Identification of novel strategies to radiosensitise tumour cells

Selvakumar Anbalagan

St Edmund Hall

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Abstract

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In this study we found that tumour cells can be radiosensitised by targeting the DNA damage response kinases, ATM and ATR. Furthermore, we highlight that Wee1 inhibitors, which are already under the clinical trials need to be further investigated in combination with radiation in the context of tumour hypoxia. In addition, we observed that induction of autophagy using STF-62247 can lead to radiosensitisation of VHL deficient RCC cells. Our studies with the rapamycin analogue temsirolimus, already in the clinic for the treatment of various cancers, can be a potential candidate as a radiosensitiser for RCC cells. Overall, these finding led us to investigate further whether autophagy inducing compounds, which are either in clinic or in clinical trials, can effect the response to radiation. From a panel of candidate drugs which are known to induce autophagy we identified an aminopeptidase inhibitor, CHR-2797. CHR-2797 induces autophagy in the oesophageal cancer cell lines FLO-1 and OE21. Although, our results with CHR-2797 demonstrate it as a potential radiosensitiser, the mechanism of its radiosensitisation needs to be established. Our results from CHR-2797-induced radiosensitisation, further led us to investigate if other aminopeptidase inhibitors have a role in radiosensitisation. Therefore, we selectively screened candidate aminopeptidase inhibitors and identified some promising effects on radiosensitivity.

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List of abbreviations

AADR	Amino acid deprivation response
Ambra	Activating molecule in Beclin-1-regulated
AML	Acute myeloid leukaemia
AMPK	AMP-activated protein kinase
ANGPT	Angiopoietin
ATG	Autophagy related gene
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related protein
53BP1	p53-binding protein 1
BER	Base excision repair
BFA	Brefeldin A
Bif-1	Endophilin B1
Bnip3	Bcl-2/adenovirus E1B 19kDa interacting protein
BRCA1/2	Breast Cancer 1 and 2
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CSA	DNA excision repair protein ERCC8
CSB	DNA excision repair protein ERCC6
CT scan	Computerised tomography
CUL2	Cullin-2
CQ	Chloroquine diphosphate
DAPI	4',6' diamino-2-phenylindole·2HCl
DBS	Double strand break
DDR	DNA damage response
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport

FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FIH	Factor inhibiting hypoxia-inducible factor
FITC	Fluorescein isothiocyanate
FoxO3	Forkhead box O3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT1/3	Solute carrier family 2, facilitated glucose transporter member 1
Gy	Gray
h	Hours
H2AX	Histone
γH2AX	Phosphorylated histone
HIF-1/2α	Hypoxia-inducible factor 1/2α
HOPS	Homotypic fusion and vacuole sorting protein
HRE	Hypoxia response element
HR	Homologous recombination
HU	Hydroxyurea
hVps34	mammalian vacuolar protein sorting 34 homologue
IGRT	Image-guided radiotherapy
IMRT	Intensity-modulated radiotherapy
IR	Ionising radiation
kDa or Da	Kilodalton, Dalton
LC3-I (MAP-LC3)	Microtubule-associated protein light chain 3-I
LC3-II (MAP-LC3)	Microtubule-associated protein light chain 3-II
LET	Linear-energy-transfer
LINAC	Linear accelerator
LOXL	Lysyl oxidase
3-MA	3-methyladenine
mins	Minutes
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
NER	Nucleotide excision repair
NHEJ	Non homologous end joining
ns	Non-significant
O_2	Oxygen
OER	Oxygen enhancement ratio

p53	Cellular tumour antigen p53
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PDGFB	platelet-derived factor B
PDK1	3-phosphoinositide-dependent kinase 1
PE	Phosphatidylethanolamine
PET	Positron emission tomography
PFA	paraformaldehyde
PHD	Prolyl hydroxylases
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphate 3-phosphatase
PtdIns3P	Phosphatidylinositol-3 phosphate
RAD51	DNA repair protein RAD51 homolog 1
RBE	relative biological effective dose
RCC	Renal cell carcinoma
RFC	Replication factor C subunit
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute
RIP1	Receptor interacting protein 1
SDS	Sodium dodecyl sulphate
SER	Sensitisation enhancement ratio
SNARE	N-ethylmaleimide-sensitive factor-attachment protein receptors
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered tween
Tris	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
TSC	Tuberous sclerosis protein
ULK1/2	unc-51-like-kinase ½
UVRAG	UV irradiation resistance-associated gene
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau
Wee1	Wee1-like protein kinase
WHO	World health organisation
XPB/D	TFIIH basal transcription factor complex helicase subunits

XPF	DNA repair endonuclease XPF
XLF	Non-homologous end-joining factor-1
XRCC4	DNA repair protein XRCC4

Chapter 1

Introduction

1.1 Cancer

Cancer is considered to be a global epidemic. In the year 2012, WHO documented that there were 14.1 million people diagnosed with cancer and 8.2 million deaths due to cancer worldwide (WHO, 2012). All cancers have certain characteristics in common, which include the ability to proliferate unconditionally, migrate to surrounding tissues and adapt to new environments and ultimately survive.

The primary causes of cancer arise as a result of genetic inheritance, exposure to carcinogens (including smoking, Schroeder, 2013) or the individual's lifestyle. Although, cancer can develop in children and young adults, it is primarily a disease associated with ageing (Balducci & Ershler, 2005). In the UK, 36% of the people diagnosed with cancer are aged 75 or over (CRUK, 2014). In the USA, it has been projected that by the year 2030 almost 70% of people, who are aged 65 or more, will be at risk of developing some form of cancer (Edwards et al, 2002).

Cellular ageing results in molecular changes leading to DNA damage, epigenetic alterations, genomic instability, immune response, premature senescence, deregulated nutrient sensing and mitochondrial dysfunction. These changes can all play a role in increasing the probability of cancer development (Balducci & Ershler, 2005; Hoeijmakers, 2012; Lopez-Otin et al, 2013).

1.1.1 The Hallmarks of Cancer

Normal cells develop into cancer cells through a cascade of events during which they acquire specific traits, and eventually become benign or malignant. The progression of a normal cell into a cancer cell is a multistep process that had been described by Hanahan and Weinberg as the Hallmarks of cancer. The six conceptual framework of

cancer hallmarks are sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death (Hanahan & Weinberg, 2000) (Figure 1.1). Over the years, as a result of extensive developments in the field of cancer biology, the emerging hallmarks (avoiding immune destruction and deregulating cellular energetics) and enabling characteristics (genome instability and mutation and tumour promoting inflammation) have been added to the original six hallmarks of cancer (Hanahan & Weinberg, 2011). Together, these concepts have helped us in the development of cancer therapy.

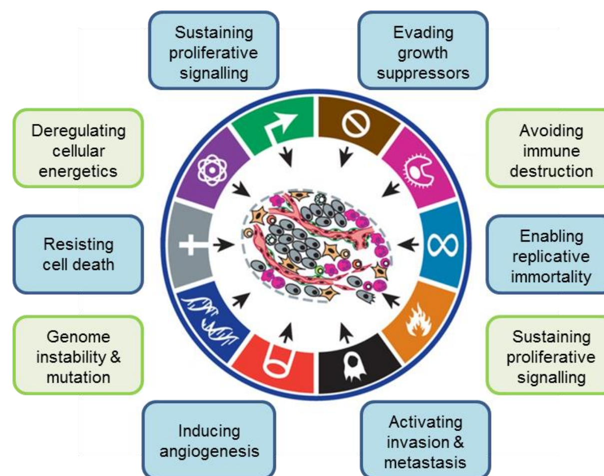

Figure 1.1 Hallmarks of cancer: the next generation. Boxes shaded in blue indicate the hallmarks proposed in 2000, whereas those in green proposed as the emerging hallmarks in 2011 [This article was published in Cell, 144, Hanahan and Weinberg, Hallmarks of cancer: The next generation, 646-674, Copyright Elsevier (2011)]

1.2 The tumour microenvironment

Tumour cells do not often exist in isolation. Instead, tumour cells exist in what is known as the tumour microenvironment. The tumour microenvironment refers to the physical characteristics of the tumour as well as the heterogeneous population of cells present in the tumour. For example, solid tumours comprise of not just one single population of clonal cancer cells but multiple cell types and extracellular matrix. This

mixture of cells in the solid tumour are integrated together and have been described to function similarly to an organ (Egeblad et al, 2010).

The tumour microenvironment comprises of the stroma, tumour cells, non-transformed cells, immune cells, lymphatic vessels and the tumour vasculature (Balkwill et al, 2012; Junttila & de Sauvage, 2013). Depending on tumour staging the biology of the tumour microenvironment varies, with alterations in the stroma, vasculature and immune responses (Bissell & Radisky, 2001; Coussens & Werb, 2002; Elinav et al, 2013; Lewis & Pollard, 2006).

The extracellular matrix (ECM) is both dynamic and complex, comprised mainly of fibrous proteins and proteoglycans. In normal tissues as well as during embryonic development the ECM architecture is tightly regulated, but in cancer it is highly disorganised and deregulated (Lu et al, 2012b). The ECM plays a critical role in tumour cell migration and is also capable of generating mechanical stress leading to regions of stiffness and relaxation within tumours (Chen et al, 2013). The mechanical stress occurs as a result of the increase in tumour mass, as it expands compressing the surrounding tumour microenvironment, which in turn generates an opposing force to counter balance. Tumour cells recognise the stiffness and generate either an opposing force leading to increased pressure inside the tumour or remodel the architecture of the ECM to enhance growth, survival and tumour invasion (Butcher et al, 2009). The increased pressure inside the tumour leads to accumulation of interstitial fluid which leads to vascular abnormalities and therefore, increased resistance to various cancer therapies (Simonsen et al, 2012).

1.2.1 Hypoxia

Hypoxia refers to any condition of insufficient oxygen and is a common occurrence in solid tumours. Reduced levels of oxygen occur in various pathological conditions such as inflammation, stroke, tissue ischaemia, wound healing and cancer. Depending on the levels of oxygen in the tissue, hypoxia can be classified as physoxia (5% O_2) where tissues homeostasis is regulated, pathological hypoxia (1% O_2) which has disrupted homeostasis due to deregulated vasculature and finally, radiobiological hypoxia ($\leq$0.4% O_2) (Hockel & Vaupel, 2001; McKeown, 2014).

Hypoxia has been shown to be a poor prognostic factor in patients with solid tumours. Irrespective of the tumour staging, patients with higher hypoxic tumours have an increased risk of mortality. This increased mortality rate is due to chemoresistance, radioresistance, angiogenesis, vasculogenesis, invasiveness, metastasis, resistance to cell death, altered metabolism and genomic instability (Semenza, 2010; Vaupel & Mayer, 2007; Wilson & Hay, 2011).

Regions of hypoxia occur in most if not all solid tumours and this is due to a mismatch in supply and demand. Rapidly proliferating tumour cells metabolise the available oxygen leaving other cells with insufficient and therefore hypoxic. This is referred to as chronic hypoxia and occurs at distances more than 100 μm from functional blood vessels. Regions of acute hypoxia also occur in tumours and this is due to the inefficiency of the tumour vasculature. Although through the process of angiogenesis tumours are able to develop their own vasculature and blood supply, the vessels are usually poorly formed and inefficient with many structural abnormalities. The result of this is blocked and non-functional vessels leading to regions of acute hypoxia, which can then be rapidly reoxygenated (Figure 1.2).

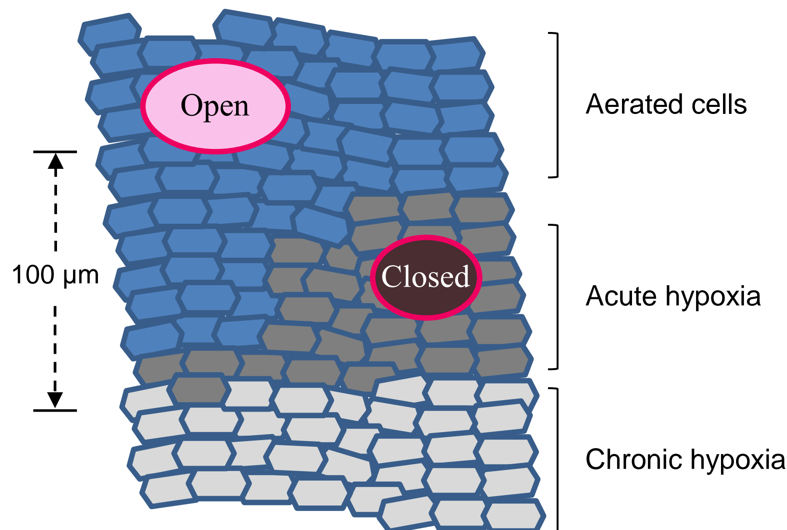

Figure 1.2 Regions of chronic and acute hypoxia occur in solid tumours. A cross-section of solid tumour representing regions of well aerated cells (open blood vessel), acute hypoxia (closed blood vessel) and chronic hypoxic regions occurring either due to their distance from the blood vessel or imbalance in oxygen supply and demand.

Lack of oxygen is not the only physical stress which contributes to the unique tumour microenvironment. Under hypoxic conditions, there is reduced mitochondrial consumption of oxygen and also ATP production in tumour cells. ATP depletion in the tumour cells can lead to disruption of the Na^+/K^+ gradient, depolarisation of membrane potential, cellular uptake of Cl^-, cell swelling, increased cytosolic Ca^{2+} concentrations and decreased cytosolic pH resulting in acidosis (Hockel & Vaupel, 2001). The increase in acidic pH is due to poor perfusion resulting from disorganised tumour vasculature. As a result of this poor perfusion the acid diffuses into the stroma and leads to remodelling of the normal cells in the proximity of the leaky site leading to local tumour invasion (Estrella et al, 2013). The survival of tumour cells under such acidic environments is through an adaptive mechanism (Wojtkowiak et al, 2012). Apart from these stress stimuli, tumour cells also undergo nutrient deprivation such as glucose and amino acids. This nutrient deprivation leads to induction of alternative metabolic pathways to fuel the metabolic demands of the tumour (Reitman et al, 2014; Roslin et al, 2003; Sun & Denko, 2014).

1.2.2 HIF transcription factors

The hypoxia inducible factors, HIF-1 and HIF-2, are heterodimeric transcription factors composed of an alpha subunit HIF-1α and HIF-2α respectively, both of which dimerise with a constitutively expressed beta subunit HIF-1β (Partch & Gardner, 2011). The levels of HIF-1/2α are stabilised in response to decreased levels of oxygen, growth factors, oncogenic activation or loss of the von Hippel-Lindau (VHL) tumour suppressor gene (Keith et al, 2012).

Under normoxic conditions, the proline residues (402 and/or 564) of HIF-1α are hydroxylated by prolyl hydroxylases (Ivan et al, 2001; Jaakkola et al, 2001). This hydroxylation initiates the binding of hydroxylated HIF-1α to an E3 ligase complex composed of VHL, Elongin and CUL2 with subsequent ubiquitination (Kamura et al, 1999). The ubiquitinated complex of HIF-1α undergoes degradation by the 26S proteosomal complex (Cockman et al, 2000; Groulx & Lee, 2002; Ohh et al, 2000).

In well-oxygenated cells the HIF-1α and HIF-2α subunits are also hydroxylated at the asparagine residues 803 and 851 respectively by factor inhibiting HIF (FIH) (Lando et al, 2002b). Similarly, to prolyl hydroxylation, the hydroxylation of asparagine residue is also catalysed by Fe^{2+} and 2-oxoglutarate dioxygenase (Hewitson et al, 2002; Lando et al, 2002a). The hydroxylation of the asparaginyl residue in the HIF-α subunits inhibits the binding of HIF subunits to the transcriptional co-activators p300/CBP (p300/CREB Binding Protein), therefore results in inactivation of HIF transcriptional activity (Freedman et al, 2002).

Under hypoxic conditions, HIF-1α is not hydroxylated by the prolyl hydroxylases (PHD) as PHD’s are dependent on oxygen for their catalytic activity. Therefore, HIF-1α is not degraded by the VHL E3 ligase complex, and accumulates. HIF-1α then translocates to

the nucleus and dimerises with HIF-1β (Depping et al, 2008; van de Sluis et al, 2010). The stabilisation of HIF-1 initiates the binding with the cofactors p300/CBP (Partch & Gardner, 2011). The HIF-1 dimer along with the p300/CBP binds to the hypoxia response element (HREs) within the promoter region of HIF-1 responsive target genes and activates transcription (Figure 1.3) (Ahn et al, 2014; Greijer et al, 2005; Ruas et al, 2005).

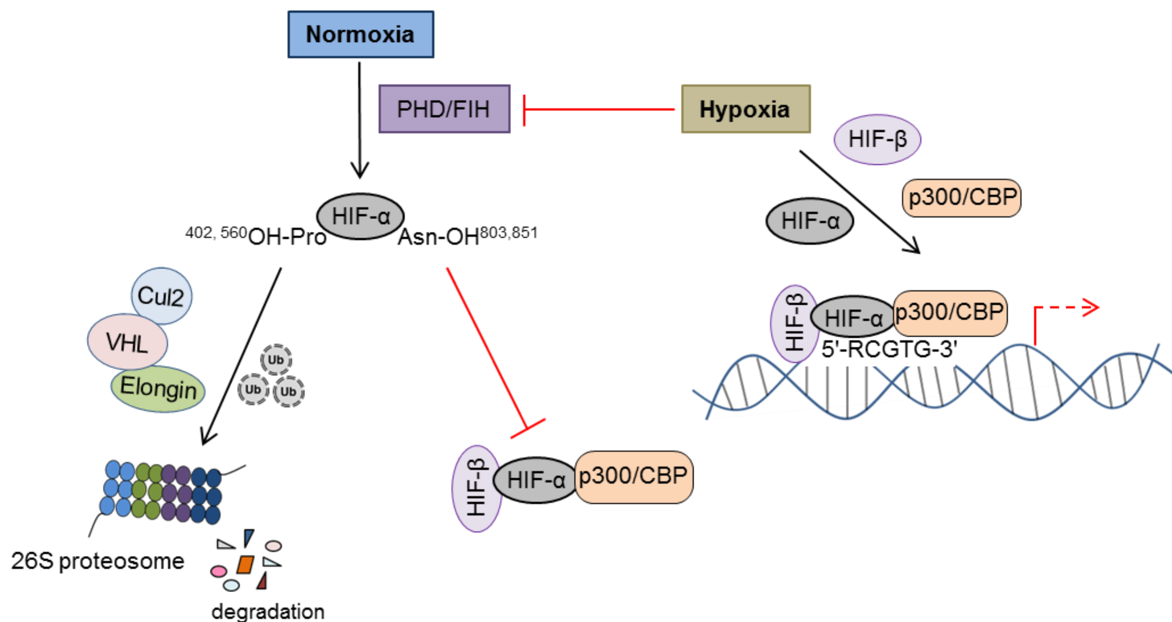

Figure 1.3 Mechanism of HIF regulation in normoxia and hypoxia. Under normoxia, hydroxylation at the proline residue (402, 560) recruits the VHL-Cul2-Elongin complex which ubiquitinates HIF-α and subsequently is degraded by the proteosomal machinery, whereas the hydroxylation at the asparagine residue (803, 851) inhibits binding of the cofactor p300/CBP with HIF subunit. The hydroxylation of HIF-α under normoxia therefore leads to inhibition of the HIF mediated gene expression. Under hypoxia, the activity of PHD and FIH are inhibited. This initiates the interaction of HIF subunit with the cofactors p300/CBP. The complex consisting HIF subunit and p300/CBP binds to the HRE region (RCGTG) of the target gene and induces the expression of various hypoxia induced HIF-mediated transcription factors. In the scheme represented above the perpendicular end of the lines represents inhibitory effect whereas the arrows are positive regulators.

The two isoforms HIF-1α and HIF-2α are overexpressed in hypoxic regions of solid tumours (Beasley et al, 2002; Talks et al, 2000). Although the HIF-α subunits are structurally similar and are regulated similarly, they have a distinct transcriptional gene

regulation pattern, in response to hypoxia (Carroll & Ashcroft, 2006; Hu et al, 2003; Sowter et al, 2003).

1.2.3 Biological consequences of hypoxia

Primary and metastatic solid tumours with regions of hypoxia exhibit increased levels of HIF expression. The increased levels of HIF expression could either be due to the intratumoural hypoxia or loss of function of tumour suppressor genes (for example, VHL, p53, TSC2, PTEN), which are involved either in increased levels of HIF or degradation of HIF (Emerling et al, 2008; Hebert et al, 2006; Lee et al, 2012; Snell et al, 2014). The functions of the hypoxia inducible genes are known to be involved in the regulation of glycolysis, cell proliferation, angiogenesis, invasion and metastasis of tumour cells (Figure 1.4).

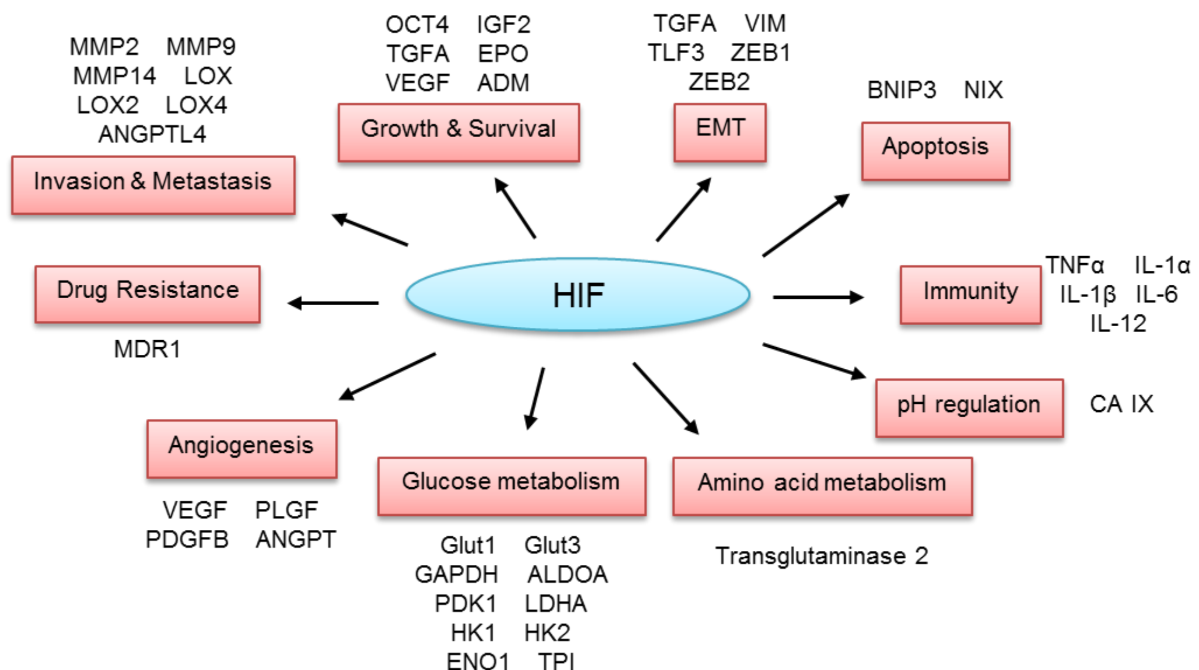

Figure 1.4 Functions of HIF. The scheme represents the various functions of HIF in response to hypoxia (Greer et al, 2012; Greijer et al, 2005; Henze & Acker, 2010; Semenza, 2003).

