X-ray in the A375, A2058 and B16-F10-LUC2 melanoma cell lines. BMS-34551 dosed at 25µm in combination with 1Gy X-ray irradiation treatment induced significant enhancement of G2/M arrest in the A375 (p0.0002), A2058 ($p < 0.0001$) and B15-F10-LUC2 (p 0.0021) respectively following 2-way ANOVA and Dunnetts multiple comparison tests. Similarly, SU567 25µm combination treatments consistently produced highly significant increases in G2/M arrest in all cell lines compared to X-rays alone ($p < 0.0001$) indicative in both cases of radio sensitisation. Corresponding with clonogenic assay data (figure 6-2) Reponses to SU182 were observed exclusively in the B16-F10-LUC2 cell line where a weakly significant increase in the S Phase population was observed at 10 µm (p0.0393) and 25µm (p0.0038) respectively.


A
sG1
G2/M
S
G1
% Cell Population
120%
100%
80%
60%
40%
20%
0%
Control
1Gy
BMS 10
BMS 10 +1Gy
BMS 25
BMS 25 + 1Gy
182 10
182 10 +1Gy
182 25
182 25 + 1Gy
567 10
567 10 +1Gy
567 25
567 25 +1Gy
SU Compound (μm)


B
sG1
G2/M
S
G1
% Cell Population
120%
100%
80%
60%
40%
20%
0%
Control
1Gy
BMS 10
BMS 10 +1Gy
BMS 25
BMS 25 + 1Gy
182 10
182 10 +1Gy
182 25
182 25 + 1Gy
567 10
567 10 +1Gy
567 25
567 25 +1Gy
SU Compound (μm)

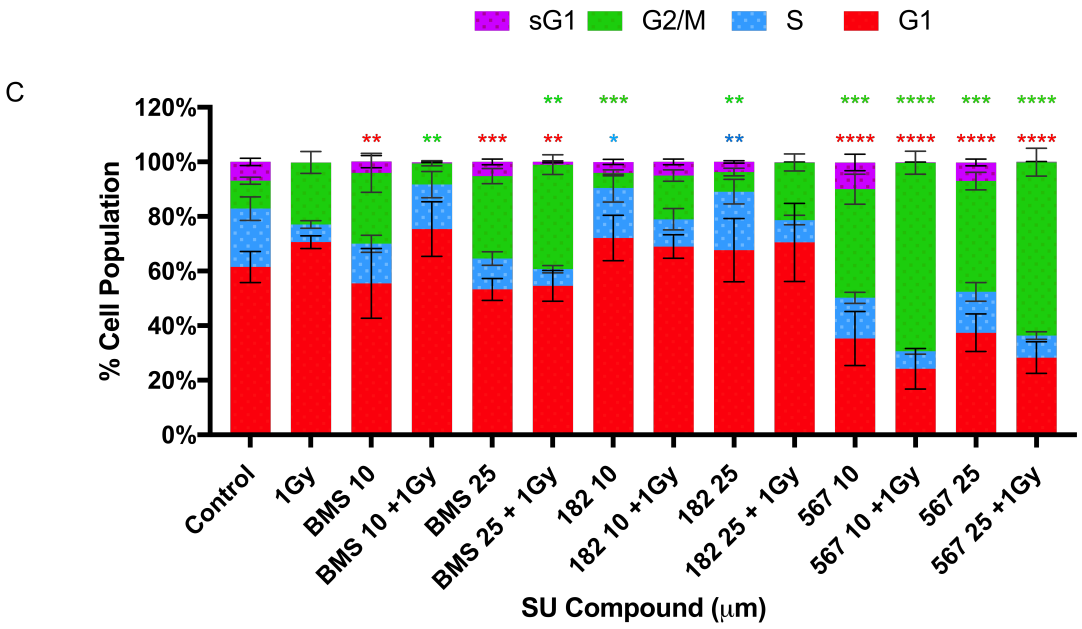
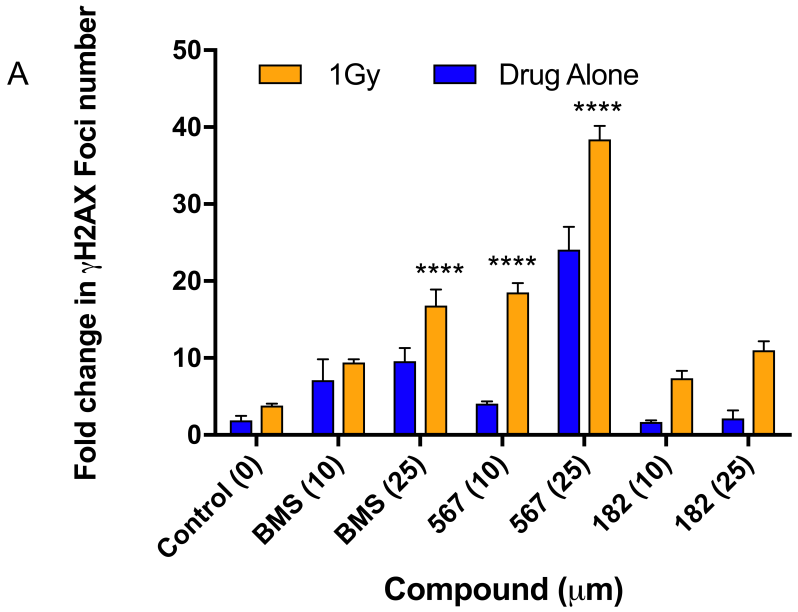