One of the hallmarks of cancer cells is the ability to proliferate unconditionally and survive. A requirement for proliferation and survival of cancer cells is the availability of supplements to fuel metabolism (Cui et al, 2012). Under hypoxic conditions, this energy metabolism is fuelled through glycolysis. HIF regulates the transcription of most of the glycolytic enzymes (for example aldolase, enolase and hexokinase) and glucose transporters (GLUT1 and 3) (Jean et al, 2006; Lambert et al, 2010; Sedoris et al, 2010). Increased tumour cell proliferation leads to increased energy metabolism demanding reorganisation of tumour vasculature so that enough nutrient supply is maintained for homeostasis (Dang, 2012). Angiogenesis plays an important role in tumour progression, by modulating the tumour vasculature. HIF-1α activates the genes involved in angiogenesis most notably VEGF, and also activates genes regulating cytokines (Carroll & Ashcroft, 2006; Doedens et al, 2013; Egger et al, 2007).

Following genetic alterations and cell proliferation, cancer cells attain the ability to invade and metastasise. The changes in extracellular matrix, intravasation, margination and the lymph node involvement determine the invasiveness and metastatic characteristics of tumour cells from their primary site. A number of genes are involved in the process of invasion and metastasis of tumour cells. Most notably the genes lysyl oxidase (LOXL) and PLOD for extracellular matrix remodelling; VEGF, angiopoietin (ANGPT) for intravasation and platelet-derived factor B (PDGFB) for lymphatic metastasis are transcriptionally regulated by HIF (Gilkes et al, 2013; Schietke et al, 2010; Schito et al, 2012; Zhang et al, 2012).

1.2.4 Hypoxia induced autophagy

Cells can adapt to hypoxic stress for their survival through the induction of autophagy (Bellot et al, 2009; Rouschop et al, 2010; Wilkinson et al, 2009). Interestingly,

hypoxia induced autophagy can be independent of HIF and nutrient availability indicating that various mechanisms regulate autophagy and its regulation is context dependent (Papandreou et al, 2008).

Autophagy is a normal cellular process used for the generation of nutrients and energy in response to stress for example, during periods of starvation. The autophagic process is deregulated in cancer and depending on the cellular context and stress, can be both pro-survival or lead to autophagic, programmed type II death (Turcotte et al, 2008b). The important role of autophagy in cancer therapy is becoming increasingly apparent. A wide variety of chemotherapeutics including approved agents such as Temozolomide and more novel compounds such as Elisidepsin (antiproliferative activity) have been shown to induce autophagy (Ling et al, 2011). The contribution of autophagy to radiation efficacy is unclear. The autophagy inhibitor chloroquine can increase the sensitivity to radiation *in vitro* and is currently being tested in clinical trials (Chaachouay et al, 2011; Solomon & Lee, 2009). However, the induction of autophagy has also been demonstrated to enhance the effects of radiation (Altmeyer et al, 2010; Chiu et al, 2010; Kim et al, 2009; Lin et al, 2010; Mukubou et al, 2010; Rajecki et al, 2009; Zhuang et al, 2011). The mechanism of autophagy will be discussed in further detail later.

1.3 Cancer therapy

Surgery is the oldest form of cancer treatment. Apart from surgery other treatment modalities include radiotherapy, chemotherapy, hormonal therapy, immunotherapy, gene therapy (for example oncolytic viruses), photodynamic therapy, hyperthermia and laser treatment. Treatment planning depends largely on the type of the tumour, the stage of the tumour, and the patient. Tumours are heterogeneous and their response to a single therapy

modality may not be sufficient therefore requiring a combination of therapies or adjuvant treatment.

1.3.1 Historical perspective of cancer treatment

Reports on cancer and its treatment date back to 1500 B.C. in ancient Egyptian inscriptions. In 1894, Halsted W.E., and Moore, C.H., were the first to pioneer and develop radical mastectomy for breast cancer (Osborne, 2007). As a result of various screening studies post World War II, a few compounds namely, mustard gas, Actinomycin-D and methotrexate were found to have antitumour effects (Farber, 1949; Jacobson & Wachowski, 1947; Pinkel, 1959; Tan et al, 1959). These discoveries transformed cancer treatment and were the basis of chemotherapy and adjuvant therapies.

The discovery of X-Rays was an important milestone in radiotherapy. Initially, X-rays were used to treat patients with dermatological disorders and also for the treatment of cancer. The invention of computerised tomography (CT scan), positron emission tomography scan (PET scan) and magnetic resonance imaging scan (MRI scan) has led to advancement in imaging diagnostics and revolutionised the delivery of radiation to the tumour.

1.3.2 Current therapies for cancer treatment

The treatment of cancer commonly involves surgery, chemotherapy or radiotherapy. Surgery is preferred if the tumour can be easily resected and accessed by the surgeon. Surgery not only helps in the removal of tumour but is also used in diagnosis and further treatment. Chemotherapy involves the use of drugs, which can be target specific but often have deleterious side effects. Another form of common treatment for cancer is radiotherapy, which commonly involves the use of X-rays (photons). Recent advances in

technology, have led to the fusion of PET, CT scan and PET, MRI scan. Overall, these advancements had led to intensity-modulated radiotherapy (IMRT), image-guided radiotherapy (IGRT) and stereotactic radiosurgery for the treatment of cancer.

1.3.3 Personalised medicine

The treatment for primary or metastatic tumours, which are unresectable by surgery is chemotherapy, immunotherapy, hormonal or radiotherapy. However, treatment planning on a patient-to-patient basis, has led to the concept of personalised medicine. Personalised medicine targets the tumour based on its genetic makeup rather than its location in the patients. This is due to the heterogeneity in the progression of the tumour as well as the genetic makeup, which varies among individual patients. The recent advances in genomics, proteomics and imaging technologies has led to the understanding of the molecular basis of disease, biomarkers and drug response. Moreover, with the sequencing of human genome becoming cost-efficient, personalised medicine can be achieved for the diagnosis and treatment of the disease.

Clinically it is well known that every cancer patient is unique and the response towards treatment differs. The advent of recent technical improvements in the field of omic’s has a great impact on tumour molecular profiling. There are numerous reports from clinical trials indicating that cancer patients who were treated with anti-cancer drugs matching their genomic targets individually have a better outcome (Black & Morris, 2012; Hollebecque et al, 2013; Tsimberidou et al, 2012; Von Hoff et al, 2010). Currently, there are 54 potential drugs for use as personalised medicines to treat cancer. Trastuzumab (Herceptin) a recombinant monoclonal antibody is used in the treatment of HER2 positive breast and gastric cancers. Trastuzumab was one of the first drugs to be used in targeted

therapy in the treatment of metastatic breast cancers overexpressing HER2 (Slamon et al, 2001; Vogel et al, 2002).

1.4 Radiotherapy

Radiotherapy is given to nearly 50% of patients with cancer during their period of treatment either alone or in combination with other treatment modalities (Begg et al, 2011). Cancer patients receive radiation either for curative or for palliative treatment. Approximately 40% of patients receiving radiation are cured from their primary tumour and lymph node metastases (Barnett et al, 2009). Cancer patients who present with advanced tumours or metastatic tumours often have palliative radiation treatments to alleviate pain, reduce tumour size, spinal compression, headaches, visual problem, haematuria, hydronephrosis, to control bleeding and enhance the quality of life (Jones et al, 2014).

1.4.1 History of radiotherapy

The invention of X-rays by Roentgen led to the treatment of cancer patients with X-rays (Moullin, 1899). In the following years the discovery of electrons, natural radioactivity and also radium led to various developments in the delivery of radiation for treating cancer. Although, initially the advent of X-rays and radium were of much interest, it became obvious within a short period of time that exposure to higher or unwanted radiation doses could also cause cancer (Johnson, 1902; Moullin, 1899).

1.4.2 Modes of radiation delivery to cancer patients

Radiation can be delivered to target tumours either using X-rays or by inserting radioactive material for eg. Cesium (brachytherapy) or through the administration of

radioactive compounds orally or by injection (systemic radiotherapy). External beam radiotherapy is most commonly used clinically, in which the tumours are specifically targeted using high-energy ionising radiation (photons and heavy particles) (Kamada, 2012; Suit et al, 2010). Brachytherapy involves the delivery of radiation inside the body through sealed catheters or radiation seeds inserted at the sites of tumour. Brachytherapy is commonly used in the treatment of prostate and gynaecological tumours (Haie-Meder et al, 2011).

External beam radiotherapy has been around for more than a century but over the years has made a lot of improvement with the advancement in imaging and delivery systems. External beam radiotherapy can deliver either single or fractionated doses of radiation. The delivery of the doses is managed through linear accelerators (LINAC) for X-rays and electrons and by cyclotron for the delivery of protons, carbon or other heavy ion particles. The recent advancement and implementation of heavy particles have led to personalised radiotherapy in treating cancer patients. Particle therapy involving protons or carbon ions are being used to target tumours by delivering large doses of radiation with steep gradients by utilising the Bragg peak (Figure 1.5). In contrast to photons (X-rays or gamma rays), radiotherapy involving protons or carbon ions have the advantage of lower entry dose, minimal dose scatter when passing through the tissue and no exit dose following its dose deposition. Similar to photons, proton and carbon ion beams can be conformed to fit the tumour target volume with high precision and therefore improving local control. This is now largely possible due to the integration of multimodality scanning system like PET, CT and MRI scans and computer algorithms and various device systems integrated with the radiation delivery unit (Thariat et al, 2013).

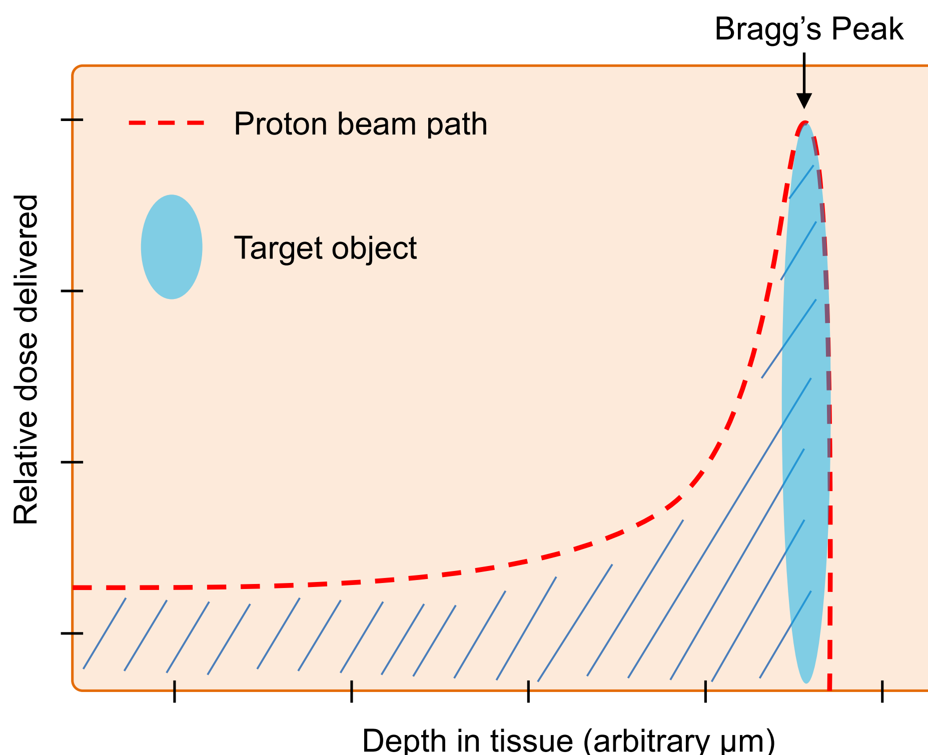

Figure 1.5 Depth-dose distribution of a heavy ion beam. An ionisation curve of a heavy ion proton beam depositing its maximum energy at an increased depth.

1.4.3 The 5 Rs of Radiation

The outcome of the tumour response to radiation is governed by the 5Rs, which are repair, repopulation, reassortment, reoxygenation and radiosensitivity. Radiation leads to DNA damage, which leads to a cellular requirement for DNA repair pathways, which are often compromised in tumours. Depending on the radiation dose the level of damage can be lethal (cells do not survive), sublethal (cells can survive) or potentially lethal (cell survival can be modulated post radiation). Unlike the tumour cells, normal cells have an intact repair mechanism to repair any sublethal damage which results from radiation (Hall & Giaccia, 2006). Therefore, the defective repair mechanism in tumour cells could be exploited by synthetic lethal methods. This synthetic lethality can be exploited in tumour

cells by targeting them with pharmacologically active compounds in combination with radiation (reviewed in Good & Harrington, 2013).

During treatment, cancer patients often receive fractionated radiotherapy. Radiation treatment is performed over a period of time by giving smaller fraction of total radiation doses. The period between each fraction of radiation dose allows normal cells to recover from the radiation-induced damage but at the same time, a certain number of cancer cells also recover. Following radiation, both the normal cells as well as cancer cells repopulate through cell division. The doubling time at which the irradiated cells repopulate depends on the type of tissue (Hall & Giaccia, 2006). The cellular repopulation is not only dependent on the tumour tissue type but also the host immune system, radiation fraction and dose schedule and secretion of growth factors in response to radiation (reviewed in Good & Harrington, 2013).

During radiotherapy, the cells are in different stages of the cell cycle. Cells in different cell cycle phases have different radiosensitivity. Cells in S-phase are more radioresistant whereas cells in the late G_2 and M phase are relatively sensitive (Seiwert et al, 2007). This distribution of radiation sensitivity throughout the cell cycle represents the reassortment in the 5Rs of radiobiology.

Cells present within a tumour contain a mixed proportion of both well oxygenated and hypoxic cells. During radiation treatment a single dose of radiation can kill cells, which are well oxygenated and to a certain extent the sub-population of cells in intermediate oxygen levels, sparing the hypoxic cells. This loss of well oxygenated cells due to radiation will lead to an increase in the proportion of hypoxic cells within the tumour. Under these circumstances, the surviving hypoxic cells may gain access to oxygen and become oxygenated. Following each fractionated radiation dose, the reoxygenated

hypoxic cells are killed leaving behind the hypoxic cells. This process of oxygenation of surviving hypoxic cells in response to radiation is termed as reoxygenation (Hall & Giaccia, 2006). Additionally, reoxygenation may occur when a large fraction of radiation is administered. Higher doses per fraction induces VEGF, triggering angiogenesis, which influences reoxygenation. The increased levels of VEGF protect the cells from radiation-induced apoptosis and therefore could contribute to the outcome of radiotherapy (Hovinga et al, 2005; Park et al, 2012).

Apart from repair, repopulation, redistribution and reoxygenation, the tumour cells have their own intrinsic radiosensitivity. The intrinsic radiosensitivity of the tumour cells depends on the steepness of the survival curve demonstrating the loss of reproductive ability of the cells due to early or late reactions in response to radiation. For determining intrinsic radiosensitivity, the survival fraction at 2 Gy (SF_2) is considered. The intrinsic radiosensitivity of tumour cells could demonstrate the clinical response of a tumour. An advantage of intrinsic radiosensitivity being a single parameter and it does not involve any assumptions of the mechanisms underlying the lethal effects of radiation. (Hall & Giaccia, 2006; Malaise et al, 1992; Steel et al, 1989; Szumiel, 2008).

1.4.4 DNA damage induced by radiation

Clinically, cancer patients undergoing radiotherapy are treated with high-energy ionising radiation such as X-rays, gamma rays, electrons, protons and other heavy ion particles. These high-energy particles when they enter into the cell have the ability to deposit their energies through ionisation or by releasing electrons on interaction with the molecules within the cell. This deposition of energy leads to a complex cluster of DNA damage directly or indirectly (Anderson et al, 2006; Goodhead, 1989). The DNA damage resulting from exposure to ionising radiation can lead to genomic instability and are

carried through to the progeny cells (Clutton et al, 1996; Kim et al, 2006). The level of damage inflicted determines the effectiveness of radiotherapy and also can have a role in radiation-induced carcinogenesis.

1.4.5 The DNA damage response

Cells are constantly exposed to various genotoxic insults, which induce different types of DNA damage. Clinically relevant doses of ionising radiation can induce 1000 SSBs, 40 DSBs and about 30000 damaged bases per Gray (Ward, 1994). These DSBs are known to be deleterious for the survival of the cells. Ionising radiation-induced DNA damage triggers a signalling cascade in order to repair the damaged DNA, control cell cycle arrest through various available checkpoint mechanisms and chromatin remodelling. Radiosensitivity is also determined by either deletion or loss of functional genes involved in DNA repair and processing of radiation induced DNA damage (for example ATM, ATR, MRE11, NBS1, BRCA1/2, Rad50, Ku70/80, DNA-PKcs, Rad51, Rad52, XRCC2, CHK1, CHK2, Wee1), the mutation of oncogenes (for example RAS, PTEN, p53) or patients with pre-disposed genetic syndromes (ATM, Blooms, Downs' syndrome, Fanconi's anaemia, Gardner, Li-Fraumeni, Nijmegen breakage syndrome, retinoblastoma, Wilm's tumour and Xeroderma pigmentosum) (Abbaszadeh et al, 2010; Beamish et al, 2002; Christensen et al, 2014; Clark et al, 2007; Ernestos et al, 2010; Lim et al, 2014; Mangoni et al, 2011; Neubauer et al, 2002; Petrovic et al, 2013; Williams et al, 2008b; Wilson et al, 2010).

In response to DNA damage, cells initiate repair mechanisms by delaying the progress of cell cycle by inducing checkpoints either before or during DNA replication (G_1/S or intra-S checkpoint) or during cell division (G_2/M checkpoint) to maintain genomic integrity (d'Adda di Fagagna et al, 2003; Gorgoulis et al, 2005). These checkpoint

mechanisms are regulated by the protein kinases ATM and ATR (Figure 1.6). Although, ATM and ATR phosphorylate the same cellular substrates, their response to DNA damage is distinct (Bakkenist & Kastan, 2003; Gamper et al, 2013; Myers & Cortez, 2006; Riballo et al, 2004). Once the checkpoints are activated the progression of cell cycle is delayed and the repair mechanism is initiated. The DSBs are repaired either by homologous recombination (HR) or non-homologous end joining (NHEJ). The decision between HR and NHEJ to repair the DBS is governed by the phase of the cell cycle (Escribano-Diaz et al, 2013; Kakarougkas et al, 2013; Karanam et al, 2012; Mao et al, 2008).

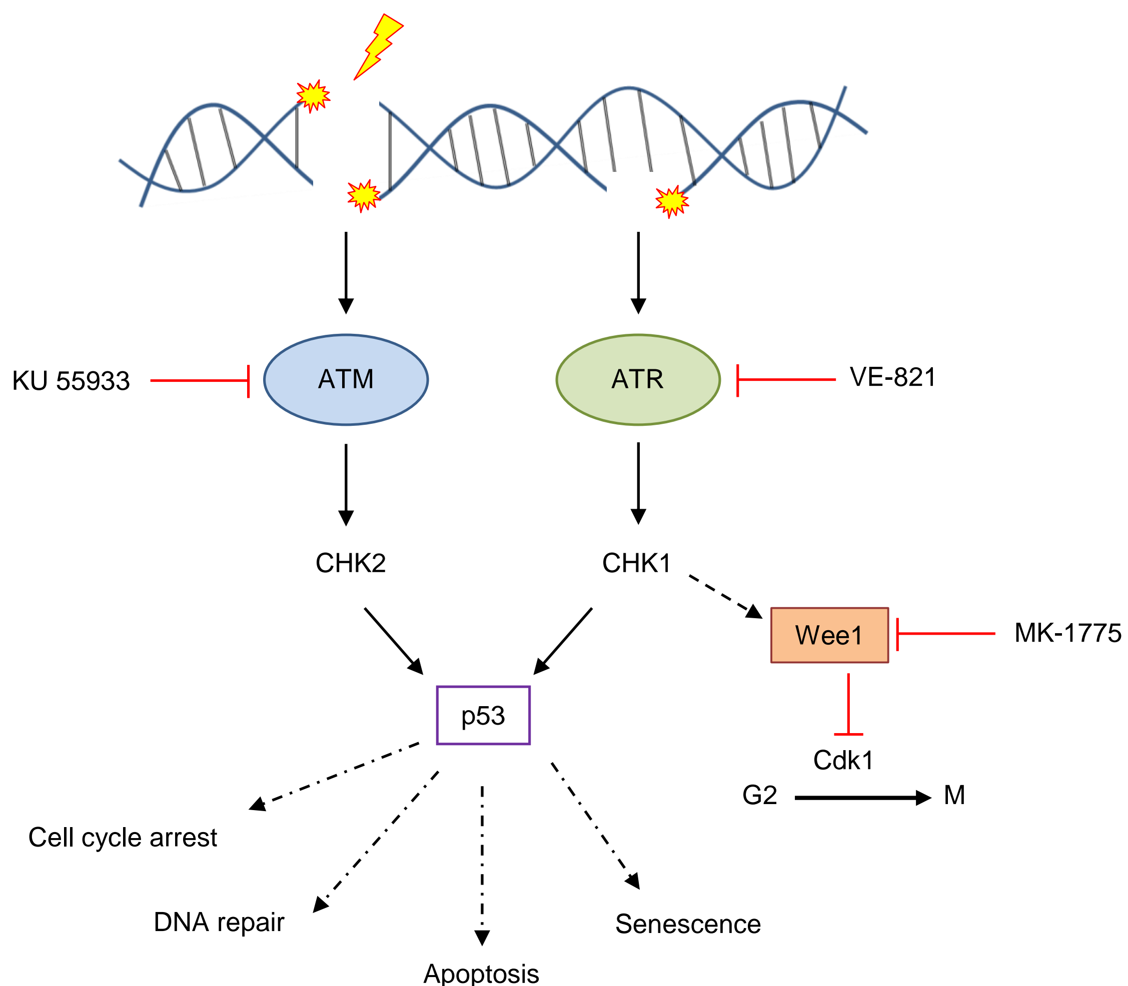

Figure 1.6 Radiation-induced ATM/ATR signalling. In response to ionising radiation cells initiate the DNA damage response pathway. Initially, the DNA damage sensors detect the occurrence of damaged sites. On detecting the sites of DNA damage, the repair machinery recruits ATM to the sites of DSB and ATR for SSBs. In response to ATM/ATR activation a cascade of downstream signalling is mediated by CHK1 and CHK2 with the

signalling converge to p53 and cell cycle phosphatases. As an outcome, cellular fate is decided by either triggering checkpoint mechanism, repair damaged DNA, entry into apoptosis, or senescence. In the scheme represented above the perpendicular end of the lines represents inhibitory effect whereas the arrows are positive regulators.

Ionising radiation-induced DSBs are sensed by ATM (Andegeko et al, 2001; Bakkenist & Kastan, 2003). In response to DSB, ATM phosphorylates a number of downstream targets involved in DNA damage response (DDR) including p53 (ser-15), Brca1, NBS1, Mdm2, Chk1, and Chk2 (Borges et al, 2004; Fabbro et al, 2004; Foray et al, 2003; Gannon et al, 2012; Gatei et al, 2003; Jazayeri et al, 2006; Melchionna et al, 2000; Momcilovic et al, 2009; Moumen et al, 2013; Zhao et al, 2008; Zhao et al, 2000). This signalling cascade decides on the cellular fate, an example being ionising radiation induced apoptosis. Radiation-induced apoptosis is initiated through ATM phosphorylation of CHK2. The activated CHK2 stabilises p53 through phosphorylation at serine residues 9 and 46 (required for apoptosis) (Saito et al, 2002). The stabilisation of phosphorylated p53 leads to accumulation inside the nucleus leading to transactivation of pro-apoptotic genes PUMA, BAX and NOXA (Kuribayashi et al, 2011; Oda et al, 2000; Toruno et al, 2014). In addition, the activated p53 regulates cellular fate by initiating cell cycle arrest or senescence (Chung et al, 2012; Delia et al, 2003; Wang et al, 2006; Webley et al, 2000). These protective mechanisms therefore safeguard the genomic integrity by determining that the mutations or the damage arising due to ionising radiation is not carried over to the progeny cells.

1.4.6 Limitations of radiotherapy

All treatments come with limitations, and radiotherapy is not exempt. The side effects that arise due to radiation largely depend on the part of body being exposed to radiation and the number of fractions and the total dose that has been delivered. The

severity of side effects depends on the area irradiated. Usually, after radiotherapy treatment the patients can experience skin erythema, tiredness, nausea, vomiting,, muscle or joint stiffness and hair loss. Radiotherapy can cause late effects leading to the development of fibrosis, sarcomas or ischemic heart disease (Bernstein et al, 2013; Darby et al, 2013; Lingos et al, 1991; Torres et al, 2013).

Radiotherapy induces clustered DNA DSBs and these DSBs can be lethal to the cells if they fail to initiate repair mechanisms (Lomax et al, 2013). Various genetic syndromes are known to have defective repair mechanisms (Alvarez-Quilon et al, 2014; Bakry et al, 2014; Chen et al, 2003; Donahue & Campbell, 2004; Langland et al, 2002; Lehmann, 2003). Therefore, cancer patients with pre-disposed genetic syndromes such as A-T, Blooms, Downs' syndrome, Fanconi's anaemia, Li-Fraumeni, Nijmegen breakage syndrome, and Xeroderma pigmentosum are not administered radiotherapy. The reason being that they cannot be administered the same conventional radiation dose, which is usually administered in the clinic as it could be lethal due to their defective repair mechanisms (Kawata et al, 2003).

One of the major limitations of radiotherapy is in targeting the tumour and delivering the maximum dose whilst sparing the normal tissues. The delivery of target dose has its own limitation due to the movement of the tumour. This tumour motion is largely dictated by the location of the tumour site. Tumours tend to move due to respiration, cardiac motion and gastrointestinal movement. Therefore, treatment planning becomes critical in dose delivery (Cole et al, 2014).

Another major drawback with radiotherapy is tumour relapse. As the tumour develops it often develops the ability to metastasis and therefore it is critical to treat the primary tumour in the initial stages. Unlike other molecular targeted therapies,

radiotherapy involves targeting of the radiation beam to a localised delineated tumour region. Tumours have the ability to metastasise and there is a higher probability that these metastases tumours could go unnoticed for radiotherapy. The other probability being that there could be radio-resistant subpopulation, which has the potential to metastasise post treatment (Lizarraga et al, 2014).

1.4.7 Radiotherapy and Hypoxia

Tumour cell repopulation and tumour hypoxia are two critical factors that determine the outcome of radiotherapy in solid tumours. The presence of oxygen determines the effect of radiation on tumour cells (Rockwell et al, 2009). This dependence on the level of oxygen is termed the oxygen enhancement ratio (OER). The OER is defined as the ratio of dose under hypoxia to well-aerated conditions necessary to produce the same biological effect. Tumour cell lines irradiated using low linear-energy-transfer (low LET) like X-rays and gamma rays have an increased OER (2.5 – 3.0) (Figure 1.7), with intermediate ionising radiation particles like neutrons the OER = 1.6 and for high LET radiation like alpha-particles results in OER = 1 indicating no significant effect (Wenzl & Wilkens, 2011). LET is the rate at which energy is lost by an ionising particle as it passes through the soft tissue.

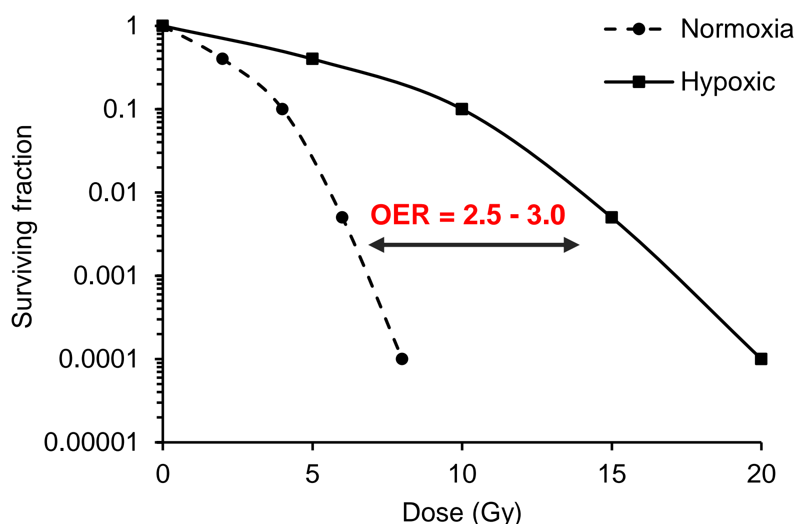

Figure 1.7 A graphical representation of oxygen enhancement ratio (OER). A schematic representation of a cell survival curve demonstrating the presence of oxygen increases radiation-induced cell death. The ratio of radiation doses between the hypoxic cells to that of oxygenated cells to produce an identical biological effect is termed the OER.

The difference in OER between the various LET radiations is due to the oxygen effect. For maximum effect oxygen must be present either during or within milliseconds of radiation delivery. When cells are exposed to radiation, the charged particles ionise the biomolecules in the cells resulting in free radicals. These free radicals are highly reactive molecules having the potency to disrupt chemical bonds in DNA. These free radicals induce damage to the DNA through the formation of peroxides due to the presence of oxygen. However, in the absence of oxygen, peroxides are not formed and the reduced biomolecules are restored from donor hydrogen in the thiol group present in cysteine and/or glutathione (Bertout et al, 2008).

The OER response of cells to the same radiation dose when exposed to different types of LET radiation is not the same. The energy deposited by the type of LET radiation largely determines the effect of cell kill. To determine the same biological effect for a specific dose of radiation irrespective of its LET, relative biological effective (RBE) dose is taken into consideration. RBE is a ratio of dose from 250 kV X-ray to that of the dose

from another source of LET radiation that produces the same biological effect (Hall & Giaccia, 2006).

1.4.8 Radiosensitisers

To target the hypoxic fractions of tumours a number of approaches have been investigated including, hyperbaric oxygenation therapy, blood transfusion and the use of substances such as perfluorocarbons to increase oxygen supply to the tumour during radiotherapy (Koch et al, 2002; Ogawa et al, 2012; Santin et al, 2003). Radiosensitisers are classified into three categories hypoxic cell radiosensitisers that specifically target the hypoxic tumour cells, bioreductive drugs, which become cytotoxic under hypoxic conditions and molecular radiosensitisers targeting specific molecular pathways (Table 1.1).

Type of radiosensitiser	Examples	References
Hypoxic radiosensitiser	Hyperbaric oxygen, nimorazole	Metwally et al, 2014; Ogawa et al, 2012
Bioreductive drugs	PR-104, TH-302, EO9	Hendricksen et al, 2012; McKeage et al, 2012; Takakusagi et al, 2014
Molecular targeted radiosensitiser	Temozolomide, cetuximab, POLQ siRNA	Higgins et al, 2010; Miqueli et al, 2009; Stupp et al, 2009

Table 1.1 The three classes of radiosensitisers. The three different classes of radiosensitisers and their examples along with references are listed.

A classic radiosensitiser increases the efficacy of radiation-induced lethality to the tumour while having no effect as a single agent. The radiosensitisation effect is largely governed by spatial cooperation, cytotoxic enhancement, biological cooperation, temporal modulation and normal tissue protection (Katz et al, 2009). Targeting the hypoxic cells in

a tumour is a challenge due to limited blood vessels and supply of oxygen. Since 50% of patients treated for cancer receive radiation during their treatment, using a hypoxic radiosensitiser or bioreductive drug or molecular targeted radiosensitiser along with radiation could enhance the radiation-induced damage.

1.5. Mechanism of Radiosensitisation

Radiation induces damage to various subcellular components. The extent of damage caused by radiation alone depends on its dose, the energy of the radiation particle, its proximity to the DNA, presence of intracellular free radical scavengers and the efficiency of DNA repair pathways within the cells. The use of radiosensitisers along with radiation can inhibit tumour cell progression through cell cycle arrest, target a tumour specific repair pathway or increase DNA damage to the genome. The DNA lesions that arise as a result of radiation alone or in combination with the radiosensitisers triggers the DNA damage response (DDR). DNA damage is repaired either by single-strand annealing, cross link repair, nucleotide excision repair, base excision repair, mismatch repair, non-homologous end joining (NHEJ), or homologous recombination. The initiation of these repair mechanism is not only dependent on the type of lesion but also on the phase of the cell cycle (Chaudhry, 2007; Escribano-Diaz et al, 2013; Frankenberg-Schwager et al, 2009; Karanam et al, 2012; Parlanti et al, 2007; Sarkar et al, 2006; Schroering et al, 2007; Vrouwe et al, 2011).

1.5.1 Base excision repair (BER)

Simple DNA lesions such as oxidation, alkylation and single stranded breaks are repaired by BER. BER involves the removal of damaged bases by the DNA glycosylases

followed by trimming of the abasic site by apurinic endonuclease (APE1). The resulting 3'-OH acts as a substrate for DNA polymerase β and the newly synthesised complementary bases are ligated by DNA ligase. PARP1 and XRCC1 act as scaffolding proteins and are important in coordinating the repair mechanism (Wallace, 2014).

1.5.2 Nucleotide excision repair pathway (NER)

The NER pathway is involved in the repair of lesions that arise as a result of exposure to UV rays (cyclobutane-pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts), chemical adducts, intrastrand crosslinks (for example chlorambucil, mitomycin C, nitrous acid, platinum drugs) and ROS generated cyclopurines. These lesions are detected by global genome NER (occurs due to structural distortion in DNA helix) or transcription-coupled NER (lesions block the RNA polymerase II activity). For GG-NER, XPA binds to the site of lesion and recruits other proteins XPB and XPD, whereas for TC-NER, the proteins CSA and CSB are recruited. Following the recruitment of the proteins XPB, XPD and CSA, CSB, the transcription factor-IIH resolves these damaged sites, initiating the incision by the XPF-endonucleases. The activity of endonuclease is coordinated in parallel with the synthesis and ligation of DNA by the replication machinery proteins PCNA, RFC, DNA Pol δ, DNA Pol ε and DNA Pol κ and DNA ligase (reviewed in Marteijn et al, 2014).

1.5.3 Non-homologous end-joining (NHEJ)

DSBs arising in the cells due to endogenous damage or genotoxic stress are commonly repaired by either NHEJ or homologous recombination (HR). NHEJ is a repair pathway that is most commonly implemented by the cells to repair DNA DSBs, irrespective of the cell cycle phase. NHEJ does not require any sequence homology, although for the initial recruitment of the NHEJ repair enzymes require terminal

microhomology. The NHEJ machinery comprises of Ku70/80 (loads to the sites of damage), DNA PKcs (phosphorylates Artemis), Artemis (nuclease activity), DNA pol μ, λ (DNA template-independent synthesis), XLF (initiates DNA ligase IV), XRCC4 (stabilises DNA ligase IV) and DNA ligase IV (ligates the DNA ends) (reviewed in Deriano & Roth, 2013).

1.5.4 Homologous recombination (HR)

Homologous recombination is one of the major mechanisms for repairing DBSs. Unlike NHEJ, HR is active only in S-phase and G_2 phase of the cell cycle. HR is an error free mechanism as it makes use of the intact sister chromatid as a template to restore the genetic sequence. This repair process involves the resection of broken ends nucleolytically leading to formation of single stranded overhangs of DNA. RAD51 a recombinase protein binds to these single stranded DNA structures giving rise to the presynaptic complex. Following the formation of presynaptic complex, the single stranded overhangs invades into the homologous sequence and subsequently DNA synthesis is processed at the invading ends resulting in D-loop formation. This D-loop structure is resolved either by synthesis-dependent strand annealing leading to a non-crossover product or through the formation of Holliday junctions. The Holliday junctions are resolved either by HJ resolvase into crossover product or by the action of helicase-topoisomerase complex to give rise to a non-crossover product (reviewed in Daley et al, 2014).

1.6 Radiation-induced cell death

One of the primary end points of radiation is cell death. Radiosensitisers are known to enhance radiation-induced cell death (Table 1.2). Radiation-induced cell death could

trigger apoptosis, autophagy, necrosis, mitotic catastrophe and senescence. The mode of radiation-induced cell death is dependent on various factors such as exposure to radiation dose, oxygen tension, mutational status of oncogenes, DNA repair efficiency, gene signature, cell cycle and type of cells (Cengel et al, 2007; Deschavanne & Fertil, 1996; Ernestos et al, 2010; Golden et al, 2014; Hall et al, 2014; Hinz et al, 2005; Lee et al, 2004; Overgaard, 2007; Rieckmann et al, 2013; Rothkamm et al, 2003; Tamulevicius et al, 2007; Thangavel et al, 2014).

Radiosensitisers	Mechanism of radiosensitisation	Reference
Hyperbaric oxygen	Generation of reactive oxygen species and nitrogen	Ogawa et al, 2012
Hyperthermia	Heat-induced inhibition of DNA repair leading to mitotic catastrophe	Horsman & Overgaard, 2007; Mackey & Ianzini, 2000
Nicotinamide with carbogen	Increases dissolved oxygen volume in plasma and blood flow	Janssens et al, 2012
Nimorazole	Depletion of nucleophiles and formation of toxic products	(Thomson et al, 2014)
Mitomycin-C	DNA crosslinker	Thind et al, 2014
Tirapazamine	Produces oxidising free radicals	von Pawel et al, 2000
Fluoropyrimidine	Deregulates S-phase checkpoints	Beveridge et al, 2012
BrdU, IdU	DNA/RNA crosslinkers, DNA repair inhibition	Wang & Lu, 2010
Gemcitabine	Inhibits cells in their S-phase	Loehrer et al, 2011
Motexafin gadolinium	Redox modulator	Bradley et al, 2013
Taxanes (paclitaxel, docetaxel)	G_2/M arrest	Brunsvig et al, 2005; van Meerten et al, 2006
Irinotecan	DNA Topoisomerase I inhibitor	Kubota et al, 2014
Temozolomide	G_2/M arrest and apoptosis	Stupp et al, 2009
PARP inhibitors	Inhibits DNA repair	Senra et al, 2011
EGFR and kinase inhibitors (cetuximab, gefitinib, vandetanib, erlotinib)	Inhibits kinases which are involved in DNA repair mechanism or necessary for cellular metabolic signalling pathways	Bonner et al, 2006; Drappatz et al, 2010; Iyer et al, 2013; Joensuu et al, 2010
Chloroquine	Inhibits autophagy	Firat et al, 2012
Temsirolimus (CCI-779), everolimus (RAD001), gossypol (AT-101)	Autophagy enhancer	Moretti et al, 2010; Sarkaria et al, 2010; Sarkaria et al, 2011
Resveratrol (natural component found in grapes and red wine)	Senescence	Luo et al, 2013

Table 1.2 A list of radiosensitisers which are either in clinical trials or in use for cancer treatment. Different types of radiosensitisers, their mechanism of radiosensitisation and references.

1.6.1 Apoptosis

Apoptosis is a highly regulated programmed cell death pathway, which is involved in embryonic development and tissue homeostasis. Cells undergoing apoptosis are characterised by cytoplasmic blebbing, fragmentation of DNA and nuclear condensation leading to cell death. Deregulation of the apoptotic machinery can lead to development or progression of cancer. In response to radiation, apoptosis can occur primarily through the activation of p53 (intrinsic pathway) but depending on the context of the environment can also occur through the activation of death receptor (extrinsic pathway) or by ceramide production (membrane stress pathway) (Ch'ang et al, 2005; Inagaki-Ohara et al, 2001; Lu et al, 2012a; Mesicek et al, 2010; Verbrugge et al, 2008)

1.6.2 Necrosis

In addition to apoptosis, high radiation doses induce necrosis. Necrotic cells have characteristic organelle swelling, dysfunctional mitochondria and permeabilised plasma membranes (Hotchkiss et al, 2009). Necrosis is not mediated by either the caspase, BCL-2 family of proteins or cytochrome c, the key regulators of apoptosis. However, necrosis is triggered by the death receptors (belonging to the tumour necrosis factor receptor superfamily), which are also known to initiate apoptosis. The Fas-associated death domain (FADD) decides on the fate of cells by initiating the caspases leading into apoptosis or mediates the activation of receptor interacting protein 1 (RIP1) for necrosis (Degterev et al, 2008; Holler et al, 2000).

1.6.3 Mitotic catastrophe

Mitotic catastrophe is another form of non-apoptotic cell death initiated in response to ionising radiation in solid tumours. Cells undergoing mitotic catastrophe are characterised as large cells containing multiple nuclei. Mitotic catastrophe occurs in tumour cells with defective checkpoint mechanisms leading to aberrant mitosis and eventually generation of aneuploidy progeny and cell death (Vitale et al, 2011). Mitotic catastrophe can occur through the inhibition of proteins involved in the G_2 checkpoint mechanism (ATM, ATR, Chk1, Chk2 and p21) and therefore increase radiosensitivity (Moding et al, 2014; On et al, 2011; Riesterer et al, 2011; Sarcar et al, 2011; Weston et al, 2010).

1.6.4 Senescence

Another characteristic form of ionising radiation-induced cell death is senescence. Senescence is a condition in which the cells undergo permanent cell cycle arrest. Senescent cells remain metabolically active even with loss of replicative potential. The senescent cells expresses senescence associated markers for example β-galactosidase (Sabin & Anderson, 2011). The activation of p53 due to genotoxic stimuli like radiation induces the activity of p21, p16, and inhibits transcription of genes involved in cell cycle progression and therefore the cells stop proliferating (di Fagagna et al, 2003). Radiation-induced chromosomal abnormalities include end-to-end fusions, a trait of telomere dysfunction. Cells with telomere dysfunction enter into senescence and this is tightly regulated by p53 (Jones et al, 2005).

1.7 Autophagy

Radiation induces damage not only to DNA but also to the cell membrane, proteins, lipids and various sub-cellular organelles leading to cellular stress and the cells counteract this stress through various mechanisms. Autophagy is one such mechanism, which is initiated in response to stress. Unlike radiation-induced apoptosis which leads to cell death, radiation-induced autophagy can be either pro-survival or pro-death depending on the context or environment (Chaachouay et al, 2011; Ito et al, 2005; Kim et al, 2011a; Paglin et al, 2001; Yao et al, 2003). Furthermore, inhibition of autophagy increases radiosensitivity but in contrast, studies by various groups have also demonstrated that promotion of autophagy can increase radiosensitivity (Altmeyer et al, 2010; Apel et al, 2008; Bristol et al, 2013; Chaachouay et al, 2011; Chiu et al, 2010; Firat et al, 2012; Fujiwara et al, 2007; Gewirtz et al, 2009; Kim et al, 2009; Ko et al, 2014; Kuwahara et al, 2011; Lin et al, 2010; Rajecki et al, 2009).

1.7.1 Autophagic flux

Autophagy involves degradation and recycling of sub-cellular organelle and proteins through highly regulated autophagic machinery. Regulation of the autophagic machinery involves various signalling pathways in response to various cellular and environmental stimuli. A characteristic of the autophagic machinery is the formation of autophagosomes, which are double-layered membrane vesicles. The autophagosomes are formed due to elongation of phagophores leading to sequestration of cytoplasm along with sub-cellular organelles or proteins (Nakatogawa et al, 2007; Nath et al, 2014; Sawa-Makarska et al, 2014). The phagophores are membrane structures which arise from endoplasmic reticulum (ER), plasma membrane, mitochondria or ER-mitochondria contact site (Axe et al, 2008; Hailey et al, 2010; Hamasaki et al, 2013; Hayashi-Nishino et al,

2009; Ravikumar et al, 2010). The autophagosomes fuse with late endosomes to form the amphisomes, which subsequently fuse with lysosomes to form the autolysosomes (Figure 1.8). These fusion events are highly regulated and mediated by Rab7, ESCRTs (endosomal sorting complexes required for transport), SNAREs proteins (N-ethylmaleimide-sensitive factor-attachment protein receptors), class C Vps, small GTPase Rab7 and HOPS (homotypic fusion and vacuole sorting protein) (Jager et al, 2004; Jiang et al, 2014; McCullough et al, 2013). The overall process involving the transformation of autophagosomes to autolysosomes is defined as autophagic flux (Castillo et al, 2013).