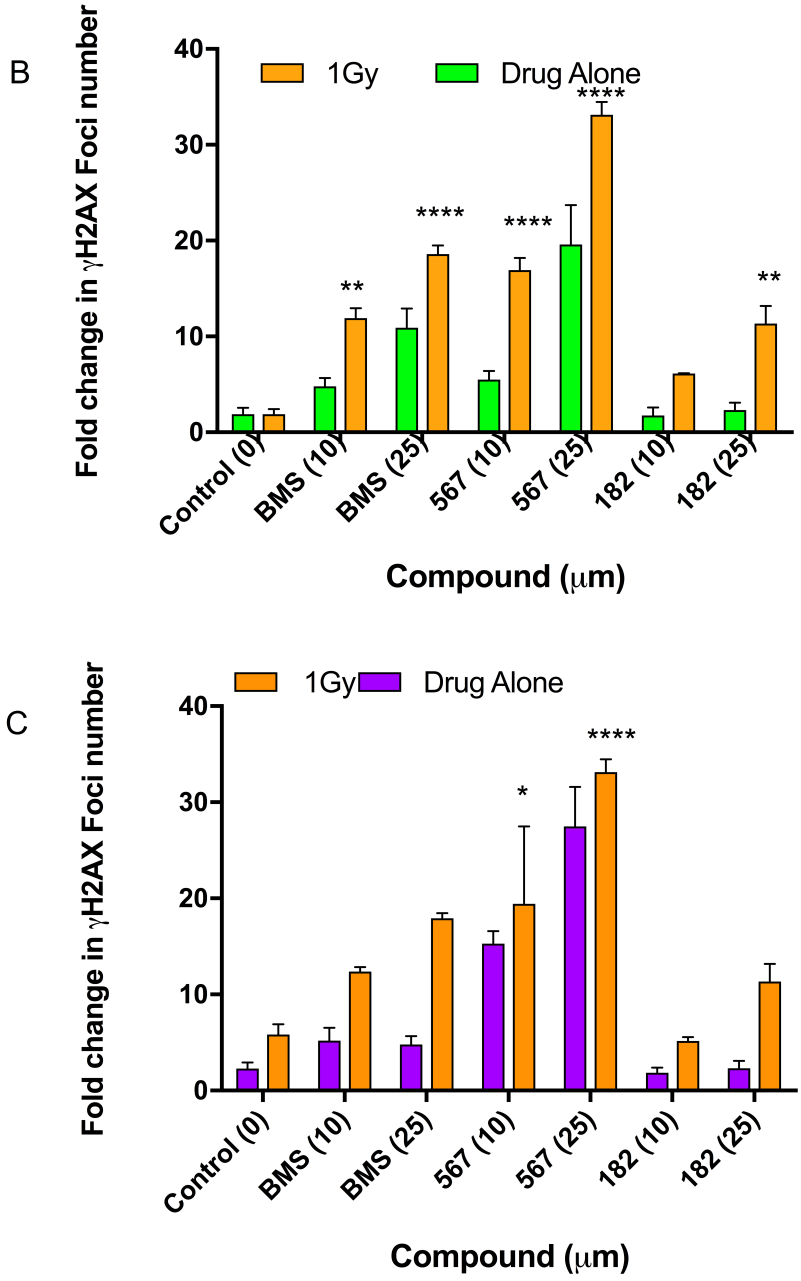

**Figure 6-3 Cell cycle analysis of A375, A2058 and B16F10-luc2 following IKKβ inhibitor treatment.**

Cell cycle analysis of A375 (A), A2058 (B) and B16F10-luc2 (C) respectively for SU compounds alone (left) and in combination with 1Gy X-rays (right). Compounds were administered 1hr prior to X ray exposure; measurements were taken 24hrs post treatment. Data presented in the table was analysed by 2-way ANOVA and dunnetts multiple comparison test. Significance is represent for each stage of the cell cycle by a corresponding coloured star with the following scheme G2/M = *, S = *, G1 = * and sG1= * Results represent the mean of 3 experiments carried out in triplicate.

### *6.3.1 γH2Ax expression in response to treatment by novel NF-κB inhibitors.*

Using FACS analysis to determine γH2AX expression of cells treated with SU compound as single agents and in combination with 1Gy irradiation it is demonstrated that SU182 moderately impaired DNA double strand break repair in the A2058 and A375 cell lines (figure 6-4 A+ B) (p0.0035) and (p 0.0455) respectively elevating γH2AX levels beyond those observed in the 1Gy alone treatments after 24hrs. whilst BMS 345541 and SU567 in the A375 and A2058 cell lines (6-4 A+C) ( indicating that treated samples have greatly impaired capacity for DNA damage repair.






| | A375 (A) | | A2058 (B) | | B16-F10-LUC2 (C ) | |
|---|---|---|---|---|---|---|
| | Significance | P value | Significance | P value | Significance | P value |
| 1Gy vs. 1Gy + BMS (10) | ns | 0.2571 | ** | 0.0017 | ns | 0.8454 |
| 1Gy vs. 1GY + BMS (25) | **** | <0.0001 | **** | <0.0001 | ns | 0.0536 |
| 1Gy vs. 1Gy + 567 (10) | **** | <0.0001 | **** | <0.0001 | * | 0.0188 |
| 1Gy vs. 1Gy + 567 (25) | **** | <0.0001 | **** | <0.0001 | **** | <0.0001 |
| 1GY vs. 1Gy + 182 (10) | ns | 0.9071 | ns | 0.7366 | ns | >0.9999 |
| 1Gy vs. 1Gy + 182 (25) | * | 0.0455 | ** | 0.0035 | ns | 0.9623 |

Figure 6-4 H2Ax expression of A375, A2058 and B16F10-Luc2 cells

H2AX expression of A375 (A), A2058 (B) and B16F10-Luc2 (C) cells respectively treated with SU Compounds alone and in combination with 1Gy X-Rays. Data presented in the table was analysed by 2-

way ANOVA and Dunnett's multiple comparison test. Compounds were administered 1hr prior to irradiation and measurements were taken 24hrs post treatment. Results are representative of 3 separate experiments and include standard deviation.

## 6.4 *Discussion.*

Aberrant NFκB signaling is widely identified in many tumours including malignant melanoma. Often associated with radiation and chemotherapeutic resistance, the upstream and downstream signally pathways involved in the NFKB family are well characterized and present an attractive target for radio sensitizing therapies.

Complexes of the I Kappa Kinase kinase protein family (IKK) govern the activity of the NFκB pathways and as such are key targets when aiming therapy against NFκB. Selective inhibitors of IKKβ such as BMS-345541 abrogate the canonical NFκB pathway and have previously been shown cytotoxicity in melanoma cell lines (Yang, Amiri et al. 2006) and to sensitize colon cancer (Jani, DeVecchio et al. 2010) and neuroblastoma cell lines (Ammann, Haag C Fau - Kasperczyk et al. 2009) to traditional treatments such as doxorubicin, TRAIL and oxaliplatin (Tapia, Gonzalez-Navarrete et al. 2007, Jani, DeVecchio et al. 2010). Breast cancer cell lines are also susceptible to BMS-345541 pre-treatment and cytoxicity has been reported in MCF7 cells following ionising radiation (Wu, Shao L Fau - Li et al. 2013), however to this researchers knowledge neither BMS-345541 nor other selective IKK inhibitors have been studied in combination with ionising radiation in malignant melanoma.

Within this chapter, the aims of this study were to characterize the effects of the novel IKKβ inhibitory compounds SU182 and SU567 compared to BMS-345541 on the A375, A2058 and B16F10-LUC2 melanoma cell lines as single agents and in conjunction with 1Gy X-ray irradiation.

Each cell line was treated with 10 μm and 25μm of either SU567, SU182 or BMS-345541 which is consistent with effective doses of BMS-345541 noted in the literature (Yang, Amiri et al. 2006). Clonogenic assays (figure 6.2) demonstrate that BMS-345541 and SU567 elicit considerable cytotoxicity as individual agents and significantly enhanced cytotoxicity when combined with 1Gy of X-rays in all melanoma cell lines. Interestingly SU182, elicited a moderate effect as a single agent, did not enhance X-ray induced toxicity in either the A375 or A2058 cell lines but did enhance toxicity in the B16-F10-LUC2 cell line in combination with X-rays, a potential explanation for this susceptibility of the B16-F10-LUC2 cell line may lie in the pigmentation status of melanoma cell lines whereby greater pigmentation has been correlated with lower levels of NFκB activation (Adini, Adini et al. 2015).

NF-κB has been reported as a regulator of DNA double strand break repair (Volcic, Karl et al. 2012) and this has been specifically attributed to IKKβ activity independent of the IKKα subunit by (Wu, Shao et al. 2011) who have shown that in cells treated with 2Gy ionising radiation BMS 345541 pre-treatment inhibited DNA double strand break repair. Therefore, DNA damage assays have been used to assess whether SU567 and SU182 as IKKβ targeting agents alongside BMS 345541 inhibit DNA damage repair.

Figure 6.4 demonstrates that both untreated controls and 1Gy treated groups across all cell lines reached a baseline γH2AX foci number 24hrs post treatment indicating

that the melanoma cells were able to efficiently repair 1Gy X-ray damage after 24hrs. γH2AX foci clearance (and thus DNA repair) is substantially reduced 24hrs post treatment with all compounds as single agents, and show that this effect is enhanced when administered pre-X-ray radiation exposure. This correlates closely with the clonogenic cell kill data, where diminishing levels of cell survival match elevated levels of γH2AX foci. The data recapitulates the inhibitory effects of BMS 345541 as reported by (Wu, Shao et al. 2011) and demonstrate this effect for the first time following SU567 and SU182 treatment.

Further evidence of the NF-κB inhibitory effects of BMS345541, SU567 and SU182 was observed in the cell cycle data (fig 6.3). NF-κB signaling is a key component of cell cycle control via the regulation of cyclin dependent kinases (CDK) (Joyce, Albanese et al. 2001) and as such alterations in normal cell cycle responses to conventional treatments act as an indicator of disrupted NF-κB pathway activation. Cell cycle analysis of melanoma cell lines demonstrated significant alterations to the cell cycle response to BMS34551, SU567 administration as single agents and prior to 1Gy irradiation.

The typical response of Melanoma cell lines to low dose irradiation is G2/M arrest (Villa, Zaffaroni et al. 1996) which was seen across all cell lines in the 1Gy treated groups. Treatment with BMS34551 and SU567 resulted in greater accumulation of G2/M arrest than radiation alone, an observation that is consistent with IKKβ specific inhibition which results in the stabilisation of p53 and concomitant p21 expression

leading to G2/M arrest and apoptosis (Yang, Huang et al. 2010). Greater levels of G1 arrest were also observed and are similarly typical of NF-κB inhibition, as NF-κB regulates cyclin D1, an essential regulator of the G1/S transition and reduction of this protein lead to G1 cell cycle arrest (Hinz, Krappmann et al. 1999), the enhanced levels of cell cycle arrest mirror the levels of cell survival reported in the clonogenic data indicating that these responses lead to greater levels of apoptosis within the cell population. Interestingly, SU182 treatments did not result in significant cell cycle alterations in the A375 and A2058 cell lines as a single agent or as a pre-treatment to 1Gy irradiation but did show a non-significant trend towards and S phase and G2/M arrest respectively in the B16-F10-LUC2 cell line which is potentially indicative of the cytoxicity observed in this cell line.