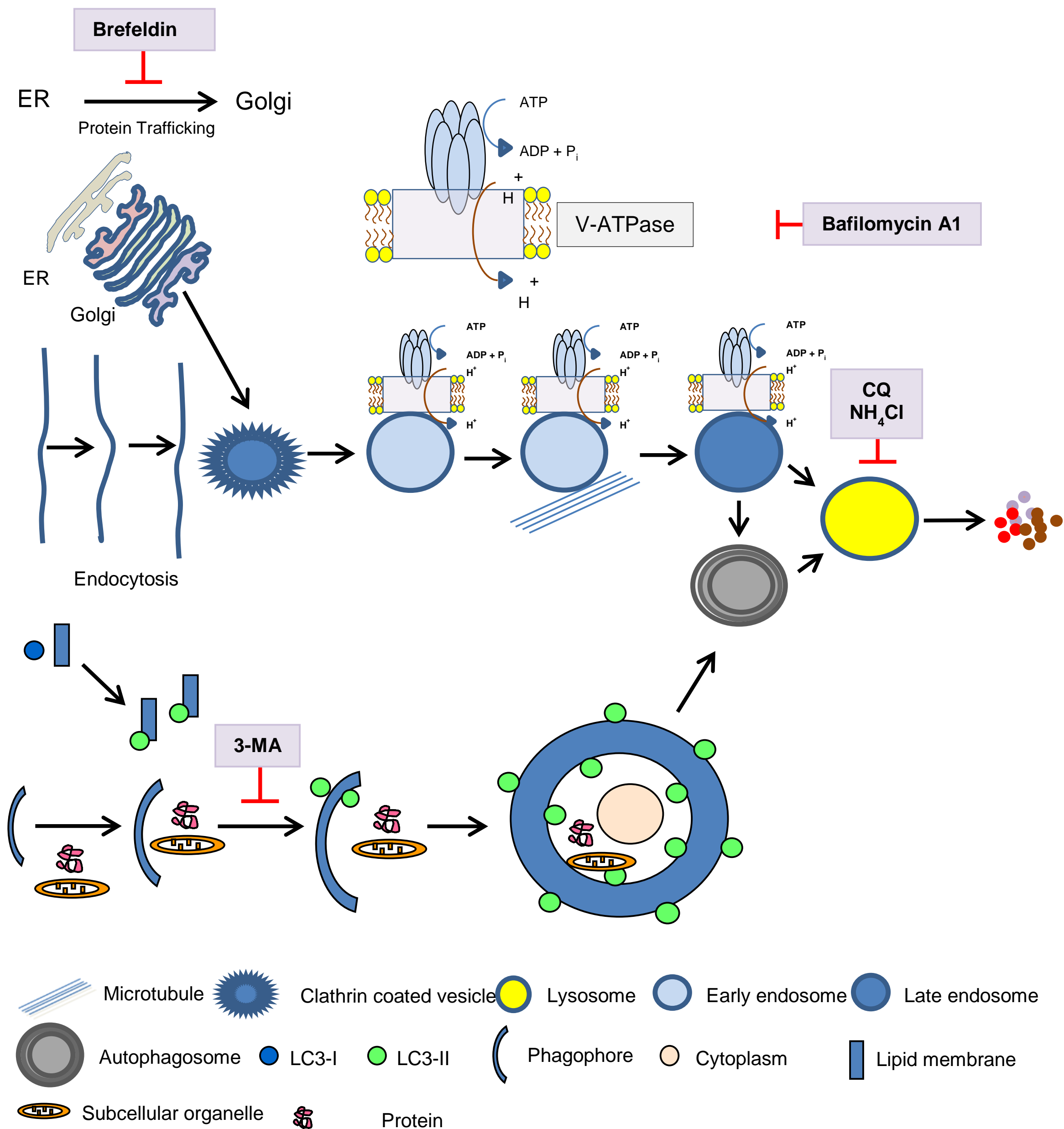

Figure 1.8 A schematic representation of autophagic flux. Autophagy involves the degradation of various subcellular organelles. A characteristic of autophagy is the formation of autophagosome a double layered membrane which subsequently fuses with lysosomes for endolytic degradation. The progress of autophagosomes transforming into autolysosomes is termed autophagic flux. Autophagic flux can be assessed by measuring the levels of LC3 with different inhibitors targeting a specific stage of autophagy as indicated in this scheme. In the scheme represented above the perpendicular end of the lines represents inhibitory effect whereas the arrows are positive regulators.

1.7.2 Types of autophagy

Autolysosomes are involved in the degradation of sub-cellular organelles and proteins into simpler biomolecules by the action of hydrolases present in the lysosome. These degraded biomolecules are further assimilated for various cellular processes. The process of sequestration and degradation by the autophagic machinery can either be non-selective or selective. Non-selective autophagy involves random sequestration of cytoplasmic structures by the autophagosomes formed as a result of basal autophagy (Hara et al, 2006; Nakai et al, 2007). However, selective autophagy is dependent on autophagy cargo-specific receptors which facilitate the sequestration of the cargo into autophagosomes (Table 1.3) (Birgisdottir et al, 2013; Kirkin et al, 2009; Lamark et al, 2009; Lin et al, 2013; Mancias et al, 2014).

Selective autophagy	Sequestered targets by autophagosomes for lysosomal degradation	Reference
Mitophagy	mitochondria	Saita et al, 2013
Pexophagy	peroxisomes	Mao et al, 2014
Lipophagy	lipids	Singh et al, 2009
Reticulophagy	endoplasmic reticulum	Ogata et al, 2006
Ribophagy	ribosomes	Kraft et al, 2008
Xenophagy	intracellular pathogens	von Muhlinen et al, 2012
Aggrephagy	protein aggregates	Thomas et al, 2013
Zymophagy	secretory granules	Grasso et al, 2011

Table 1.3 Selective autophagy and their targets for lysosomal degradation. The table depicts different types of autophagy and their target specificity for lysosomal degradation.

1.7.3 Autophagy regulation by cell signalling pathways

Autophagy is regulated by multiple cellular signalling pathways. The autophagic signalling cascade is classified as mTOR-dependent (nutrient, growth factor, stress and energy sensing) or mTOR-independent pathway (inositol signalling, $Ca2^{+}$/calpain, cAMP, JNK1, small molecules) (Figure 1.9). Depending on the context of autophagic induction, mTOR plays an important role. The serine/threonine protein kinase mTOR complex (nutrient sensing kinase) is a negative regulator of autophagy. The mTOR complex consists of two subunits, mTORC1 and mTORC2. mTOR inhibitors, such as rapamycin and analogues, have the potential to inhibit the activity of mTORC1 and induce autophagy. The autophagic machinery depends on the action of several autophagy-related (Atg) proteins. The initial steps of autophagy involve the coordination of two separate ubiquitin-like conjugation system. The first system being the conjugation of Atg5-Atg12 which is mediated by Atg7 (E1, ubiquitin-activating enzyme-like) and Atg10 (E2, ubiquitin-conjugating enzyme-like). The Atg5-Atg12 complex conjugates with Atg16L1 to form a larger complex involved in the phagophore elongation until the formation of autophagosomes (Matsushita et al, 2007; Romanov et al, 2012). In the second system, the conjugation of microtubule associated protein 1 light chain 3 (LC3-I) to phosphatidylethanolamine (PE) is mediated by Atg7 (E1-like) and Atg3 (E2-like) resulting in LC3-II which is bound to the autophagosomes (Kabeya et al, 2004). The formation of LC3-1-PE complex can occur as a result of cross talk between the two systems through the E3 enzyme-like activity of Atg12-Atg5-Atg16L1 complex and localises LC3-II to the autophagosomal membrane (Fujita et al, 2008). Subsequently LC3-II binds with p62/SQSTM1 (polyubiquitin-binding protein) to the autophagosomes, for cargo selection and subsequent degradation by fusion with lysosomal compartment (Bjorkoy et al, 2005; Pankiv et al, 2007).

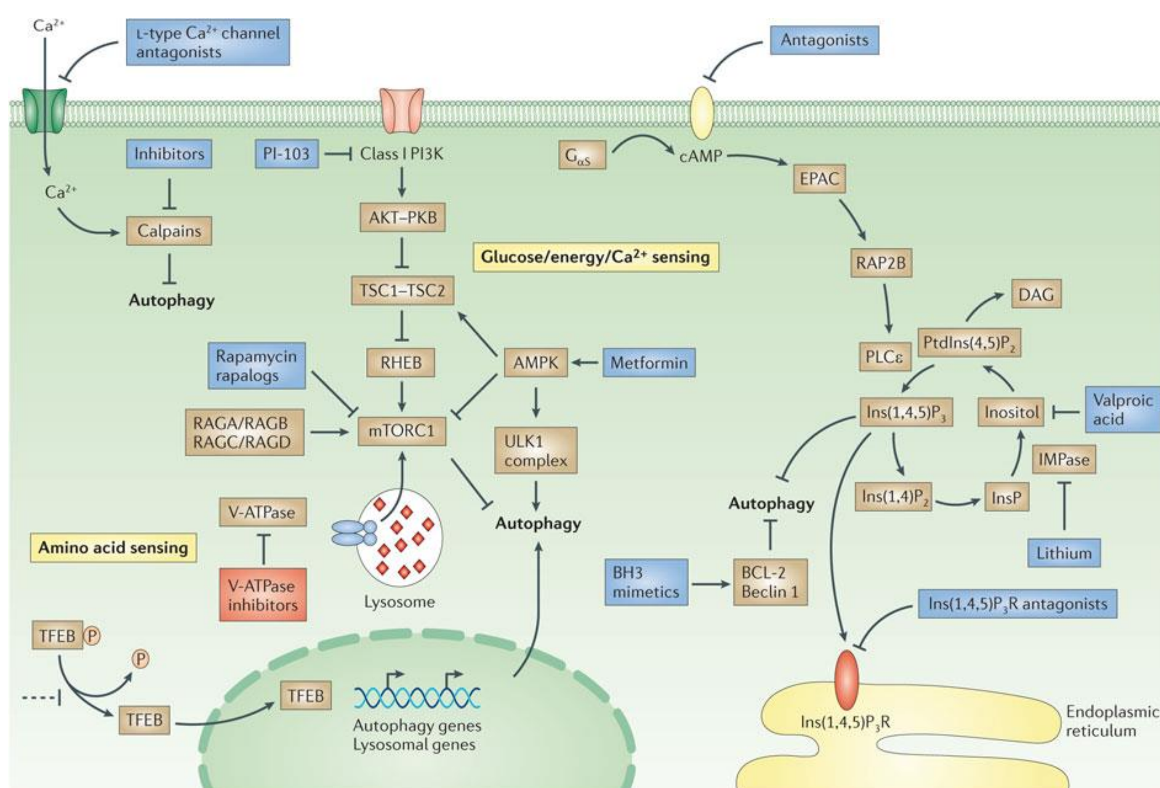

Figure 1.9 Regulation of Autophagy.
Representation of various mechanisms involved in the regulation of autophagy that can be mTOR dependent or independent of mTOR activity. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery (Rubinsztein et al, 2012), copyright 2012.

1.7.4 Autophagosomes biogenesis

The initiation of autophagosomes biogenesis requires the ULK kinases (ULK1, 2; unc-51-like kinase). ULK1, 2 is activated in response to nutrient starvation or mTOR inhibition. Although, ULK1 and ULK2 share 78% homology in their protein kinase domain, the role of ULK1 is critical in the regulation of autophagy (Lee & Tournier, 2011). ULK1 forms a stable complex with ATG13, ATG101 and FIP200 (focal adhesion kinase family-interacting protein of 220 kDa) (Ganley et al, 2009). The following step involves the vesicle nucleation of autophagosomes by class III PI3K (hVps34, a mammalian vacuolar protein sorting 34 homologue) and the integral membrane protein

Atg9 (Puri et al, 2014). The lipid kinase hVps34 is a part of the autophagy-initiating complex containing Beclin-1, Atg14L and hVps15. The interaction of hVps34 with Beclin-1 enhances the phosphorylation of phosphatidylinositol to phosphatidylinositol-3 phosphate (PtdIns3*P*), which are important for the autophagosomes biogenesis (Axe et al, 2008; Furuya et al, 2005).

1.7.5 Maturation of autophagosomes

Cells when exposed to conditions of starvation or treatment with mTOR inhibitors leads to activation of ULK1. The activated ULK1 phosphorylates Beclin-1 at Ser-14 residue enhancing the Atg14L-Vps34 complex inducing autophagy (Russell et al, 2013). Apart from this the binding of GTPase Rab5 to Beclin-1 and hVps34 complex is also known to enhance the induction of autophagy (Ravikumar et al, 2008). The Beclin-1 interacting proteins ATG14L, Ambra (activating molecule in Beclin-1-regulated autophagy), UVRAG (UV irradiation resistance-associated gene) and Bif-1 (endophilin B1) promote the maturation of autophagosomes, whereas binding of Rubicon, Bcl-2 (B-cell lymphoma 2), Bcl-X_L and Bim negatively regulate the maturation of autophagosomes (Fimia et al, 2007; Liang et al, 2008; Luo et al, 2012; Matsunaga et al, 2009; Pattingre et al, 2005; Takahashi et al, 2007).

1.7.6 Role of AMPK/TSC/mTOR in the regulation of autophagy

In conditions with compromised energy and stress the AMPK/TSC pathway is initiated. Cellular uptake of nutrients is converted into energy through the glycolytic pathway or by mitochondrial respiration and stored in the form of ATP. AMPK (energy sensing kinase) is activated when there is a decreased level of ATP/AMP (reviewed in Hardie et al, 2012). Metabolic stress leads to the phosphorylation of AMPK by LKB1 (a serine/threonine kinase). The activated AMPK phosphorylates Rheb (Ras homologue

enriched in brain) and Raptor (regulatory-associated protein of mTOR, co-subunit of mTORC1), which dephosphorylates mTOR. This dephosphorylation of mTOR leads to its inactivation and therefore dissociates from ULK1. In addition, AMPK phosphorylates serine 317 and 777 of ULK1 leading to the initiation of autophagosomes formation (Ganley et al, 2009; Kim et al, 2011b). Furthermore, AMPK directly phosphorylates TSC2 (tuberous sclerosis protein 2), which regulates mTOR by inhibiting its subunit mTORC1, therefore inducing autophagy (Inoki et al, 2006; Inoki et al, 2003).

1.7.7 Role of growth factors in the regulation of autophagy

Growth factors such as insulin are known to regulate autophagy by activating the PI3KC1a complex. Activation of PI3KC1a complex leads to accumulation of PtdIns(3,4,5)*P*3 which recruits Akt and 3-phosphoinositide-dependent kinase 1 (PDK1) to the membrane. PDK1 phosphorylates Akt at the Thr-308 residue and mTORC2 phosphorylates at Ser-473 (Najafov et al, 2012). Activated Akt phosphorylates mTOR as well as TSC2. Akt mediated phosphorylation of TSC2 enhances the binding of Rheb to mTORC1 resulting in the inhibition of autophagy (Cai et al, 2006; Zhang et al, 2003). PTEN (phosphatase and tensin homolog deleted on chromosome 10) a phosphatase inhibits the PI3KCIa/AKT/mTOR pathway by dephosphorylating PtdIns(3,4,5)*P*3 in insulin-dependent cell signalling, leading to the activation of autophagy (Errafiy et al, 2013; Ueno et al, 2008). FoxO3 (forkhead box O3), a nuclear transcription factor, is one of the downstream effector targets of activated Akt. FoxO3 is involved in the transcriptional regulation of the autophagosomes proteins LC3, Bnip3 (Bcl-2/adenovirus E1B 19kDa-interacting protein), Vsp34 and ULK1 (Mammucari et al, 2007; Warr et al, 2013; Zhao et al, 2007).

1.7.8 Radiation induced autophagy

In response to DNA damage, FoxO3 detaches from the DNA and interacts with ATM regulating DNA repair (Tsai et al, 2009). Both ATM and FoxO3 have been shown to have a role in the regulation of autophagy when cells are exposed to genotoxic and oxidative agents (Mammucari et al, 2007; Tripathi et al, 2013; Warr et al, 2013). Activation of ATM inhibits the activity of mTORC1 through the AMPK/TSC2 pathway inducing autophagy whereas in response to radiation-induced DNA damage, autophagy is regulated through the MAPK14 pathway (Alexander et al, 2010; Liang et al, 2013). Ionising radiation has been demonstrated to induce autophagy in certain cancer cell lines but the mechanism of autophagic induction has not been studied extensively (Bristol et al, 2012; Chaachouay et al, 2011; Ito et al, 2005; Kuwahara et al, 2011; Lomonaco et al, 2009). Independent of p53's classical function it is also involved in the induction of autophagy through the activation of AMKP, PTEN and DRAM (damage-regulated autophagy modulator) but contradictory studies from Kroemer's group have reported that deletion of p53 can lead to induction of autophagy (Crighton et al, 2007; Tasdemir et al, 2008a; Tasdemir et al, 2008b).

Radiotherapy is an effective and generally well-tolerated method of cancer therapy. However, as discussed several limitations occur including both tumour hypoxia and the need to reduce damage to surrounding normal tissues. The development of radiosensitisers, which are designed to specifically target hypoxic cells, is an ideal approach (Cazares-Korner et al, 2013). Most importantly, it is paramount that novel approaches to radiosensitise tumours are tested in conditions, which mimic the tumour microenvironment and in particular tumour hypoxia.

AIM: The aim of this thesis was to investigate the possibility of using either inhibitors of the DDR or compounds known to increase autophagy as novel means of radiosensitising tumour cells *in vitro*.

Chapter 2

Materials and Methods

2.1 Cells and Culture conditions

2.1.1 Cell maintenance

Cells were grown in T75 flasks (Corning) and when confluent were passaged by rinsing with sterile PBS followed by treatment with Trypsin-EDTA (Sigma-Aldrich, T3924) in normal tissue culture conditions (37°C in humidified air containing 5% CO_2). Trypsinised cells were resuspended with culture media as listed in the Table 1 containing either Dulbecco's Modified Eagle's Medium, DMEM (Sigma-Aldrich, #D5796) or Roswell Park Memorial Institute medium, RPMI (Sigma-Aldrich, #1640) supplemented with 10% Foetal Bovine serum (Sigma-Aldrich), 100 units/ml Penicillin, 100 µg/ml Streptomycin (Sigma-Aldrich P4333), and cells were seeded at the required density. Cells were maintained under normal tissue culture conditions at 37°C in humidified air containing 5% CO_2.

Cell line	Tissue	Disease	Origin	Culture media
786-O and 786-O/VHL	Renal	Adenocarcinoma	Gift from Prof. Peter Ratcliffe, Univ. of Oxford	DMEM
A549	Lung	Carcinoma	ATCC	DMEM
DLD1	Colon	Adenocarcinoma	Gift from Dr. Fred Bunz, Baltimore	DMEM
FLO-1	Oesophageal	Adenocarcinoma	Gift from Dr. Ricky Sharma, University of Oxford	DMEM
H1299	Lung	Carcinoma	ATCC	DMEM
HCT116	Colon	Carcinoma	CRUK	DMEM
HeLa	Cervix	Adenocarcinoma	ATCC	DMEM
IGR39	Skin	Melanoma	Gift from Dr. Eric O'Neill, Univ. of Oxford	DMEM

MDA-MB-231	Breast	Adenocarcinoma	Gift from Dr. Amanda Coutts	DMEM
MDA-MB-468	Breast	Adenocarcinoma	Gift from Dr. Eric O'Neill, Univ. of Oxford	DMEM
OE21	Oesophageal	Squamous Carcinoma	Gift from Dr. Ricky Sharma, University of Oxford	RPMI
OE33	Oesophageal	Carcinoma	Gift from Dr. Ricky Sharma, University of Oxford	RPMI
RCC4 and RCC4/VHL	Renal	Carcinoma	Gift from Prof. Peter Ratcliffe, Univ. of Oxford	DMEM
RKO	Colon	Carcinoma	ATCC	DMEM
RT-112	Bladder	Carcinoma	Gift from Dr. Anne Kiltie, Univ. of Oxford	RPMI
SKmel28	Skin	Melanoma	Gift from Dr. Eric O'Neill, Univ. of Oxford	DMEM
SQ20B	Head & neck	Squamous carcinoma	Gift from Dr. Eric O'Neill, Univ. Of Oxford	DMEM
U2-OS	Bone	Osteosarcoma	Gift from Dr. Eric O'Neill, Univ of Oxford	DMEM
U87-MG	Brain	Glioblastoma	Gift from Prof. Adrian Harris, Univ of Oxford	DMEM
VmCub1	Bladder	Carcinoma	Gift from Dr. Anne Kiltie, Univ of Oxford	DMEM

Table 2.1 Human cancer cell lines and specifications. The table illustrates the cell lines used in this study along with the information involving the origin of tissue, disease, the source and culture media used.

2.1.2 Cryopreservation of cells

For cryopreservation, cell were stored in liquid nitrogen in cryovials (Thermo Scientific Nunc, 363401) at a cell concentration of approximately 1 – 2 x 10^6 cells/ml. Exponentially growing cells that had reached 70% confluence were trypsinised and resuspended in complete media and centrifuged at 300 g for 5 min. The pellet obtained was resuspended in freezing mixture containing 10% DMSO and 90% FBS and aliquoted into cryovials. The cryovials were placed in Mr. Frosty (Nalgene, #CRY-OFF-700C) containing 2-propanol for 24 – 48 h in -80°C and later transferred to liquid nitrogen.

2.1.3 Thawing cells

Frozen aliquots of cells were removed from liquid nitrogen and thawed rapidly at 37°C in a water bath. Cells were resuspended in 5 ml of complete media and centrifuged at 300 g for 5 min. The pellets were then resuspended in complete media and transferred to a T25 flask (Corning Inc.,) and placed under normal tissue culture conditions.

2.2 Mycoplasma Testing

Cells grown in antibiotic free media were harvested when 90% confluent and heated for 15 min at 100°C. 50 µl of the above suspension was added to 200 µl of HEK-Blue cell (1 - 3.5 x 10^5 cells/ml) in HEK-Blue™ detection medium. The experiment was performed in duplicate in a 96-well plate, along with a positive and negative control provided by the manufacturer (PlasmoTest™, InvivoGen). Plates were incubated under normal tissue culture conditions and observed over 24 h for a colour change. The appearance of blue colour indicating a positive result for presence of lipoprotein expressed

by all types of bacteria and mycoplasma. A pink colour indicated a negative result. Mycoplasma testing was carried out routinely once every 6 months.

2.3 List of drugs and compounds with their solubility and concentrations

Name	Company	Solubility	Stock solution	Working concentration
STF-62247	Cayman Chemical (#CAY13084)	DMSO	10 mM	0.15 - 1.25 µM
Temsirolimus (CCI-779)	Sigma-Aldrich (#PZ0020)	DMSO	500 µM	1 - 100 nM
3-methyladenine	Sigma-Aldrich (#M9281)	DMSO	500 mM	1 - 2 mM
Chloroquine hydrochloride	Sigma-Aldrich (C6628-25G)	dH_2O	50 mM	2 – 4 µM
VE-821	Vertex Pharmaceuticals	DMSO	10 mM	1 µM
MK-1775	Selleckchem (#S1525)	DMSO	2 mM	0.2 µM
KU-55933	Tocris (#3544)	DMSO	100 mM	10 µM
Valproic acid	Sigma-Aldrich (#P4543-10g)	dH_2O	0.5 M	1 mM
Clonidine	Sigma-Aldrich (C7897-100mg)	dH_2O	1 mM	1 µM
Minoxidil	Sigma-Aldrich (#M4145-25g)	DMSO	100 mM	1 µM
Tosedostat (CHR-2797)	Tocris (#3595)	DMSO	10 mM	0.01 – 2 µM
Niclosamide	Sigma-Aldrich (#N3510-50G)	DMSO	10 mM	10 µM
Actinonin	Santa Cruz Biotechnologies (sc-201289)	DMSO	10 µM	5 – 100 µM
Bestatin	Sigma-Aldrich (B8385)	dH2O	10 mM	0.5 – 5 µM
SC 5471A	Sigma-Aldrich (PZ0110)	DMSO	10 mM	2.5 – 50 nM
Leuhistin	Enzo lifescience	DMSO	4 mM	0.05 – 1 µM

2.4 Colony Survival

For clonogenic survival studies, exponentially growing cells were trypsinised and counted using a haemocytometer. The plating efficiency of different cell lines was determined (Table 2.2 and 2.3) and cells were seeded such that at least 100 colonies were obtained on the control plates. Seeded cells were allowed to adhere for 2 h under normal tissue culture conditions pre-treatment. Post treatment colonies were allowed to form under normal tissue culture conditions. Cells were stained with 0.5% (w/v) crystal violet in 50% methanol and 20% ethanol after 8 - 14 days depending on the cell type. Colonies containing at least 50 cells were counted using a colony counter. The survival fraction was calculated by considering [(number of colonies formed after treatment) / (number of cells seeded x plating efficiency)] x 100, where plating efficiency is the number of colonies obtained / number of cells seeded in the untreated control. The data were fitted using Graphpad Prism v6.03.

<table>
<tr><th>Cell lines</th><th colspan="2">Seeding density of cells</th><th>Plating efficiency (%)</th></tr>
<tr><td>786-O and 786-O/VHL</td><td>400</td><td>6 cm dishes</td><td>29.5 (786-O), 53.58 (786-O/VHL)</td></tr>
<tr><td>A549</td><td>250</td><td>6-well plate</td><td>79.2</td></tr>
<tr><td>DLD1</td><td>250</td><td>6-well plate</td><td>95</td></tr>
<tr><td>HCT116</td><td>500</td><td>6 cm dishes</td><td>70</td></tr>
<tr><td>HeLa</td><td>500</td><td>6-well plate</td><td>41</td></tr>
<tr><td>IGR39</td><td>250</td><td>6-well plate</td><td>54.52</td></tr>
<tr><td>MDA-MB-231</td><td>500</td><td>6 cm dishes</td><td>32</td></tr>
<tr><td>MDA-MB-468</td><td>500</td><td>6 cm dishes</td><td>21.6</td></tr>
<tr><td>RCC4 and RCC4/VHL</td><td>300</td><td>6-well plate</td><td rowspan="2">39.8 (RCC4), 55.2 (RCC4/VHL)</td></tr>
<tr><td>RCC4 and RCC4/VHL</td><td>500</td><td>6 cm dishes</td></tr>
<tr><td>RKO</td><td>250</td><td>6 cm dishes</td><td>71.2</td></tr>
<tr><td>RT-112</td><td>250</td><td>6-well plate</td><td>45</td></tr>
<tr><td>SKmel28</td><td>250</td><td>6-well plate</td><td>43.2</td></tr>
<tr><td>SQ20B</td><td>200</td><td>6-well plate</td><td>56.65</td></tr>
</table>

U2-OS	400	6 cm dishes	67
U87-MG	400	6-well plate	79
VmCub1	500	6 cm dishes	54.8
H1299	250	6-well plate	41.2
FLO-1	200	6-well plate	69
OE21	250	6-well plate	58
OE33	200	6-well plate	32.33

Table 2.2 A list of seeding densities used for clonogenic assay in various tumour cell lines at 0 Gy. The table illustrates the different tumour cell lines along with their seeding densities used for clonogenic assays performed in this study.

Cell line	Seeding density of cells per 6 cm dish for the indicated doses in Gy							
	0	0.5	1	1.5	2	4	6	8
RKO	250	300	400	450	500	750	1000	1250
H1299	500	-	-	-	750	1000	1250	1500
FLO-1	200	-	-	-	400	600	800	1000
OE-21	250	-	-	-	500	750	1000	1250
OE-33	200	-	-	-	400	600	800	1000

Table 2.3 A list of seeding densities used for clonogenic assay of various tumour cell lines exposed to radiation. The table illustrates the seeding densities of various tumour cell lines used with their corresponding radiation dose.

2.5 Hypoxia treatment

Cells were exposed to hypoxia at the indicated oxygen tension using the hypoxia chambers (Invivo$_2$ Hypoxia Workstation 400, Ruskinn or Bactron, Shell labs). For irradiation of cells under hypoxic conditions, custom made airtight Perspex plastic boxes were used (Figure 2.1). These boxes were equilibrated inside the hypoxia chamber for 4 h at the required oxygen tension. For studies involving irradiation the boxes were sealed with the treated cells inside the chamber and irradiated. The hypoxia boxes were initially validated by assessing the OER of cells exposed to radiation under hypoxic conditions, to

indicate that the boxes were airtight and no reoxygenation occurred during radiation (Figure 3.2A). For studies involving comets, cells were seeded in glass petri dishes to reduce oxygen retention usually observed in plastic petri dishes.

Figure 2.1 Custom made air-tight Perspex hypoxia box. The figure illustrates an air tight Perspex box which was used for radiation studies involving hypoxia.

2.6 Irradiation studies

Cells were irradiated at the required doses using Cs-137 irradiator (GSM: GSR D1) at a dose rate of 1.7 Gy/min. All irradiations were carried out in room temperature. Cells irradiated in hypoxic conditions were returned to normal tissue culture conditions 10 min post irradiation and allowed to develop colonies and stained as previously described (2.4). The surviving fractions were calculated as mentioned above (2.4) and the data was fitted according to the linear quadratic model using the formulae $SF=e^{-(\alpha D + \beta D^2)}$ using Graphpad Prism v6.03.

2.7 Cell proliferation

Cells were seeded in 6-well plates as indicated, allowed to adhere for 12 h and subsequently treated with either DMSO or STF-62247 (0.62 µM) or CHR-2797 (0.5 µM) as indicated. Post treatment every 24 h cells were harvested by trypsinisation and the cell number was determined using a haemocytometer.

2.8 Protein lysate preparation

Cells were washed once with PBS, pelleted and resuspended in 60 µl protein lysis buffer (9 M Urea, 75 mM Tris-HCl pH 7.5, 0.15 M 2-mercaptoethanol). The lysed cells were sonicated briefly (20 s) and pelleted at 300g for 15 min at 4°C. Protein concentration was determined from the supernatant using Nanodrop1000 (Thermo Scientific) and samples were stored at -20°C for further studies.

2.9 Western Blotting

Protein samples were solubilised in 2x Laemmli sample buffer (120 mM Tris-HCl pH 6.8, 10% (w/v) SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.02% (w/v) bromophenol blue) and heated at 100ºC for 5 min. Prepared samples were loaded onto SDS-PAGE gels (Table 2.4) along with pre-stained molecular weight markers (Precision Plus Kaleidoscope, Bio-Rad). Electrophoresis was carried out in a SDS-PAGE electrophoresis running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at a constant current of 120 V for 60 - 90 min depending on the molecular size of the protein of interest. Post electrophoresis, the gel was transferred to nitrocellulose membrane (Bio-Rad, #162-0115) sandwiched between Whatmann 3MM filter paper and transferred at a constant voltage of 100 V current for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Nitrocellulose membranes containing the transferred protein were treated with blocking buffer solution (LI-COR Biosciences) for 1 h at room temperature on a belly dancer.

Depending on the primary antibody (Table 2.5) the membrane was probed by incubation at room temperature for 1 h or overnight at 4ºC on an oscillator. Non-specific binding of the primary antibody was removed with washing the membrane in TBS-T for 10 min (3x). The probed membrane was incubated in secondary antibody (Table 2.6) for 1

h at room temperature in dark and washed with TBS-T for 10 min (2x) and further 10 min with TBS. The membranes were scanned using the LI-COR Odyssey Imager and images were acquired with the Odyssey software.

	Stacking gel (2x)	Resolving gel (2x)	
		7.5%	15%
dH_2O	6.1 ml	7.4 ml	3.75 ml
0.5 M Tris pH 6.8	2.5 ml	-	-
1.5 M Tris pH 8.8	-	3.75 ml	3.75 ml
Acrylamide/Bis	1.3 ml	3.65 ml	7.25 ml
10% SDS	100 µl	150 µl	150 µl
10% APS	100 µl	75 µl	75 µl
TEMED	20 µl	18 µl	18 µl

Table 2.4 Preparation of SDS-PAGE gels. Table describing the volume of various reagents and solutions required for casting of SDS gels.

Protein	*Company*	*Mol. wt*	*Dilution Used*
HIF-1α	BD Biosciences	120 kDa	1:500
HIF-2α	Novus Biologicals	120 kDa	1:500
LC3	MBL Medical & Biological Laboratories Co., Ltd., Clone: 51-11, # M115-3	16, 18 kDa	1:1000
VHL	BD Biosciences, #556347	21-30 kDa	1:1000
β-Actin (Ac-15)	Santa Cruz Biotechnology, Inc, #sc-69879	43 kDa	1:100000

Table 2.5 List of primary antibodies. Table describing the primary antibodies and their corresponding molecular weight along with the dilutions used.

Secondary Antibody	*Company*	*Dilution Used*
Alexa Fluor 680 goat anti-mouse IgG	Invitrogen, #A21057	1:10000
IRDye 800CW donkey anti-mouse IgG	LI-COR, #926-32212	1:5000
IRDye 800CW donkey anti-rabbit IgG	LI-COR, #926-32213	1:5000

Table 2.6 List of secondary antibodies. Table describing the secondary antibodies and the dilutions used.

2.10 Electron Microscopy

Cells were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer for 15 min at room temperature, scrapped and centrifuged at 200 g for 5 min. Pellets were resuspended in 500 µl of fixative (1% (w/v) osmium tetroxide in 0.15 M cacodylate buffer). Samples were then sent to Dr. David J. P. Fergusson for processing as previously described (Bertog et al, 2000).

2.11 Immunofluorescence

Cells ($1x10^5$) were seeded on glass coverslips overnight and treated with the required drug concentration or exposed to irradiation. Post treatment, cells were fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature and washed in PBS. Cells were permeabilised with ice-cold methanol for 10 min at -20°C and washed with PBS. Coverslips were incubated with primary antibody (Table 7) for 1 h at 37°C. Coverslips were washed with PBS for 5 min (3x) and incubated with secondary antibody (Table 7) for 1 h at room temperature in the dark. Post staining, coverslips were washed with PBS for 5 min (3x) and mounted with Prolong Gold antifade reagent with DAPI (Invitrogen, #P36935) and stored at 4°C. Cells were examined using Nikon 90i fluorescence microscope and images were acquired using NIS Elements software.

	Protein	*Company*	*Dilution Used*
Primary Antibody	LC3	nanoTools antikoerpertechnik, #5F10	1:50
	53BP1	Novus Biologicals, #NB100-904	1:500
Secondary Antibody	Alexa Fluor 488 goat anti-mouse	Invitrogen, #A11017	1:250
	Alexa Fluor 488 goat anti-rabbit	Invitrogen, #A11070	1:250

Table. 2.7 List of antibodies for immunofluorescence. Table describing the primary and secondary antibodies and their corresponding molecular weight and the dilutions used.

2.12 Fluorescence activated cell sorting (FACS)

Cells (2 x 10^6) were seeded overnight and treated with the required concentrations of the drug, post treatment cells were processed for propidium iodide staining (PI), and BrdU staining as described below:

2.12.1 PI Staining

Cells were harvested and fixed in 500 µl of 70% ethanol in ice for 1 h and centrifuged at 300 g for 5 min. The pellets were resuspended in PBS containing propidium iodide (10 µg/ml; Sigma-Aldrich, #P4864) and RNAse A (100 µg/ml; Sigma-Aldrich, #R6513) and incubated at 37°C for 30 min. Analysis was carried out for 10000 cells per experimental condition using BD FACSort and data was analysed using ModFit LT v3.2.

2.12.2 BrdU Staining

Cells were labelled with 20 µM BrdU for 1 h at 37°C prior to harvesting and fixed in 70% ethanol overnight at -20° C. These fixed cells were permeabilised with 0.1 mg/ml pepsin in 2 M HCl for 20 min at room temperature. Cells were rinsed twice with 2% FBS/PBS and stained with a BrdU monoclonal antibody (1:100; BD clone 44, #347580) incubated at room temperature for 90 min with occasional mixing. Cells were rinsed with 2% FBS/PBS and stained with secondary antibody AF488 (1:200; Invitrogen) by

incubating at room temperature in dark for 60 min with occasional mixing. After staining, cells were centrifuged and rinsed with PBS, followed by incubation at 37°C for 30 min in 1 ml PBS containing propidium iodide (10 µg/ml), and RNAse A (100 µg/ml). Analysis of the stained cells was carried out using BD FACSort with 10000 cells per experimental sample and the data was plotted using BD CellQuest Pro v6.0.

2.13 Single cell gel electrophoresis – Comet

Comet assay is a technique that detects the level of DNA damage and repair kinetics at the level of a single cell. This technique involves the detection of SSB, alkali-labile damage as well as DSB using the alkaline assay (sensitivity > 0.1 Gy), whereas detection of DSB is by neutral comet assay (sensitivity > 5 Gy). In principle, cells are lysed and their nuclei is salt-extracted and subjected to electrophoresis. Following electrophoresis the nuclei appears with the characteristic appearances of a comet. The tail of the comet is a direct correlation to the level of damage in the DNA (Olive, 1999; Olive et al, 1990).

2.13.1 Alkaline Comet Assay

The comet assay was performed as described previously (Parsons et al, 2012), with some modifications. 6.5 x 10^4 cells were seeded in glass petri dishes and allowed then to adhere for 12 h and then exposed to hypoxia and / or Hydroxyurea (Hu) treatment. The treated cells were trypsinised and embedded in 1% (w/v) low-melting agarose in PBS. The slides were then lysed in the lysis buffer (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris base, pH 10.5) for 1 h at room temperature for normoxic or inside the pass box of the hypoxic chamber in the dark. All solutions used for hypoxic treatment were equilibrated to the required O_2 tension. The slides were removed and washed, followed by incubation in cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA and 1% DMSO,

pH > 13) for 30 min. Electrophoresis was carried out at 1.0 V/cm, 300 mA for 25 mins. After electrophoresis the slides were washed with neutralisation buffer (0.5 M Tris-HCl, pH 8.0). Finally the slides were stained with SYBR Gold (Invitrogen, S11494). At least 50 comets per slide were included in the analysis using Komet 5.5 image analysis software (Andor Technology) in duplicate for each condition and each experiment was performed three times. Graphs were plotted for percentage tail DNA using Graphpad Prism v6.03.

2.13.2 Neutral comet assay

Exponentially growing RKO cells, 0.65 x 10^5 were plated in glass petri dishes and allowed to adhere for 12 h and then exposed to the indicated conditions. Neutral comet assays was performed with slight modification from (Olive & Banath, 2006). Cells were embedded as for the alkaline comet assay (see 2.12.1). Coated slides were submerged in lysis buffer pH 8.3 (30 mM EDTA, 0.5% (w/v) sodium dodecyl sulphate, 0.5 mg/ml Proteinase-K) for 16 h in the dark at 37°C (4°C for 2 h and then transferred to 37°C for IR studies). In case of treatment involving hypoxic studies all the slides were incubated at 37°C inside the hypoxic chamber with pre-equilibrated solutions. Following lysis, slides were rinsed with TBE buffer pH 8.5 (90 mM Tris, 90 mM Boric acid, 2 mM EDTA) for 16 h and electrophoresed at 1.0 V/cm for 25 min, with fresh TBE buffer. Post electrophoresis slides were washed with double distilled water and stained with SYBR gold for 30 min. Stained slides were washed with double distilled water and dried overnight. At least 50 comets per slide were analysed for comet using the Komet 5.5 image analysis software (Andor Technology) in duplicate for each condition. Graphs were plotted for percentage tail DNA using Graphpad Prism v6.03.

2.14 Statistical Analysis

Standard errors were calculated from the data obtained. Unless indicated each data point indicates means ± standard errors from three independent experiments in triplicate. A statistical significance of $p<0.05$ was calculated using one-tailed student t-test.

Chapter 3

Radiosensitisation through inhibition of the DNA damage response

3.1 Introduction

The level of oxygen in tumours plays a critical role in determining radiosensitivity. Cells exposed to severe hypoxia are significantly more radioresistant than those in normal oxygen conditions (normoxia). Severe levels of hypoxia or radiobiological hypoxia is a physiological stress to which cells respond by activating the DDR.

The hypoxia-induced DDR is initiated by replication stress, which occurs as a result of nucleotide imbalance (Pires et al, 2010b). Both ATM and ATR are induced in response to hypoxia and in turn phosphorylate many of the well-characterised downstream, targets (Hammond & Giaccia, 2004). Previous work has demonstrated that inhibition or knockdown of key members of the DDR leads to increased sensitivity to hypoxia/reoxygenation. For example knock down of either ATR or Chk1 leads to significant loss of viability in hypoxia (Cazares-Korner et al, 2013; Fokas et al, 2012; Pires et al, 2012). The numbers of kinases within the DDR has made this an attractive pathway to target therapeutically and more and more specific inhibitors are now available. Although many of these have been tested as potential radiosensitisers this preclinical testing is rarely carried out in conditions, which mimic the tumour microenvironment. As tumour hypoxia is a major barrier to successful radiotherapy it is important to determine the potential of these agents for use in hypoxic conditions.

AIM: To investigate the potential of inhibitors of ATM, ATR or Wee1 as radiosensitisers in hypoxic conditions.

3.2 Results

3.2.1 Hypoxia does not induce detectable damage

The level of radiation-induced killing is determined by the amount of DNA damage induced. Therefore, before using the DDR inhibitors we determined the level of DNA damage induced in response to hypoxia alone. Previous reports, using a number of assays have shown that even prolonged exposure to hypoxia does not result in the accumulation of DNA damage. However, we noted that neutral comet assays had not been carried out and therefore decided to use this assay to verify the lack of hypoxia-induced damage (Olive & Banath, 2006). RKO cells were exposed for 16 h either in normoxia (21% O_2) or severe hypoxia (<0.1% O_2) or as a control were exposed to radiation (5 or 20 Gy) and processed for neutral comet (Figure 3.1). As expected we observed that there was no increased DSB damage in hypoxic cells in comparison to the normoxic samples in the absence of radiation. In contrast, significant levels of damage were seen in the irradiated cells.

A

0 Gy | 21% O_2

21% O_2 | <0.1% O_2 | 5 Gy | 20 Gy

B n=1

Figure 3.1 Hypoxia does not induce DNA damage detectable by neutral comet.
RKO cells were seeded and exposed either to normoxia (21% O_2) or hypoxia (<0.1% O_2) for 16 h or indicated doses of irradiation under normoxia and processed for neutral comet assay and at least 100 cells were scored for each conditions. (**A**) Images of the cells exposed either to 21%, <0.1% O_2 or to the indicated doses of irradiation at 21% O_2. (**B**) % Tail DNA for the indicated conditions. Significance value: '*' p-Value <0.05.

3.2.2 Validating the hypoxia boxes and the period of reoxygenation required post radiation

In order to investigate the response to radiation in hypoxic conditions a system is required which allows the hypoxic environment to be maintained during the delivery of radiation. For this purpose we had custom-built hypoxia boxes built (Figure 2.1). These boxes were placed into the hypoxic chamber in order to equilibrate and then dishes of cells were sealed inside the boxes and transported to the caesium source. We investigated the integrity of our custom hypoxic boxes by determining the OER. RKO cells were exposed either to normoxia (21% O_2) or hypoxia (<0.1% O_2) and irradiated (0 – 6 Gy) under the same conditions. Irradiated cells were reoxygenated 10 mins post radiation and allowed to form colonies under normal tissue culture conditions (Figure 3.2A). We observed an OER_{37} of 2.36, suggesting the hypoxic boxes were airtight and no apparent reoxygenation occurred prior to radiation.

Reoxygenation following hypoxia has been shown to induce DNA damage (Freiberg et al, 2006). However, we did not know how the period of time between irradiation and reoxygenation effected colony survival. To address this we exposed RKO cells to hypoxia (2% O_2) for 16 h and irradiated (4 Gy) under the same conditions. Post radiation, cells were reoxygenated after various periods of time (0 – 120 mins) and the cell viability was determined by colony survival assay (Figure 3.2B). We observed no significant change in cell viability following reoxygenation for different periods of time in hypoxic cells exposed to radiation. Together, these data suggest that our hypoxic boxes are suitable for radiation studies in hypoxic conditions and that the time period between radiation and reoxygenation does not impact colony survival.

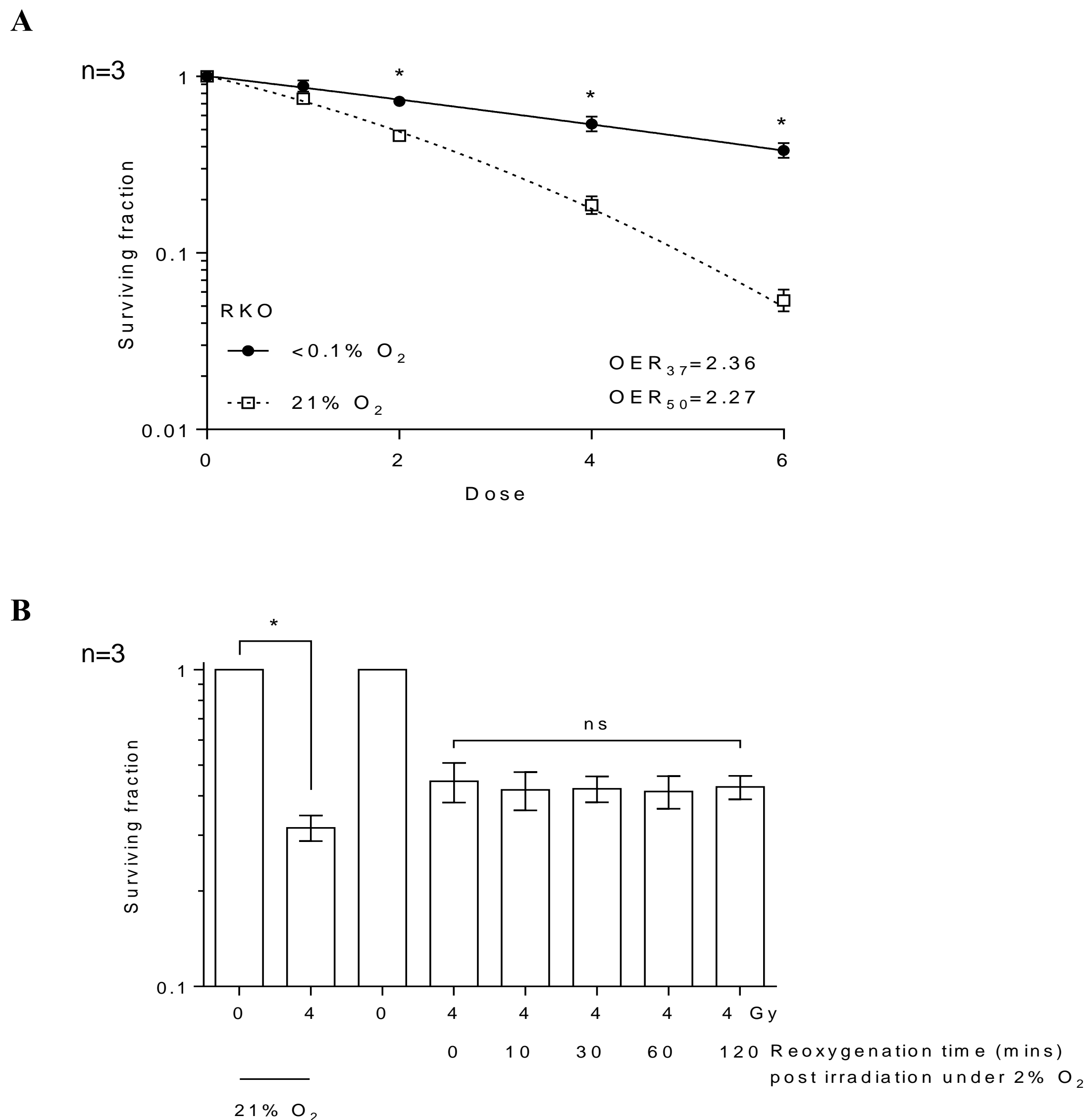

Figure 3.2 Validation of hypoxia boxes for irradiation under hypoxic conditions.
(**A**) RKO cells were pre-treated for 6 h either in normoxia (21% O_2) or severe hypoxia (<0.1% O_2) and exposed to radiation (0 – 6 Gy) under the same conditions. Post-irradiation cells were reoxygenated (10 mins) and allowed to form colonies under normal tissue culture conditions and the surviving fractions were calculated. (**B**) RKO cells were exposed to 2% O_2 for 16 h, and irradiated (4 Gy) under the same conditions using hypoxia boxes. Post-irradiation cells were reoxygenated at indicated time periods and allowed to form colonies under normal tissue culture conditions and the surviving fractions were calculated. Significance value: ‘*’ p-Value <0.05, ‘ns’ non-significant.