Preliminary data from western blot assays for IKKβ protein in the A375 cell line (appendix A1) indicate that BMS345541 and SU567 both decrease IKKβ protein levels at a concentration of 25µm, these data also indicate that SU182 may be a weaker IKKβ inhibitor than both BMS345541 and SU567 as blots demonstrated a higher amount of IKKβ protein following SU182 treatment, which is indicative of the low toxicity and γH2AX response of the A375 cell line to SU182 treatment. Further, preliminary results from superoxide dismutase (SOD) assays (Appendix A2) investigating the activity of MnSOD, a cellular antioxidant directly regulated by NF-κB activity (Maehara, Hasegawa T Fau - Isobe et al. 2000) via SOD mediated inhibition of WST-1 to WST-1 formazine, demonstrate slightly diminished MnSOD activity following BMS 344541 and SU567, treatment but do not indicate a reduction of

MnSOD activity following SU182 treatment in the A375 or A2058 cell lines. This weak observation warrants Further study, in order to draw definitive conclusions.

These data demonstrate the cytotoxic action of the novel compounds SU567 and SU182 in melanoma cell lines. Although identified as specific IKKβ inhibitors with the data presented in this chapter closely indicating this activity correct in SU567, the effects of SU182 are less clear. Of the three melanoma cell lines assessed, SU182 demonstrated activity in the B16-F10-LUC2 cell line only. This observation requires further investigation into the nature of SU182 activity and the level of NF-κB activity B16-F10-LUC2 cell line and therefore it is important that further work is directed towards confirming IKKβ inhibition as the primary mode of action for both compounds. Further analysis via western blot encompassing all cell lines assayed would focus on both the IKKβ and IKK$\alpha$ subunits and include downstream components of both canonical and non-canonical NF-κB pathways.

# Chapter 7

# Conclusions and Future Perspectives.

Malignant melanoma is historically regarded as radio resistant and as such radiation therapy is often overlooked as a treatment option (Little, Hahn et al. 1973). Recent studies however have concluded that a spectrum of radiation resistance exists and that when applied correctly melanoma tumours can be vulnerable to radiation therapy (Rofstad 1986, Skowronek, Matecka-Nowak et al. 1998, Rofstad, Mathiesen et al. 2004, Stevens and McKay 2006)

The main objective of this investigation was to interrogate the potential use of targeted radiotherapy and external beam irradiation alongside novel anti-cancer agents in the treatment of malignant melanoma.

To this end, there were three primary aims:

- To assess the effectiveness of the melanin binding radionuclide [$^{131}$I]MIP1145 in the treatment of malignant melanoma in vitro and in vivo.
- To investigate whether malignant melanoma cell lines and xenografts can be rendered susceptible to [$^{131}$I]MIBG radionuclide therapy via transfection in vitro with noradrenaline transporter (NAT) and via gene delivery in vivo with the HSV1716/NAT vector.
- To screen novel DNA repair and IKKβ Inhibitors in combination with X-Ray radiation to determine suitability for future targeted radiotherapy/drug combination therapy approaches.

These aims were successful and four main conclusions can be drawn.

Malignant melanoma is susceptible to targeted radiotherapy treatment options. The radiopharmaceutical [$^{131}$I]MIP1145 is preferentially retained in melanoma cells natively expressing the biopolymer melanin, inducing significant cytotoxicity *in vitro* and substantially reducing tumour burden in SK-MEL-3 tumour nude mouse xenograft models *in vivo*.

The noradrenaline transporter gene (NAT) can be successfully introduced to and expressed by malignant melanoma cells, rendering them susceptible to uptake of the noradrenaline analogue [$^{131}$I]MIBG which resulted in marked decreases in cell survival *in vitro. In vivo* delivery of the NAT gene can be achieved via through use of the oncolytic virus HSV1716/NAT as a delivery vector which acted to reduce tumour burden via successfully sensitizing melanoma tumours to [$^{131}$I]MIBG and via oncolysis of the tumour mass.

The MRE11 inhibitor Mirin enhances cytotoxicity and only weakly abrogates DNA repair as a pre-treatment to external beam X-ray irradiation *in vitro*.

Novel IKK$\beta$ inhibitors SU567 and SU182 are cytoxic to melanoma cell lines, enhance X-ray induced toxicity and inhibit the double DNA damage response.

**[$^{131}$I]MIP1145 is preferentially retained in melanoma cells natively expressing the biopolymer melanin, inducing significant cytotoxicity *in vitro* and substantially reducing tumour burden in SK-MEL-3 tumour nude mouse xenograft models *in vivo*.**

The present study has explored the potential of melanin as a native target for radiotherapy by utilising three melanoma cell lines, A375 amelanotic, A2058 pheomelanotic and SK-MEL-3 eumelanotic cell lines which represent differing melanin states found with in melanoma tumours in order to investigate the effectiveness of the novel benzamide [$^{131}$I]MIP1145 on melanoma cells both *in vivo* and *in vitro.*

The data presented in Chapter 3 demonstrates that [$^{131}$I]MIP1145 is selectively retained by melanin containing melanoma cell lines and elicits greater DNA damage responses as assayed by ϒH2AX foci formation than amelanotic controls. Similarly, clonogenic cell kill was greatly enhanced in melanotic cell lines treated with escalating doses of [$^{131}$I]MIP1145 when compared to amelanotic controls further demonstrating the melanin specific properties of [$^{131}$I]MIP1145. Significant anti-tumour activity of [$^{131}$I]MIP1145 was observed upon administration to an SK-MEL-3 nude mouse xenograft model whereby single, double and triple doses of the compound over consecutive weeks resulted in considerably greater reductions in tumour mass than dacarbazine controls. These data closely mirror the results from other benzamide compounds currently in development such as the radiolabelled quinoxaline derivative [$^{131}$I]ICF01012 which has been shown to be effective at

inducing tumour growth delay in melanotic xenograft models (Chezal et al., 2008, Bonnet et al., 2010, Bonnet-Duquennoy et al., 2009).

Future work [$^{131}$I]MIP1145 produced seemingly conflicting results in the A375 cell line which may be explained through observations on nonspecific uptake with using benzamide compounds (Mansard, Papon et al. 2005), it would be interesting to note any differences if the clonogenic assays were repeated with PBS washes throughout incubation to facilitate efflux in non melanotic lines. In order to confirm the absence of melanin in the A375 and UVW cell lines, melanin assays could have been performed (Hu 2008), which would confirm unexpected evidence of [$^{131}$I]MIP1145 retention in these amelanotic cell populations as a result of the experimental techniques used and not an unreported source of cellular melanin.

Taking advantage of melanin as a means of delivering targeted radiotherapy continues to prove an effective approach to treating melanoma. The data presented with in this study demonstrated the potential of [$^{131}$I]MIP1145 as a viable therapeutic for treating malignant melanoma. Subsequently [$^{131}$I]MIP1145 has been reassessed and investigated in conjunction with radiosensitising topoisomerase inhibitor topotecan, the PARP-1 inhibitor AG014699 and the proteasome inhibitor bortezomib in spheroid models (Hutchison, Rae et al. 2013). High specificity for melanin containing cells was reported in line with *in vitro* data generated by this investigation which successfully translated to results in amelanotic and melanotic spheroid models.

Encouragingly, combination treatments with topotecan and AG014699 showed significant enhancement of [$^{131}$I]MIP1145 and growth delay in combination groups. Interestingly, it was reported that bortezomib failed to provide benefit in the A2058 spheroid model. This was discussed as potentially being related to bortezomib's action to induce reactive oxygen stress (ROS) up to a threshold level which is saturated upon exposure to radiation negating any additive or synergist effect of the combination treatment. It would be interesting to investigate whether this effect was seen if [$^{131}$I]MIP1145 were combined with other ROS generating compounds such as Elesclomol, which have previously shown efficacy in melanoma models (Kirshner, He et al. 2008).

Additionally, a more recent compound [$^{131}$I]-BA52, being studied therapeutically in metastatic melanoma patients with good tolerance and low systemic toxicity demonstrating impressive 2 year long term survival rates (Mier, Kratochwil C Fau - Hassel et al. 2014) and have been taken forward to highly encouraging combination studies with the novel DNA repair inhibitor coDbait, a novel synthetic DNA molecule which sequesters DNA repair machinery, rendering a cell open to radiation induced damage (Viallard, Chezal et al. 2016) and serves to demonstrate that melanin seeking radionuclides have great scope as both single agent and combination therapies.

**The introduction of the noradrenaline transporter (NAT) gene renders melanoma cells susceptible to [$^{131}$I]MIBG. In vivo delivery of the NAT gene via the oncolytic virus HSV1716/NAT reduces tumour burden via successfully sensitizing melanoma tumours to [$^{131}$I]MIBG and via oncolysis of the tumour mass.**

One of the main boundaries to targeted radiotherapies is finding a specific molecular characteristic which renders a target cell susceptible to therapy. Although it was found that in the case of malignant melanoma cells there is a native target in melanin and a potential delivery molecule in MIP1145, due to the heterogeneous and variable melanin status both within and between melanoma tumours, the potential for tumour to be amelanotic in nature could limit the use of this targeted approach.

As such, exploring the delivery of [$^{131}$I]MIBG via the introduction of the non-native NAT gene could provide a viable alternative to treating amelanotic cells and tumours. When exploring the potential use of [$^{131}$I I]MIBG in the treatment malignant melanoma one must consider that melanoma cells do not natively express the noradrenaline transporter and therefore investigate methods for the introduction and stable expression of the NAT gene.

The data presented in Chapter 3 (figures 3.1 To 3.3) demonstrated for the first time that successful transfection and expression of the noradrenaline transporter gene (NAT) driven by the Cytomegalovirus promoter (CMV) enables uptake of [$^{131}$I]MIBG by melanoma cell lines and produces cytotoxicity *in vitro* and in *in vivo* xenograft models. Following the encouraging results of chapter 3, the concept of introducing

the noradrenaline transporter as a targetable feature was further built upon in chapter 4, where the use of a gene delivery vector for the NAT gene was explored.

This investigation applied a modified 1716 strain, carrying the noradrenaline transporter (NAT) in a preclinical model of malignant melanoma. This HSV1716/NAT vector has previously shown efficacy in malignant glioma by facilitating targeted radiotherapy treatments via uptake of [$^{131}$I]MIBG (Quigg, Mairs et al. 2005) demonstrating similar efficacies though out this investigation.

In summary, biodistribution data (figure 4-1) following viral inoculation shows that HSV1716/NAT infection was limited only to tumour sites, with no sign of viral infection in any organ systems. [$^{131}$I]MIBG uptake studies determined that treatment of either SK-MEL-3 melanoma or UVW Glioma xenografts with the HSV1716/NAT vector successfully enabled the uptake of [$^{131}$I]MIBG when compared to the HSV1716 parental vector and PBS control.