3.2.3 Hypoxia pre-treatment leads to increased radiosensitivity

DNA repair pathways have been shown by a number of groups to be repressed under hypoxic conditions (Bindra et al, 2005; Chan & Bristow, 2010; Crosby et al, 2009; Koshiji et al, 2005; Meng et al, 2005). Decreased levels of DNA repair in hypoxic cells suggest that hypoxic cells would be more radiosensitive compared to normoxic cells. However, hypoxic cells exposed to radiation have decreased levels of damage due to depleted levels of oxygen, thereby rendering them radioresistant. This led us to hypothesise that hypoxic cells would actually be more radiosensitive than normoxic counterparts if the same amount of radiation-induced damage was delivered.

To investigate our hypothesis, RKO cells were exposed to hypoxia (1% O_2) for 48 h in order to repress the DNA repair pathways and reoxygenated (5 mins) under normal tissue culture conditions. Post reoxygenation, hypoxia pre-treated cells and the normoxic control cells were exposed to radiation (0 – 6 Gy) in parallel. Post radiation cells were plated and allowed to form colonies (Figure 3.3). We observed that hypoxia pre-treated cells had decreased cell survival in response to radiation. Our result suggests that hypoxic cells could be radiosensitised if the level of DNA damage is either increased or on par with the damage seen in their counterpart normoxic cells. A caveat of this experimental set up is the hypoxic cells experience additional reoxygenation-induced DNA damage, which may account for their increased sensitivity.

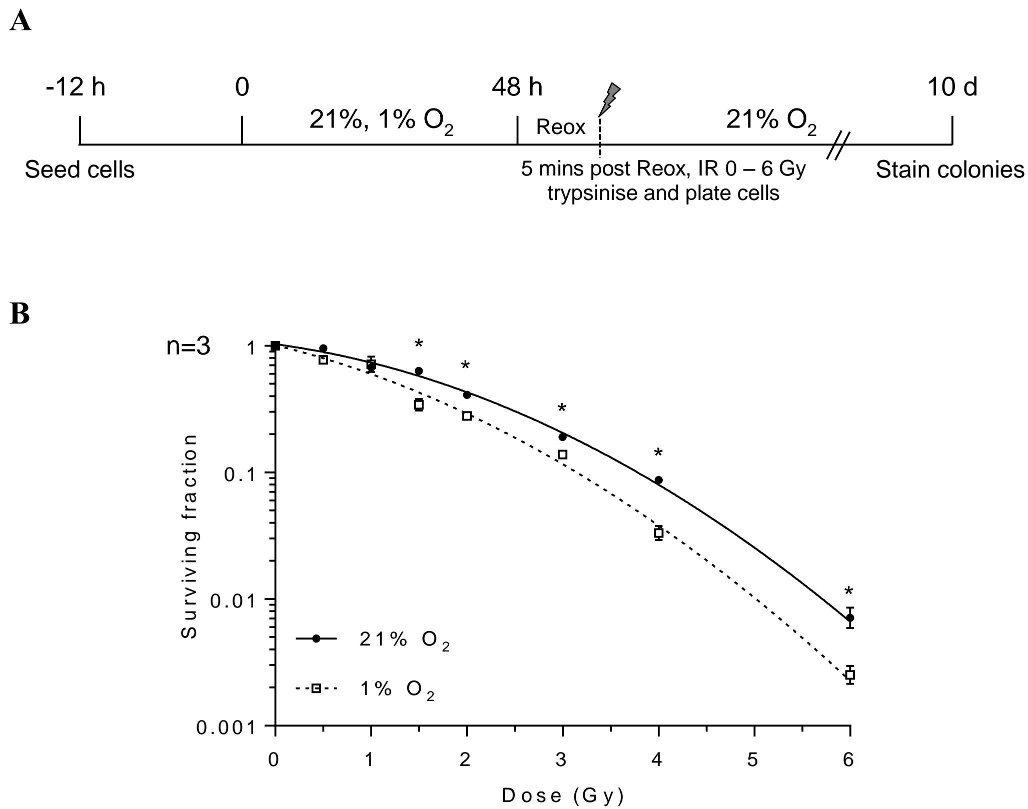

Figure 3.3 Hypoxia pre-treatment radiosensitises RKO cells.
(**A**) Scheme of RKO cells pre-treated with hypoxia and irradiated post reoxygenation. All radiations were carried out under normoxic conditions. (**B**) Clonogenic survival of RKO cells in response to hypoxia pre-treatment (1% O_2, 48 h) followed by reoxygenation for 5 mins under normal tissue culture conditions. Post reoxygenation cells were exposed to a range of irradiation doses (0 – 6 Gy). Post radiation cells were trypsinised, plated and allowed to form colonies and the surviving fractions were calculated. Significance value: ‘*’ p-Value < 0.05.

3.2.4 Targeting radiation resistant hypoxic cells through ATM inhibition

Tumour hypoxia leads to activation of ATM (Bencokova et al, 2009). It is well known that ATM loss leads to increased radiosensitisation but this has not been shown in hypoxia (Cox et al, 1981; Houldsworth & Lavin, 1980; Kuhne et al, 2004; Morgan et al, 1968; Nagasawa & Little, 1983). We hypothesised that hypoxic tumour cells can be radiosensitised by inhibiting ATM *in vitro*. Hickson et al., have described how the specific ATM inhibitor KU-55933 chemosensitised and also radiosensitised HeLa cells in normoxic conditions (Hickson et al, 2004). Initially, we investigated the effect of KU-55933 on cells exposed to hypoxia, both mild and severe. RKO cells were treated with KU-55933 (10 μM) or DMSO as a control and exposed to either hypoxia (2% O_2) or severe hypoxia (<0.1% O_2) over a period of time (0 - 24 h). Post hypoxic treatment cells were reoxygenated for 2 h and fresh media was added to the cells and allowed to form colonies (Figure 3.4). We observed a significant decrease in cell viability in RKO cells treated with KU-55933 under hypoxia.

Furthermore, we investigated if KU-55933 could radiosensitise hypoxic cancer cells. RKO cells were treated either with KU-55933 (10 μM) or DMSO as a control and exposed to severe hypoxia (<0.1% O_2) for 6 h and irradiated (0 – 6 Gy) under the same conditions. Post radiation cells were reoxygenated for 6 h under normal tissue culture conditions. Subsequently, fresh media was added to the cells and cell viability was assessed by colony survival assay (Figure 3.5). We observed the treatment with KU-55933 increased radiation-induced loss of cell viability in hypoxia (SER_{37}=2.1). Our results suggest that hypoxic cells can be radiosensitised by inhibiting ATM.

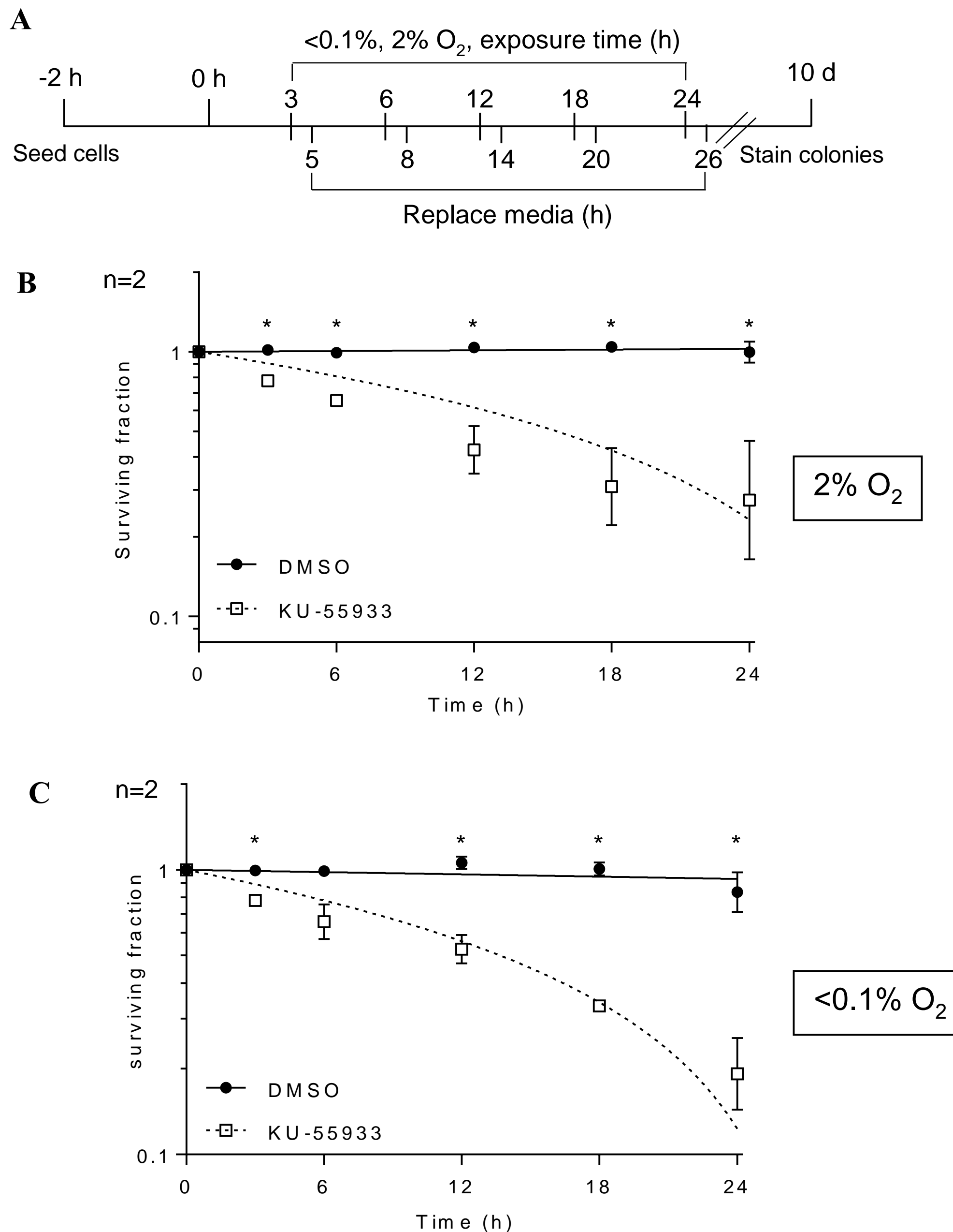

Figure 3.4 KU-55933 decreases cell survival in hypoxia.
(**A**) Scheme of RKO cells treated with KU-55933 under hypoxia. RKO cells were treated either with DMSO or KU-55933 (10 µM) and exposed to the indicated period of time in hypoxia at (**B**) 2% O_2, (**C**) <0.1% O_2. Post hypoxic treatment, cells were reoxygenated for 2 h under normal tissue culture conditions. Following reoxygenation fresh media was added to the cells and allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05.

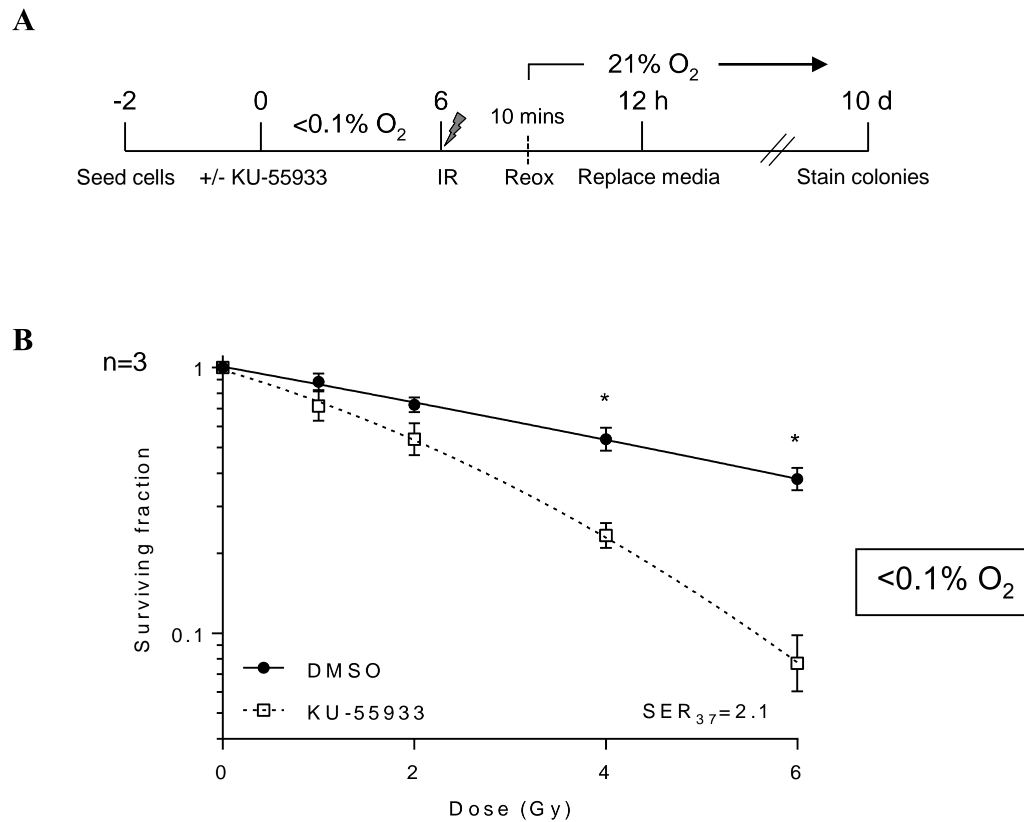

Figure 3.5 KU-55933 radiosensitises RKO cells in hypoxia.
(**A**) Scheme of RKO cells in hypoxia radiosensitised by KU-55933 pre-treatment. (**B**) RKO cells were treated either with DMSO or KU-55933 (10 µM) for 6 h in hypoxia (<0.1% O_2) and irradiated under hypoxic conditions at the indicated doses. Post radiation cells were reoxygenated for 6 h under normal tissue culture conditions. Post reoxygenation (6 h) fresh media was added to the cells and allowed to form colonies and the surviving fraction was calculated. Significance value: '*' p-Value <0.05.

3.2.5 Targeting radiation resistant hypoxic cells through ATR inhibition

We have shown that ATM inhibition radiosensitises hypoxic tumour cells *in vitro* (Figure 3.5). We hypothesised that hypoxic cells could be radiosensitised through the inhibition of ATR. Initially, in this study we combined the ATR inhibitor VE-821 with radiation in a range of cancer cell lines. The breast (MDA-MB-231, MDA-MB-468), cervical (HeLa), head & neck (SQ20B), renal (RCC4), bladder (VmCub1), colorectal (RKO, HCT116, DLD1), melanoma (Skmel28, IGR39) and lung (H1299) cells were pre-treated for 6 h with VE-821 (1 μM), and exposed to radiation doses (0 – 8 Gy) and cell viability was assessed by colony survival assay (Figure 3.6 – 3.9). We observed an increase in radiation-induced loss of cell viability in cancer cells treated in combination with VE-821. These results suggest that the radiosensitisation effect of VE-821 under normoxic conditions is independent of tumour type.

As the outcome of radiotherapy is partly dependent on the level of tumour hypoxia, we evaluated the combination of radiation and VE-821 in hypoxic conditions (Begg et al, 2011). We investigated the effect of radiosensitivity of VE-821 in the oesophageal cancer cell lines FLO-1, OE21 and OE33 under normoxia and hypoxia. The oesophageal cancer cell lines FLO-1, OE21 and OE33 were treated in the presence or absence of VE-821 (1 μM) for 6 h either in normoxia (21% O_2) or hypoxia (2% O_2) and exposed to radiation under the same conditions. Post radiation cells were allowed to form colonies under normal tissue culture conditions and the viability of the cells were assessed by colony survival (Figure 3.10). We observed VE-821 radiosensitises OE21 and OE33 in both normoxia and hypoxia (Table 3.1). However, VE-821 did not radiosensitise the FLO-1 cells in normoxia but radiosensitised the hypoxic cells (Table 3.1). The difference in response to radiation to VE-821 treatment in FLO-1 cells under hypoxia and normoxia requires further investigation.

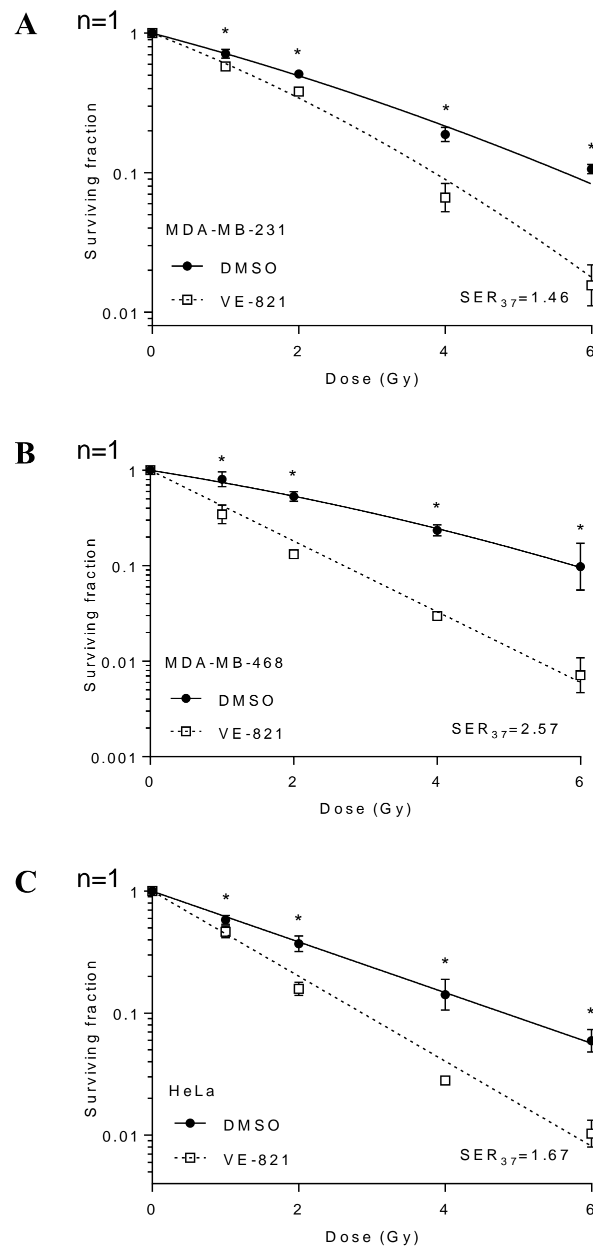

Figure 3.6 VE-821 radiosensitises breast and cervical cancer cell lines.
The breast cancer cell lines (A) MDA-MB-231, (B) MDA-MB-468 and cervical cancer (C) HeLa cells were treated either with DMSO or VE-821 (1 μM) for 6 h and then exposed to a range of radiation doses. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05. Published in Br J Cancer, Pires et al (2012).

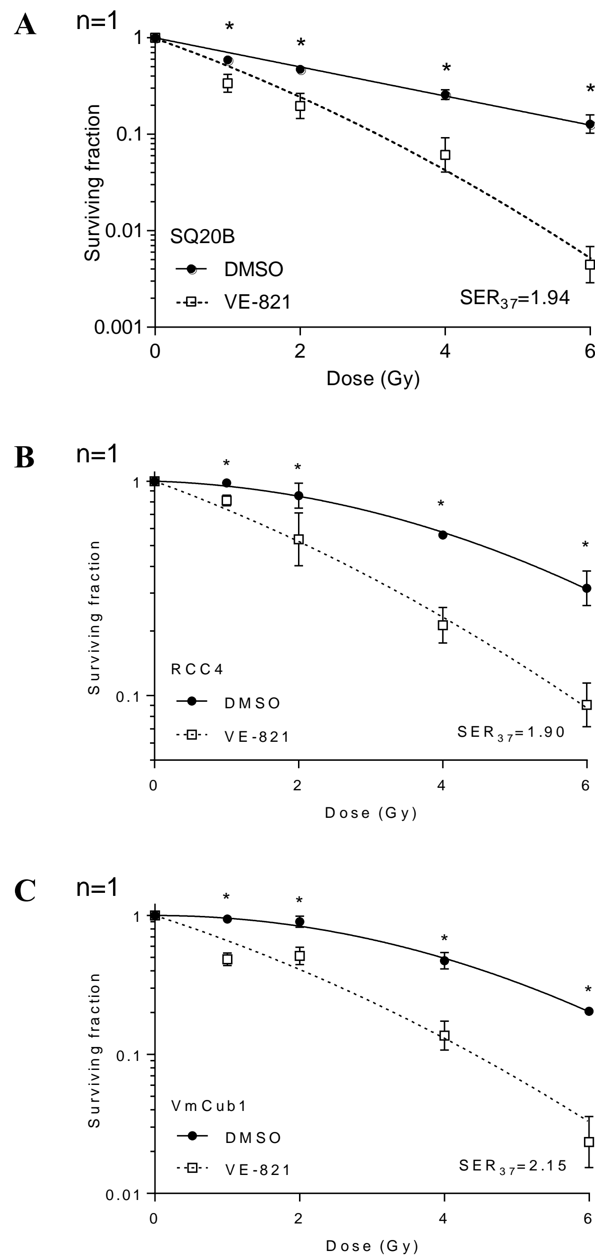

Figure 3.7 VE-821 radiosensitises head & neck, renal and bladder cancer cell lines. The head and neck (A) SQ20B, renal (B) RCC4, and bladder (C) VmCub1 cancer cells were treated either with DMSO or VE-821 (1 μM) for 6 h and then exposed to a range of radiation doses. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05. Published in Br J Cancer, Pires et al (2012).

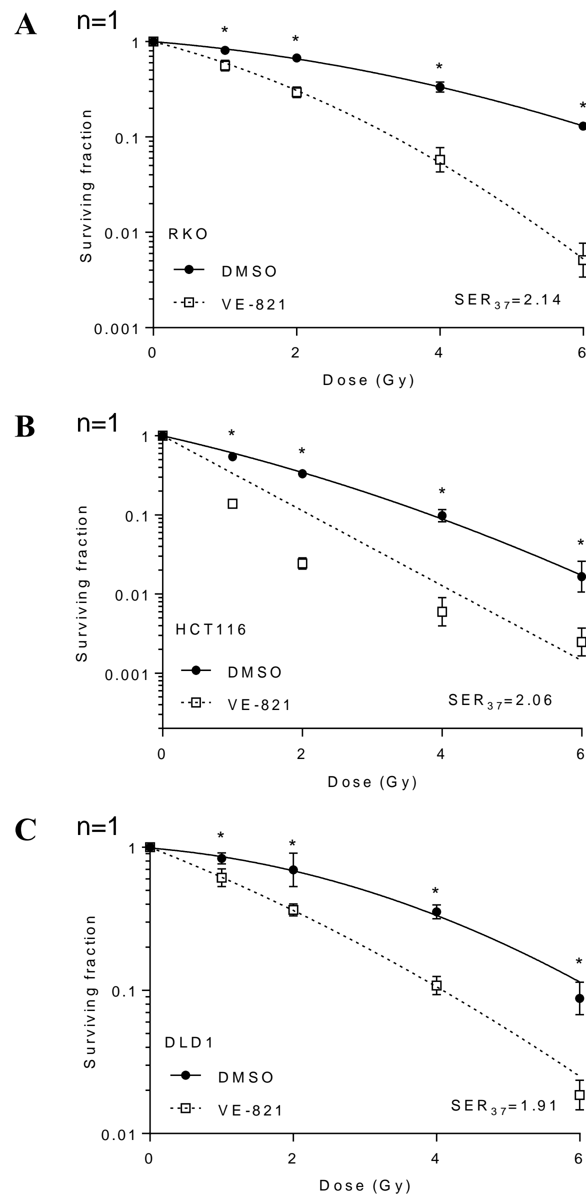

Figure 3.8 VE-821 radiosensitises colorectal cancer cell lines.
The colorectal cancer cell lines (A) RKO, (B) HCT116 and (C) DLD1 were treated either with DMSO or VE-821 (1 μM) for 6 h and then exposed to a range of radiation doses. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05. Published in Br J Cancer, Pires et al (2012).