Anti-tumour activity was noted in both viral groups, owing to the oncolytic nature of the HSV1716 vector, however this activity was significantly enhanced compared to that of the wild type vector in tumours treated with the HSV1716/NAT virus after both groups were combined with [$^{131}$I]MIBG treatment. Scheduling experiments demonstrated that an inoculation of HSV176/NAT 24hrs prior to [$^{131}$I]MIBG resulted in a doubling of the accumulation of the radiopharmaceutical when compare to simultaneous injection intratumourally. This effect may be as a result of the earlier infection window lending time for the virus to replicate, induce expression of the NAT

gene (and thus [$^{131}$I]MIBG uptake) in infected cells and reduce tumour volume via oncolysis during the replication cycle.

Importantly, intravenous injection schedules also resulted in high tumour specificity/low organ infection, demonstrating that this HSV1716 strain is a potential candidate for treating metastatic disease.

The combination of the HSV1716/NAT vector and [$^{131}$I]MIBG has great clinical potential, future work could pursue whether the vector/radiation treatment system could be utilised in combination therapies. Recent studies have shown that treatment with the standard HSV1716 vector can improve the effectiveness of Anti-PD-1 immunotherapy via enhancement of t-cell activation and accrual (Chen, Wang et al. 2017), and there is growing evidence that radiation therapy can enhance PD-1 and CTLA-4 based therapies through upregulation of surface markers, immunogenic cell death and T-cell attracting cytokine release (Van Limbergen, De Ruysscher et al. 2017). The nature of the HSV1716/NAT - [$^{131}$I]MIBG combination potentially exploit both of these effects and thus presents an interesting avenue for further investigation.

**The MRE11 inhibitor Mirin enhances cytotoxicity but only weakly abrogates DNA repair as a pre-treatment to external beam X-ray irradiation *in vitro*.**

Clonogenic Survival assay experiments assessing Mirin as a single agent and in combination with 2Gy X-irradiation (Figure xx) demonstrate that as a single agent, mirin did not significantly affect clonogenic survival. It was however observed that when administered as a pre-treatment 2hrs prior to irradiation, Mirin at concentrations of 75µm-100µm significantly enhanced radiation induced cytotoxicity. This encouraging result was followed by FACS analysis for H2AX foci number demonstrating no significant effect on foci clearance after 5hrs in Mirin treated groups either as a single agent or as a pretreatment with 2Gy X-irradiation. Weak but significant inhibition of yH2AX foci clearance was observed after 24hrs when treated with 75µm or 100µm of Mirin in both compound only and radiation combination groups.

Whilst these results consistently demonstrate some degree of efficacy for mirin via clonogenic cell kill and inhibition of yH2AX clearance alongside a 2Gy external beam irradiation high doses, the results are by no means conclusive. As Mirin inhibits the activation of the homologous recombination repair pathways (Dupre, Boyer-Chatenet et al. 2008) the expected outcome from FACS analysis would be a considerable abrogation of yH2AX foci removal over time in Mirin treated groups. Taken together with the clonogenic data, this suggests that mirin is either inducing cytotoxicity via a mechanism separate to ATM mediated repair abrogation or, that

the surviving cell population available for yH2AX foci analysis was able to effectively overcome mirin induced reduction of HRR.

Concurrent *in vivo* investigations failed to demonstrate any additional benefit to a 50mg/kg Mirin pre-dose in A375/NAT bearing nude mouse xenografts when exposed to 6Gy X-ray external beam radiation, this requires much deeper investigation as *in vivo* investigations of Mirin are scarce in the literature. Future investigations in vivo should involve a full pilot study in order to determine toxicity and dose response to be coupled with both external beam and targeted radiotherapies in order to assess any differences in action.

In order to investigate these observations, future work should consist of mechanistic assays such as western blots to determine phospho- ATM levels pre and post Mirin treatment and cell cycle analysis to determine whether Mirin treatments resulted in an expected abrogation of the G2/M checkpoint (Dupre, Boyer-Chatenet et al. 2008). Further investigations into DNA-PK and the role of other DNA repair pathways as potential therapeutic targets, such as Non-Homologous End joining, nucleotide excision repair and base excision repair directed under ATR (Stiff, O'Driscoll et al. 2004, Golding, Morgan et al. 2009) to thoroughly assess the effected targets of mirin and to ascertain cellular repair responses to Mirin treatment should also be included. Additionally, this deeper mechanistic data can be coupled with yH2AX assays which determine the number foci formed and cleared over extended time periods to give a better indication of the dynamics as with previous chapters such as the UVW line, to discount the possibility of a melanoma specific resistance to Mirin activity.

**Novel IKKβ inhibitors SU567 and SU182 are cytoxic to melanoma cell lines, enhance X-ray induced toxicity and inhibit the double DNA damage response.**

The NFκB signaling cascade is constitutively active in melanoma tumours, contributing to therapy resistance by enhancing anti-apoptotic signaling, promoting cell proliferation and aiding in tumour invasion and metastasis (Amiri and Richmond 2005). This has driven considerable interest into developing methods to diminish NFκB activity. This study has explored two agents in the A375, A2058 and B16F10_LUC2 cell lines designated SU567 and SU182, designed to abolish NFκB signaling by targeting of the IKK subunit IKKβ, which is the master regulator of the canonical NFκB pathway (Liu, Xia et al. 2012).