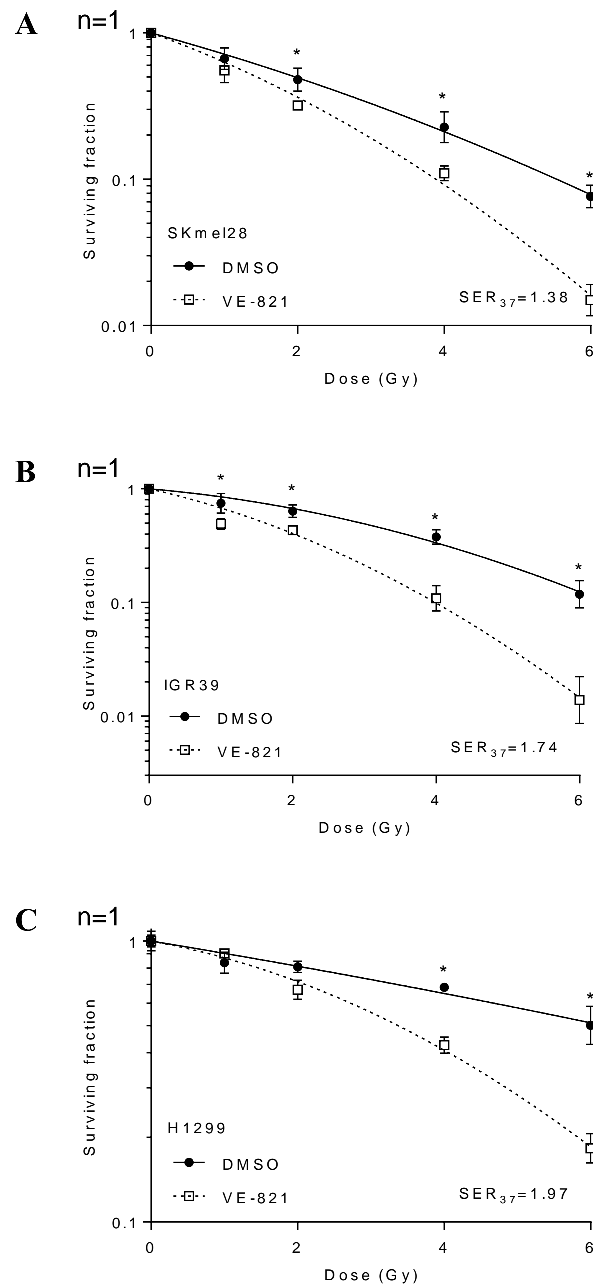

Figure 3.9 VE-821 radiosensitises melanoma and lung carcinoma cells.
Melanoma cells, (A) SKmel28, (B) IGR39 and the lung carcinoma cell line (C) H1299 were treated either with DMSO or VE-821 (1 μM) for 6 h and then exposed to a range of radiation doses. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05. Published in Br J Cancer, Pires et al (2012).

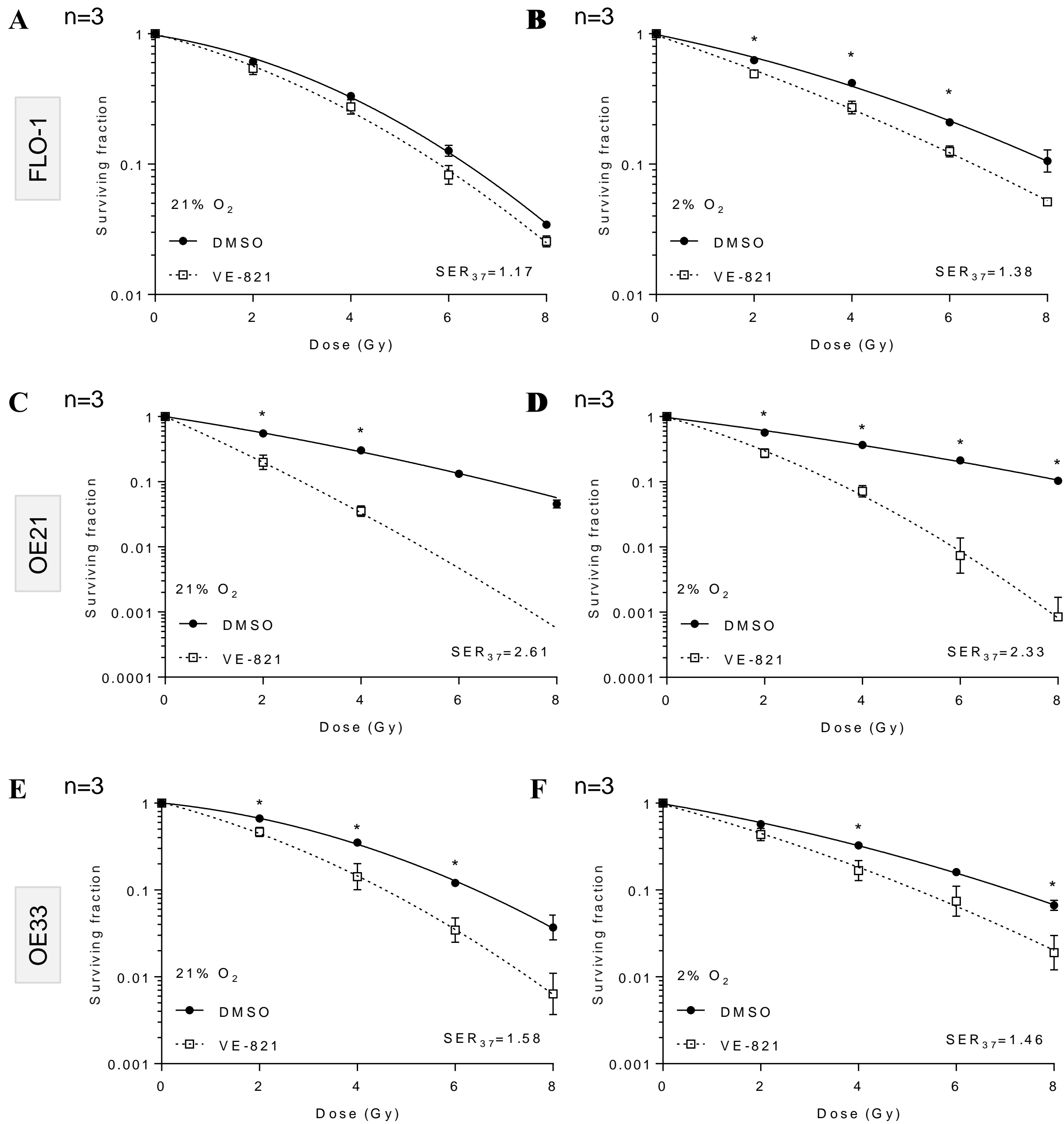

Figure 3.10 Radiosensitivity of oesophageal cell lines in response to VE-821 pre-treatment.

The oesophageal cancer cells (**A, B**) FLO-1 (adenocarcinoma), (**C, D**) OE21 (squamous cell carcinoma), (**E, F**) OE33 (adenocarcinoma) were treated either with DMSO or VE-821 (1 µM) for 6 h either in 21% or 2% O_2 and then exposed to the indicated doses of irradiation under the same conditions. Post irradiation cells were allowed to form colonies under normal tissue culture conditions and the surviving fractions were calculated. '*' p-Value <0.05.

Cancer cell line	SER_{37}	
	21% O_2	2% O_2
FLO-1	1.17	1.38
OE21	2.61	2.33
OE33	1.58	1.46

Table 3.1 Sensitisation enhancement ratio (SER) for various oesophageal cancer cell lines in response to radiation and VE-821 treatment

3.2.6 Effect of Wee1 inhibition on cell survival and radiosensitivity in hypoxic conditions

Severe hypoxia has been shown to induce replication stress triggering ATM/ATR mediated DDR signalling (Hammond et al, 2007). Inhibition or loss of ATR or CHK1 has been demonstrated to sensitise hypoxia/reoxygenation of cancer cell lines (Figure 3.10, (Cazares-Korner et al, 2013; Fokas et al, 2014; Hammond et al, 2004). Cancer cells with a defective G_1-checkpoint accumulate DNA damage and are more reliant on the G_2-checkpoint compared to normal cells (Dixon & Norbury, 2002). Apart from ATR and CHK1, Wee1 is involved in the maintenance of G_2-checkpoint and is also regarded as the gatekeeper of G_2-checkpoint (De Witt Hamer et al, 2011; Sorensen & Syljuasen, 2012).

The Wee1 inhibitor, MK-1775 is currently under clinical trials but its efficacy in response to radiation in hypoxia has not yet been studied. We hypothesised that MK-1775 could radiosensitise in hypoxia. We choose H1299 cells because they are p53 null. Cells with a loss of functional p53 will be more reliant on the G_2-checkpoint mechanism (Levine, 1997).

Initially, we assessed cell survival in response to MK-1775 under different hypoxic conditions by colony survival assay. H1299 cells were treated in the presence or absence of MK-1775 (0.2 μM) over a period of time (0 – 24 h) at different oxygen tensions (21%,

1% and <0.1% O_2) and reoxygenated. Following reoxygenation, fresh media was added to the treated cells and their cell viability was assessed by colony survival assay (Figure 3.11). We observed an increased sensitivity in response to MK-1775 from 16 h onwards in normoxic treatment, whereas under hypoxia (1% O_2), MK-1775 sensitised cells at 24 h. Under severe hypoxia (<0.1% O_2) we did not observe any significant sensitisation for the periods of treatment.

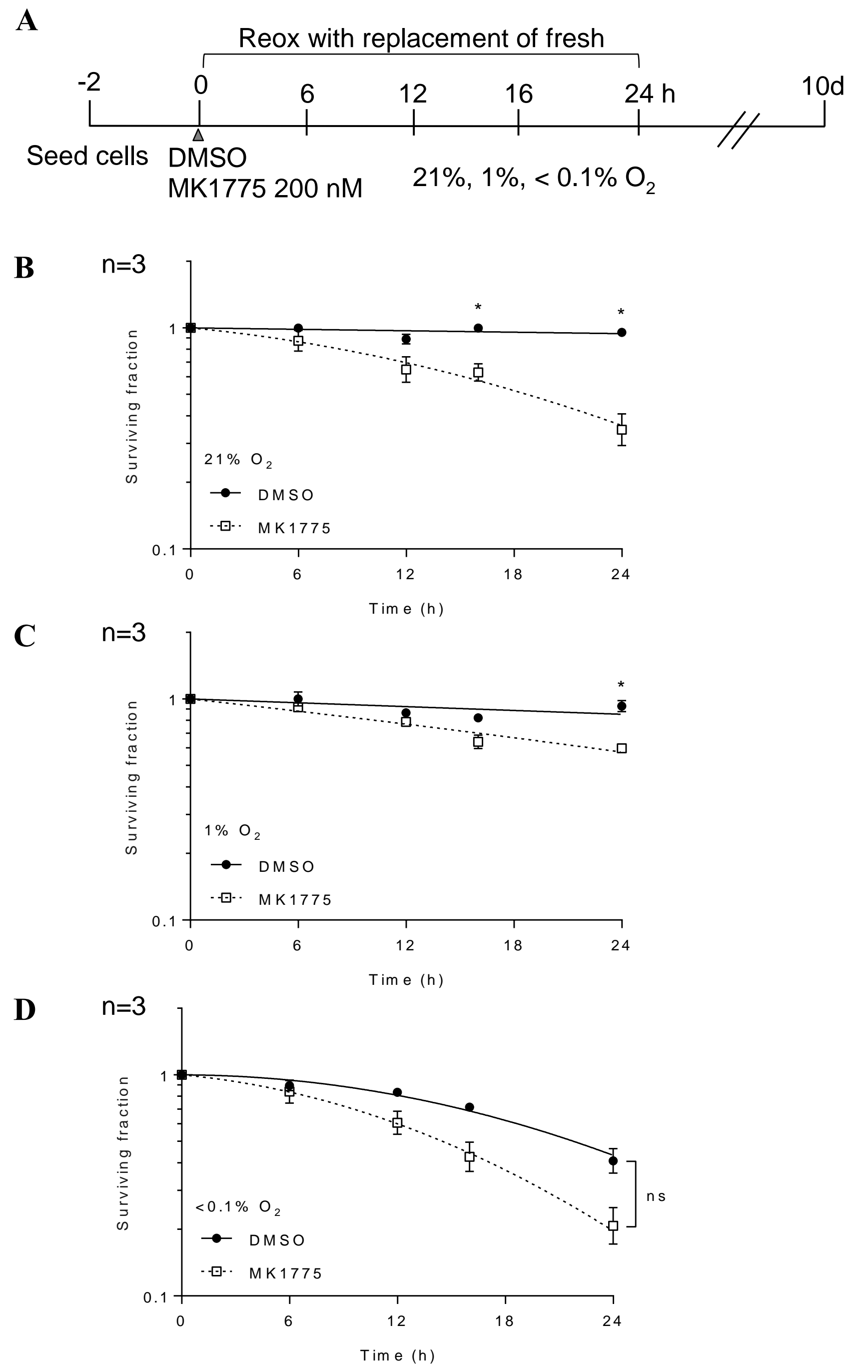

Figure 3.11 Effect of MK-1775 in H1299 cells exposed to various hypoxic tensions. (**A**) Scheme of MK-1775 treatment in H1299 cells under different oxygen tension. H1299 cells were treated either with DMSO or MK-1775 (0.2 μM) for the indicated time periods at different oxygen tensions (**B**) 21% (**C**) 1% and (**D**) <0.1% O_2 and reoxygenated. Post reoxygenation fresh media was added to the pre-treated cells and allowed to form colonies and the surviving fractions were calculated. Significance values: '*' p-Value <0.05, 'ns' non-significant. Published in Tumour microenvironment and Therapy, Vol 1, O' Brien et al., (2013).

Recent studies have demonstrated MK-1775 radiosensitises glioblastoma cell lines (Bridges et al, 2011; Sarcar et al, 2011). Therefore, we wanted to assess if MK-1775 could increase the radiation-induced cell kill under severe hypoxia. To address this, we pre-treated H1299 cells for 3 h either in severe hypoxia (<0.1% O_2) or normoxia (21% O_2) in the presence or absence of MK-1775 (0.2 μM) and exposed to radiation (0 – 8 Gy) under the same conditions. Post radiation (21 h), fresh media was added and the cell viability was assessed by colony survival assay (Figure 3.12). As expected, under normoxic conditions there was an increase in the radiation induced cell kill due to MK-1775 treatment (SER_{37}=1.40). However, under severe hypoxia we did not observe any increase in radiation-induced cell kill in response to MK-1775 treatment (SER_{37}=1.18).

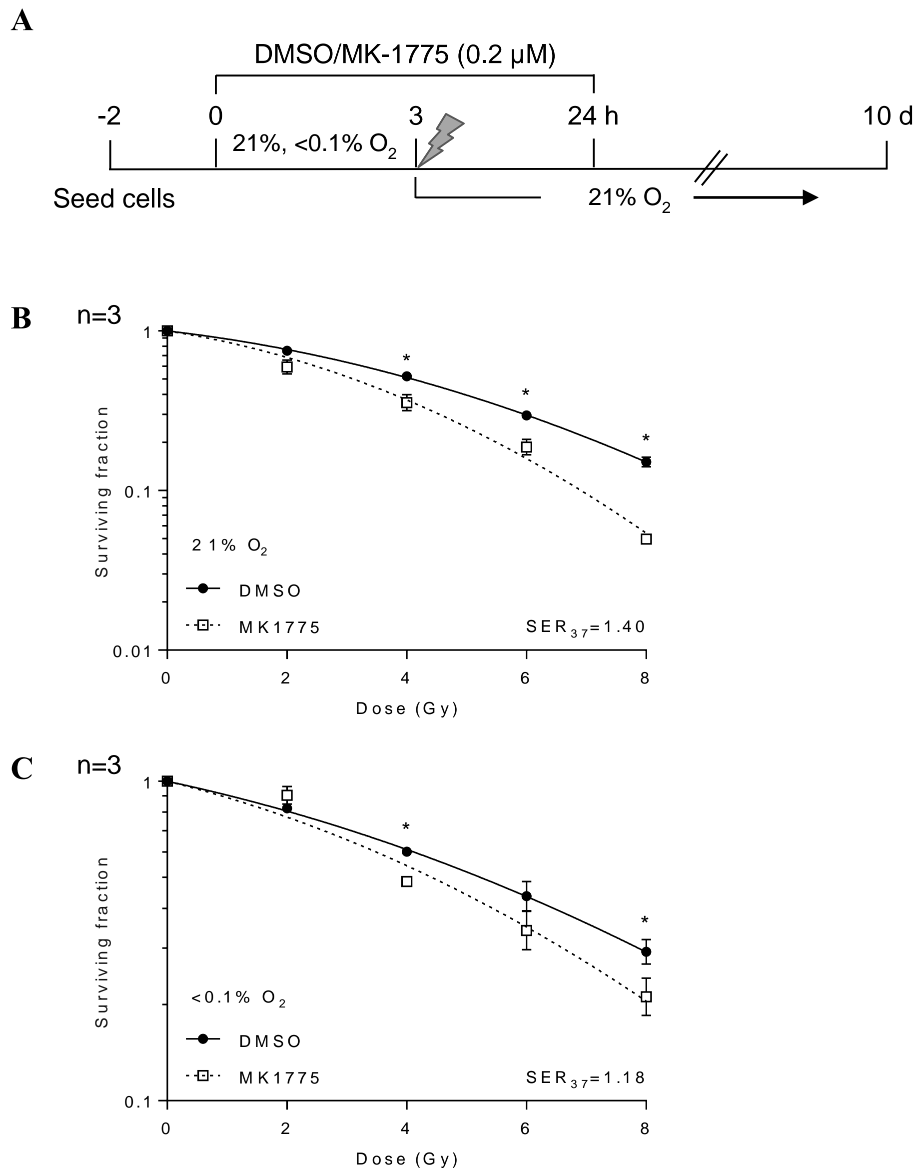

Figure 3.12 Effect of MK-1775 radiosensitivity in H1299 is oxygen dependent.
(**A**) Scheme of H1299 cells pre-treated with MK-1775 under different oxygen tensions and exposed to radiation. H1299 cells were pre-treated for 3 h either with DMSO or MK-1775 (0.2 µM) at (**B**) 21% (**C**) <0.1% O_2 and then exposed to a range of radiation doses under the same conditions. Post radiation the treated cells were returned to normal tissue culture conditions. Fresh media was added 21 h post radiation to the pre-treated cells and allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05. Published in Tumour microenvironment and Therapy, Vol 1, O' Brien et al., (2013).

3.3 Discussion

Our finding that inhibition of ATM significantly increases the radiosensitivity of tumour cells lines was unsurprising given the exquisite radiosensitivity associated with loss of ATM in AT patients. However, our study using the Wee 1 inhibitor MK-1775, clearly demonstrate the importance of testing agents in conditions which mimic tumour hypoxia. In agreement with the published literature we observed increased radiosensitivity in normoxia with MK-1775. However, we were surprised to discover that this effect was not apparent in hypoxic conditions.

Studies in fission yeast (S. pombe) and Xenopus suggest that Wee1 is a direct target of Chk1 (Lee et al, 2001; OConnell et al, 1997). This raised the possibility that Wee1 signalling could be mediated by hypoxia-induced Chk1 activity. We have previously demonstrated that loss of ATR/Chk1 signalling is an effective way to increase sensitivity to hypoxia/reoxygenation (Fokas et al, 2012; Pires et al, 2012). Therefore, we asked if Wee1 inhibitors might be effective at sensitising cells to hypoxia/reoxygenation and could also improve the radiation response of hypoxic cells. We investigated the use of Wee1 inhibitors as a monotherapy for hypoxic cells we found that those cells at the most severe levels of hypoxia were generally less sensitive. In addition to its effect as a single agent in p53 null cells, Wee1 inhibition has been shown to sensitise cells to radiation therapy (Bridges et al, 2011; Wang et al, 2001). Here, although in normoxic conditions MK-1775 was shown to significantly sensitise cells to radiation therapy, this effect was less significant in conditions of severe hypoxia. In previous studies, MK-1775 has been shown to sensitise cells to different chemotherapeutics (Hirai et al, 2010; Hirai et al, 2009; Mizuarai et al, 2009; Rajeshkumar et al, 2011). It remains to be established whether these effects will also be observed in conditions of hypoxia. Here, we have for the first time assessed the impact of Wee1 inhibition under hypoxic conditions. Most importantly, we

have highlighted the need to carry out preclinical testing of potential cancer therapeutics in conditions, which resemble the tumour microenvironment as closely as possible. Although Wee1 inhibition remains an attractive target, caution should be taken regarding the clinical application of this novel compound, as the potential combination with other anticancer therapies might not be effective.

Our study clearly demonstrates the potential benefit of combining an ATR inhibitor with radiotherapy for effective targeting of cancer cells in physiological conditions. Significantly, we observed a positive effect of combining VE-821 and radiation over a range of oxygen concentrations, and not just under severe hypoxia (<0.1% - 2% O_2) (Pires et al, 2012). Given the previously described role of ATR in the response to hypoxia-induced replication arrest in severely hypoxic conditions, this was not surprising (Hammond et al, 2002; Hammond et al, 2004). An analogue of VE-821, VE-822 also an ATR inhibitor has been demonstrated to have a significant tumour growth delay in pancreatic tumour xenograft models when combined with radiation or radiation and gemcitabine without significant toxicity to the normal tissues (Fokas et al, 2012). Following the outcome of the results from preclinical evaluation of VE-822, it is currently in a phase I clinical trial.

Chapter 4

Investigation of a novel radiosensitiser

4.1 Introduction

Patients presenting with advanced renal cell carcinoma (RCC) have a poor prognosis due to the relative chemo and radioresistance of this disease (Hollingsworth et al, 2006a; Hollingsworth et al, 2006b). Radiotherapy is rarely used to treat primary RCC although carbon ions have recently been used with some success (Nomiya et al, 2008). The majority of patients with stage I or II disease undergo surgical resection and this is largely curative although associated with future health issues such as renal insufficiency and cardiovascular problems (Huang et al, 2009). Up to 80% of RCC cases are associated with loss of function of the VHL tumour suppressor gene. VHL is the E3 ubiquitin ligase for HIF-1α and therefore loss of function leads to high levels of HIF-1 and HIF-1-dependent signalling. Due to the high levels of HIF-1, targeted therapies for RCC include the use of angiogenesis, mTOR inhibitors and tyrosine kinase inhibitors (TKI's) for example sutent.