Of the two compounds SU567 demonstrated the most significant activity across all cell lines tested, producing marked clonogenic cell kill both as a single agent and as a pretreatment with external beam radiation (figure). This effect was recapitulated in cell cycle analysis (figure) where by a shift towards G2/M and G1 arrest was observed consistent with both radio sensitization and abrogation of NFκB activity (Villa, Zaffaroni et al. 1996, Joyce, Albanese et al. 2001). DNA double strand break repair inhibition was observed, which is indicative of IKKβ inhibition (Wu, Shao et al. 2011).

SU182 failed to produce significant effects in the A375 and A2058 cell lines only producing cytotoxicity and DNA repair inhibition in the B16-F10-LUC2 cell line. Although this study did not confirm the underlying reasons behind this discrepancy,

this may have been due to weaker or differing activity of SU182 when compared to SU567 or potentially a difference in baseline NFκB expression in the B16-F10-LUC2 cell line and warrants further investigation as discussed in section 6.4.

The significant enhancement of X-ray induced cytotoxicity by both SU567 and SU182 is highly encouraging for the development of radiation/ IKKβ combination therapy treatments. Further toxicity experiments with both compounds over a broader dose range would enable median effect and combination index data to be calculated as described by (Chou and Talalay 1984) and enable scheduling experiments with a range of IKKβ/X-ray radiation dose combinations. These double combination schedules could be developed further based upon the DNA repair abrogation demonstrated by both compounds, whereby triple therapy combinations could be explored including DNA repair pathway specific inhibitory molecules such as Mirin in an effort to enhance overall effectiveness.

NFκB inhibitors have been successfully utilised in conjunction with $^{131}$I in the treatment of thyroid cancers (Pozdeyev, Berlinberg et al. 2015), demonstrating that NFκB pathway abrogation enhances $^{131}$I toxicity. This suggesting that these novel IKKβ inhibitors may also prove effective in conjunction with radiopharmaceuticals such as [$^{131}$I]MIBG and that these compounds could be utilised alongside therapies such as the HSV1716/NAT vector in malignant melanoma to further enhance antitumor activity.

# Chapter 8

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Cancer development and progression is a complex process that involves a host of functional and genetic abnormalities. Genomic perturbations and the gene expression they lead to, can now be globally identified with the use of DNA microarray. This relatively new technology has forever changed the scale of biological investigation. The enormous amount of data generated via a single chip has led to major global studies of the cellular processes underlying malignant transformation and progression. The multiplicity of platforms from different proprietors has offered investigators flexibility in their experimental design. Additionally, there are several more recent microarrays whose designs were inspired by the nucleotide-based technology. These include protein, multi-tissue, cell, and interference RNA microarrays. Combinations of microarray and other contemporary scientific methods, such as, laser capture microdissection (LCM), comparative genomic hybridization (CGH), single nucleotide polymorphism analysis (SNP) and chromatin immunoprecipitation (ChIP), have created entirely new fields of interest in the more global quest to better define the molecular basis of malignancy. In addition to basic science applications, many clinical inquiries have been performed. These queries have shown microarray to have clinical utility in cancer diagnosis, risk stratification, and patient management.

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# Chapter 9

# Appendix

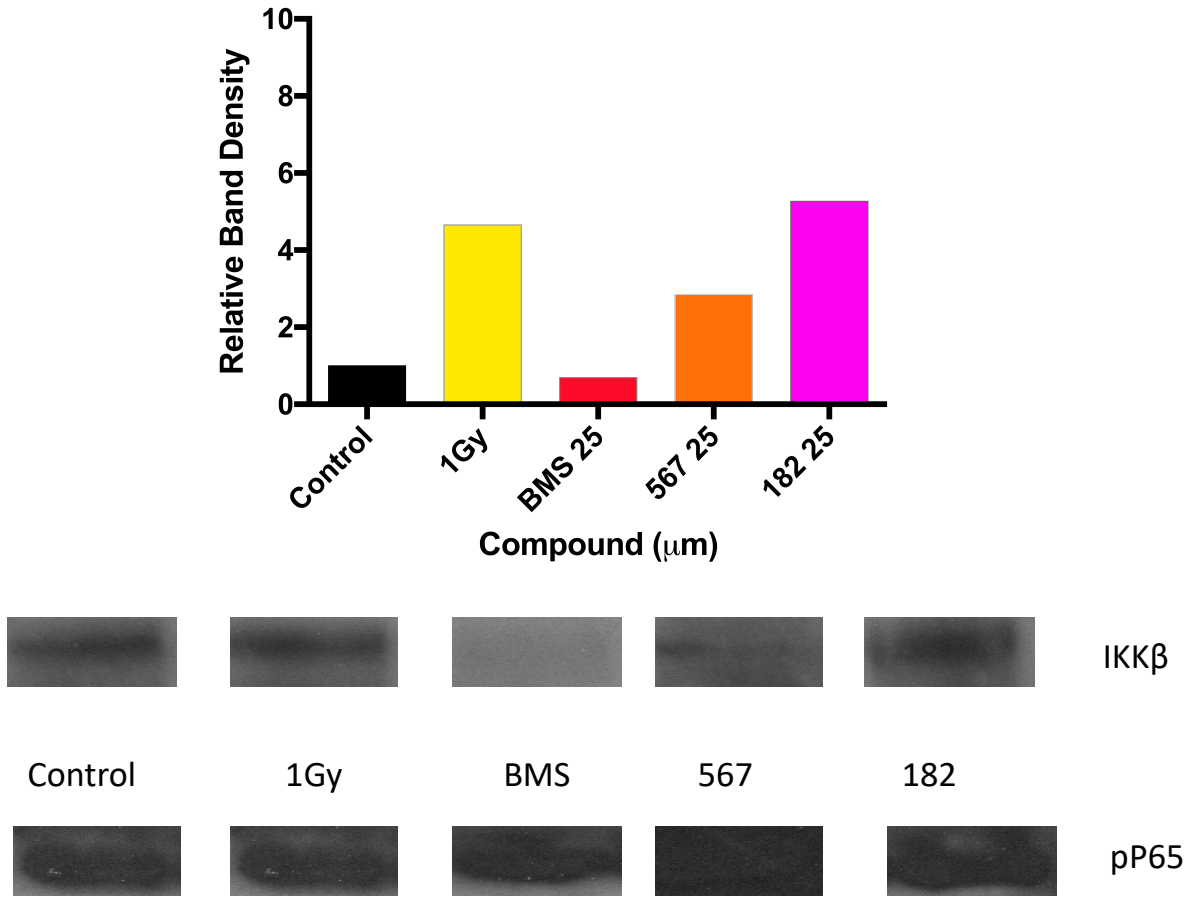


Figure A-1 Western blotting data for IKKβ expression

IKKβ expression in the A375 cell line treated with SU compounds in combination with 1Gy X-Rays. Compounds were administered 1hr prior to irradiation and measurements were taken 24hrs post treatment. pP65 was used as loading control. Control samples are untreated A375 cells.

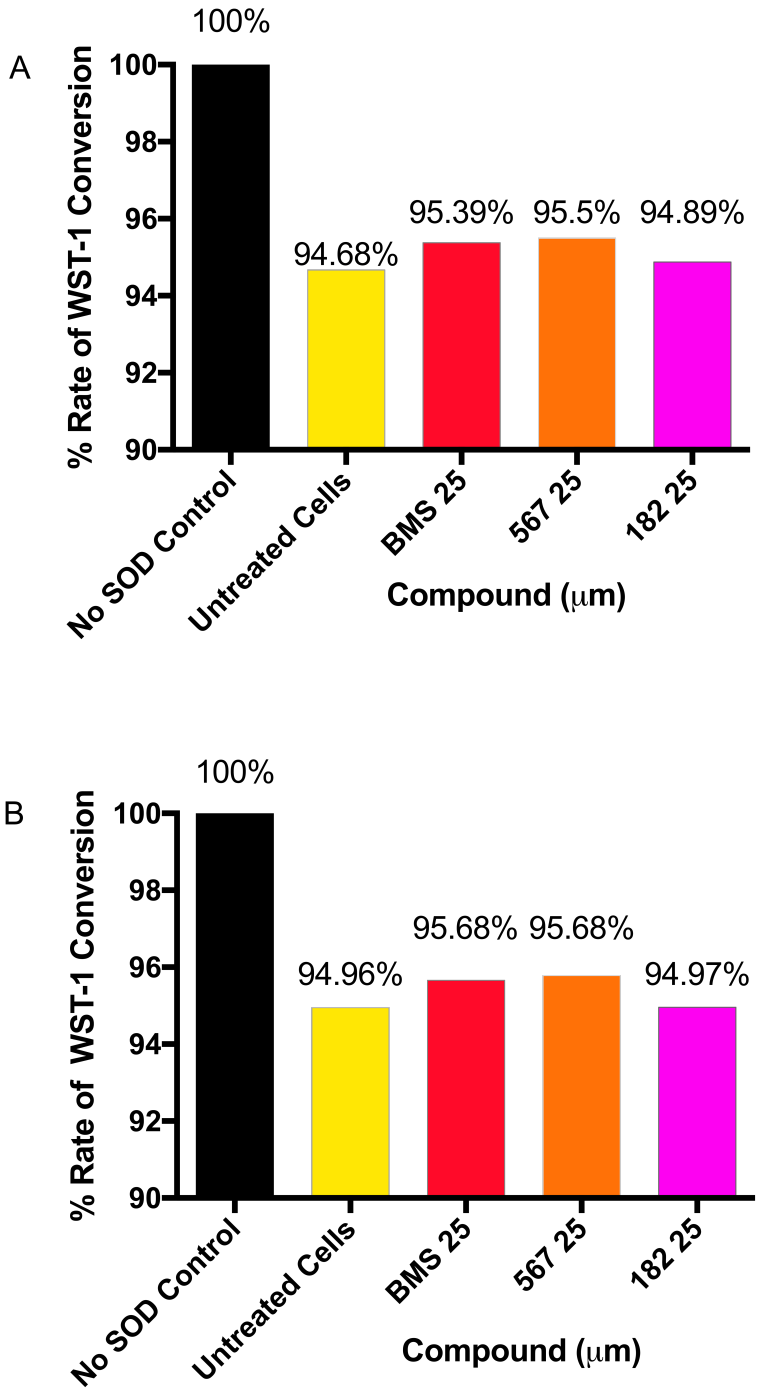


**Figure A-2 Superoxide dismutase assay for MnSOD.**

A375 (A) and A2058 (B) cells were treated with BMS 345541, SU567 and SU182 for 1hr. Graphs represent % rate of WST-1 conversion. The conversion rate of treated cells shows very weak increases in the rate of conversion vs. untreated controls in the SU 567 and BMS 345541 treated samples. Sample with no superoxide dismutase was used as a positive control.

# Related Conference Attendance and Poster Presentations

| | |
|---|---|
| 2011 | Strathclyde University Research Open Day - Poster Presentation |
| 2010 | RICAS Research Day - Invited Poster Presentation |
| 2010 | Strathclyde Biology Expo - Poster presentation |
| 2010 | Radiation Research Society - Poster Presentation |
| 2010 | Association for Radiation research - Poster presentation |
| 2009 | Association for Radiation research - Oral presentation |