Current treatments for renal cancer include the targeted agents everolimus and temsirolimus, both of which are rapamycin analogues. The induction of autophagy through mTOR inhibition by rapamycin is well characterised and may contribute to the benefit of this strategy for the treatment of renal cancer (Pirrotta et al, 2011). Temsirolimus induces autophagy and is selectively toxic to VHL-deficient cells compared to VHL-proficient cells (Thomas et al, 2006; Yazbeck et al, 2008). Recently, Prof Giaccia and colleagues took the novel approach of screening for small molecules that are synthetic lethal to the loss of VHL (Sutphin et al, 2007). A number of compounds were identified including ChA3, STF-62247 and STF-31, all three depend on the status of VHL (Chan et al, 2011; Turcotte et al, 2008a). However, ChA3 and STF-31 are both dependent of HIF-1, while STF-62247 functions independently of HIF-1.

The compound STF-62247 induces autophagy, which is pro-death in VHL-deficient cells. This agent therefore shows great selectivity for RCC compared to normal

tissue. Here, we tested the hypothesis that the induction of pro-death autophagy by treatment with STF-62247 increases the radiosensitivity of VHL-deficient RCCs whilst having little effect on cells with normal VHL function.

AIM: To test the hypothesis that STF-62247 alters the radiation sensitivity of VHL-deficient RCC cells.

4.2 Results

4.2.1 Expression levels of HIF and VHL in the RCC cells

In order to test our hypothesis, the RCC cell lines RCC4, 786-O VHL-deficient and RCC4/VHL, 786-O/VHL (truncated, 1-115 amino acids) VHL-proficient cells were used (Maxwell et al, 1999). The VHL status along with their HIF expression levels in the RCC cell lines were verified by western blot analysis (Figure 4.1).

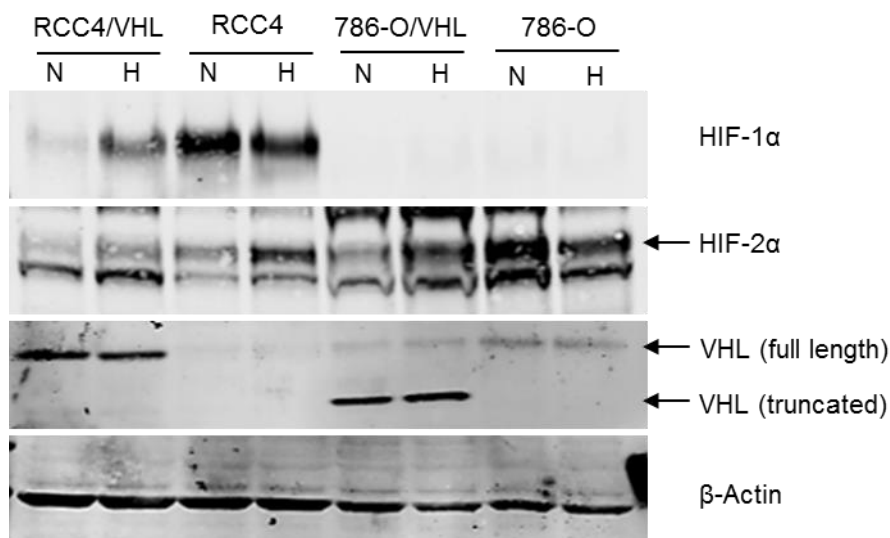

Figure 4.1 Expression levels of HIF-1α, HIF-2α, and VHL in the RCC cells
The RCC cell lines RCC4, RCC4/VHL, and 786-O, 786-O/VHL were exposed to 21% and 2% O_2 for 24 h. Western blotting was carried out for HIF-1α, HIF-2α, VHL and β-Actin (loading control). N, normoxia (21% O_2), H, hypoxia (2% O_2).

4.2.2 STF-62247 induces pro-death autophagy in a VHL-dependent manner

Previous studies from Prof Giaccia and colleagues have shown that STF-62247 induces autophagy in a VHL dependent manner (Turcotte et al, 2008a). Before embarking on combining STF-62247 with radiation we verified some of their key findings. Treatment with STF-62247 induced autophagy, which was significantly more pronounced in the

RCC4 cells compared to RCC4/VHL cells as determined by both cleavage of LC3 on a western blot (Figure 4.2A) and the appearance of cytoplasmic LC3 punctate staining (Figure 4.2B). We further confirmed their findings in an alternative pair of RCC cell lines. We also verified the LC3 cleavage by western blot analysis and the appearance of cytoplasmic LC3 punctate staining in an alternative pair of cells lines, 786-O and 786-O/VHL (Figure 4.3). From these results we were able to confirm the findings as reported earlier in the RCC4 and RCC4/VHL cells. In addition, we were able to demonstrate LC3 cleavage and the appearance of LC3 punctate in an alternate pair of RCC cell lines and also established a working model for our hypothesis.

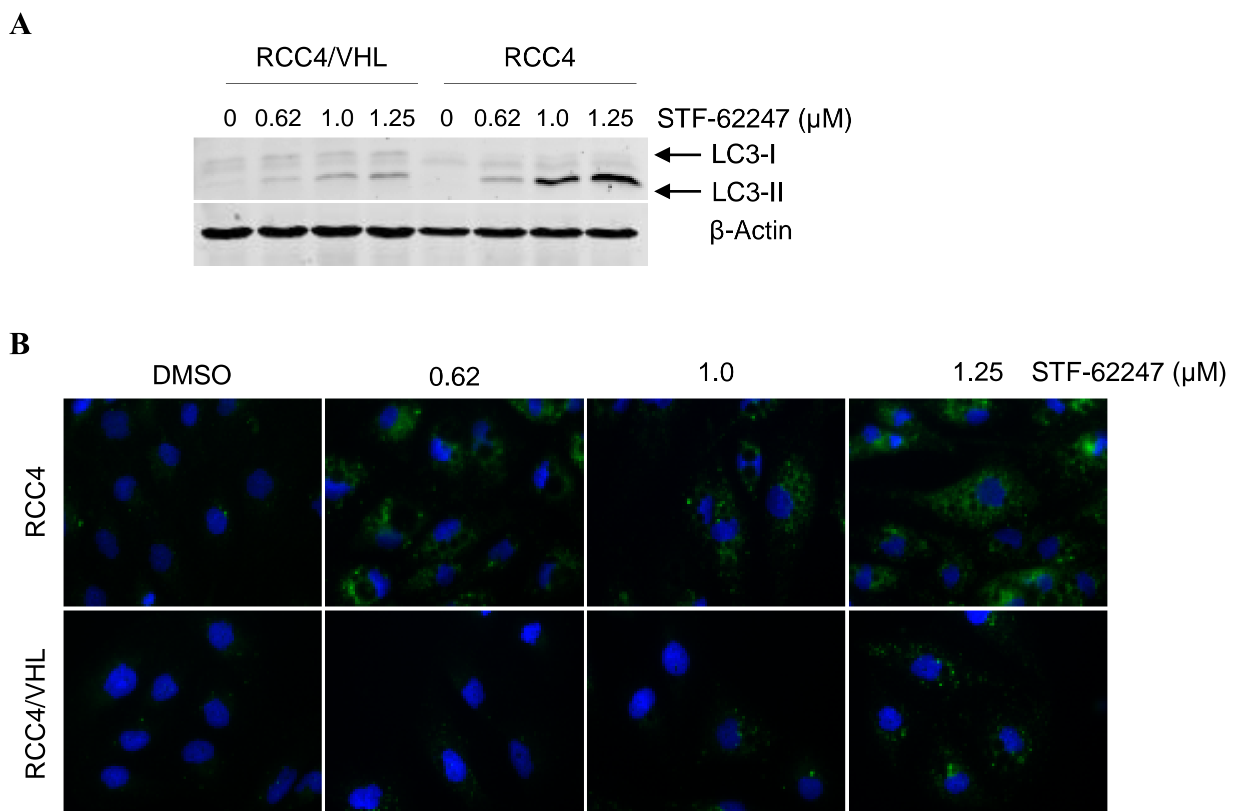

Figure 4.2 STF-62247 treatment induces autophagy
RCC4 and RCC4/VHL were treated for 24 h with the indicated concentration of STF-62247. (**A**) Western blotting was carried out for LC3 and β-Actin (loading control). (**B**) Immunofluorescence staining of cells stained for LC3 puncta (green). DAPI (blue) was used to visualise the nucleus. Magnification = 10X.

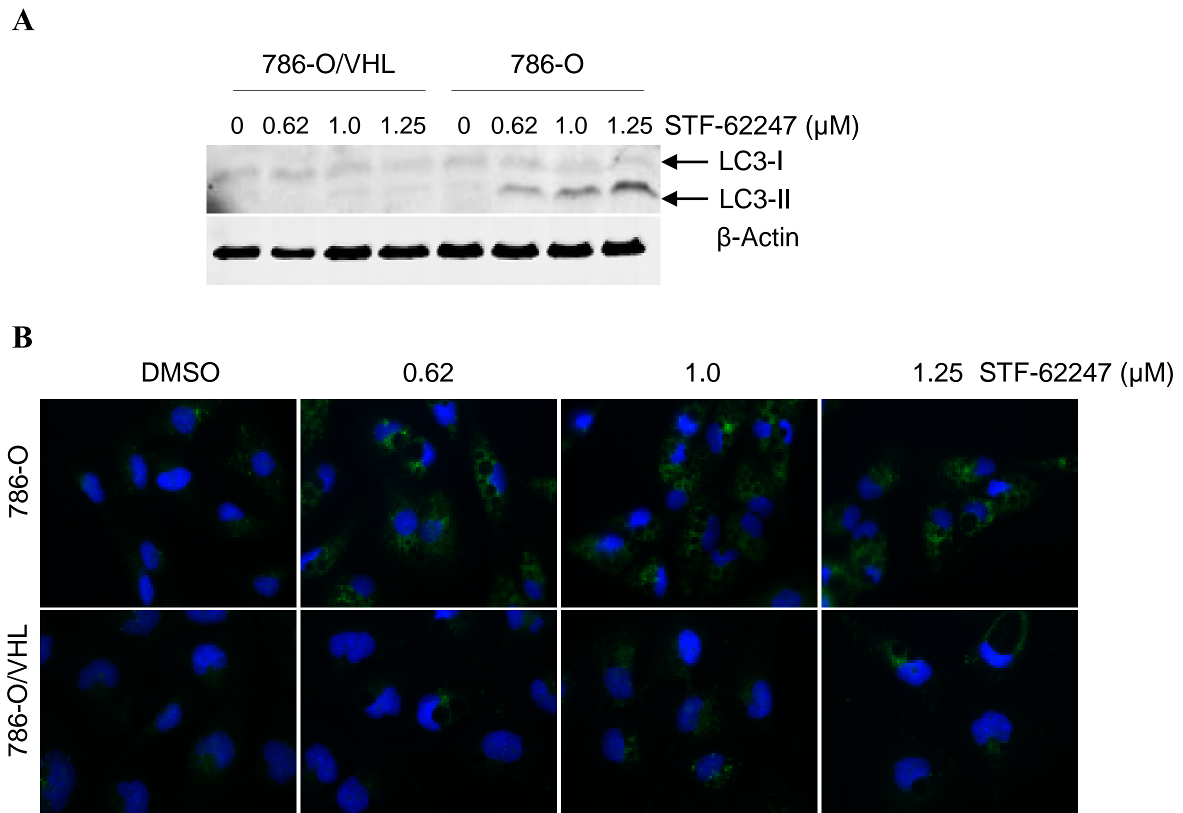

Figure 4.3 STF-62247 treatment induces autophagy in 786-O cells
786-O and 786-O/VHL were treated for 24 h with the indicated concentration of STF-62247. (**A**) Western blotting was carried out for LC3 and β-Actin (loading control). (**B**) Immunofluorescence staining of cells stained for LC3 puncta (green). DAPI (blue) was used to visualise the nucleus. Magnification = 10X.

4.2.3 Assessing the autophagic flux in RCC4 cells exposed to STF-62247

Treatment with STF-62247 has shown that there is an increase in LC3 cleavage, indicating the induction of autophagy (Figure 4.2 and 4.3). We wanted to validate if STF-62247 has role in autophagic flux. The initiation of autophagy is through the formation of autophagosomes, which requires class III PI3-kinase activity (Devereaux et al, 2013; Matsunaga et al, 2010). These autophagosomes are double layered vesicles which engulf cytoplasmic content or subcellular organelles. The autophagosome is formed as a result of binding of LC3 to the expanding phagophore (Mizushima et al, 2011). Autophagic flux is a process, which involves the synthesis and fusion of autophagosomes to the lysosomes termed as autolysosomes. Autolysosomes are involved in the degradation of the contents

present from the fused autophagosomes. Increased levels of autophagosomes and/or LC3 can be the result of autophagy induction or due to its impairment (Mizushima et al, 2010). In order to validate that STF-62247 treatment does not impair autophagy, our strategy was to use various inhibitors targeting different stages of autophagy (Figure 1.8).

RCC4 cells were treated either with STF-62247 or in combination with 3-methyladenine (3-MA), ammonium chloride or chloroquine diphosphate (CQ) and assayed for the differences in the LC3 levels. Combined treatment with 3-MA, a class III PI3-kinase inhibitor, led to a decrease in the levels of LC3 cleavage (Figure 4.4A). Our next target was inhibiting the lysosomal machinery, since the turnover of LC3 degradation happens in the autolysosomes. Inhibiting the lysosomes with lysosomotropic reagents such as ammonium chloride and CQ will result in increased LC3 cleavage (Klionsky et al, 2012; Mizushima et al, 2010; Rubinsztein et al, 2009). We observed increased levels of LC3 in RCC4 cells treated in combination with STF-62247 along with either ammonium chloride or CQ compared to the single treatment alone (Figure 4.4B and C). These results suggest that STF-62247 induces autophagy and does not inhibit the autophagy machinery.

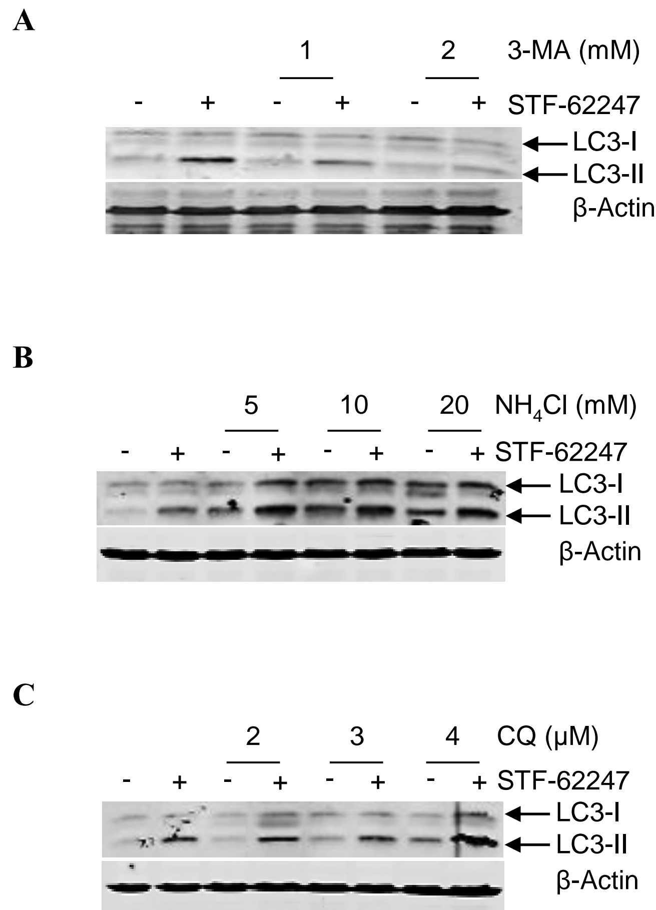

Figure 4.4 Assessing the autophagic flux in STF-62247 treated RCC4 cells
RCC4 cells were treated with the indicated doses of (**A**) 3-methyladenine (3-MA) (**B**) ammonium chloride (NH_4Cl) (**C**) chloroquine hydrochloride (CQ) either in combination or alone with STF-62247 (0.62 μM) for 24 h. Western blotting was carried out for LC3 and β-Actin (loading control).

4.2.4 STF-62247 exposure to RCC cells induces translucent intra-cytoplasmic vacuoles

In response to STF-62247, we observed the rapid (less than 2 h) formation of numerous lucent, cytoplasmic vacuoles (Figure 4.5A). The vacuoles were more prevalent in RCC4 and 786-O cell lines compared to the matched VHL controls, suggesting this was also VHL-dependent. Interestingly, primary cells taken from a RCC (Fuhrman grade 3)

showed the same response, although we were not able to verify the VHL status due to limited sample (Figure 4.5 A). In order to determine the nature of these vacuoles and specifically to determine if they were part of the autophagy process, we analysed the cells by electron microscopy. An increased number of RCC4 cells exposed to STF-62247 for 16 h contained numerous cytoplasmic vacuoles when compared to control cells (Figure 4.5B i, ii). A number of these vacuoles had the double membrane structure characteristic of autophagic vacuoles although the majority were enclosed by a single membrane with electron lucent contents with little debris or a few vesicular structures (Figure 4.5B ii, iii). The presence of extremely large vacuoles in some cells suggests that the small vacuoles form in response to STF-62247 treatment and then fuse (Figure 4.5B ii, iv). It is not clear if these vacuoles are involved or related to STF-62247-induced autophagy, although the high levels of LC3 staining seen by immunofluorescence suggests that this might be the case (Figure 4.2B).

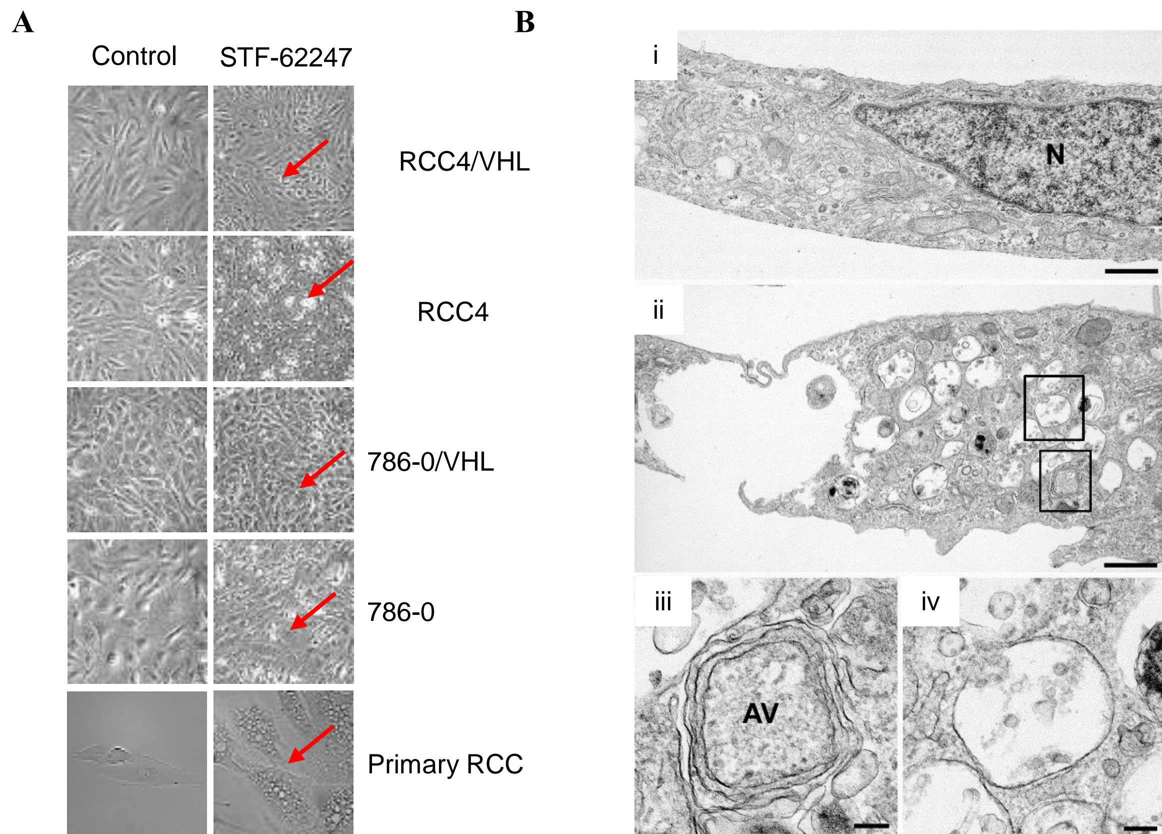

Figure 4.5 STF-62247 induces vacuoles, which are distinct from autophagosomes
(**A**) The cell lines indicated were treated with STF-62247 (0.62 µM) for 24 h and then photographed (magnification = 10X). (**B**) RCC4 cells were analysed by electron microscopy after 16 h exposure to 0.62 µM STF-62247 or DMSO treated control (i). Control cell showing nucleus (N) and normal appearing cytoplasm with few vacuoles. Bar = 1 µm. (ii) STF-62247 treated cell showing a number of cytoplasmic vacuoles. Bar = 1 µm. (iii) Lower box detail of cytoplasm enclosed by a double membrane representing an autophagic vacuole (AV). Bar = 100 nm. (iv) Upper box detail of lucent vacuole with a few vesicular structures enclosed by a single membrane. Bar = 100 nm. EM carried out by Dr. David J. P. Fergusson.

4.2.5 STF-62247 treated VHL-deficient RCC4 cells have decreased cell viability

STF-62247 has been shown to be selectively toxic in RCC cells with deficient VHL under normoxia, but it has not been studied for its selective toxicity under physiologically relevant oxygen tensions. We verified that STF-62247-induced autophagic

cell death in conditions relevant to tumour physiology. Colony survival assays with increasing concentrations of STF-62247 under 3% O_2 showed a significant loss of viability of RCC4 cells but little effect on RCC4/VHL cells (Figure 4.6A). Furthermore, we checked the proliferation rates of the RCC4 and RCC4/VHL cells treated with STF-62247 under normoxic conditions. RCC4 cells treated with STF-62247 had lower cell proliferation rates than the VHL-proficient RCC4/VHL cells (Figure 4.6B). These data verify the selectivity of this agent for cells lacking VHL function.

4.2.6 Exposure to STF-62247 does not alter cell cycle progression

As radiosensitivity is in part dependent on cell cycle phase, agents which arrest cells in more sensitive phases can radiosensitise (Seiwert et al, 2007). We sought to determine whether STF-62247 has an effect on cell cycle progression, which could in turn affect radiosensitivity. RCC4 and RCC4/VHL cells were exposed to STF-62247 for up to 24 h followed by BrdU/PI labelling and FACS analysis (Figure 4.7). STF-62247 did not significantly change the distribution of cells throughout the cell cycle compared to DMSO controls or between the cell lines. In addition, there was no evidence of an increase in the sub-G_1 population. These data indicate that any effect on radiosensitivity is not likely due to changing cell cycle transit.

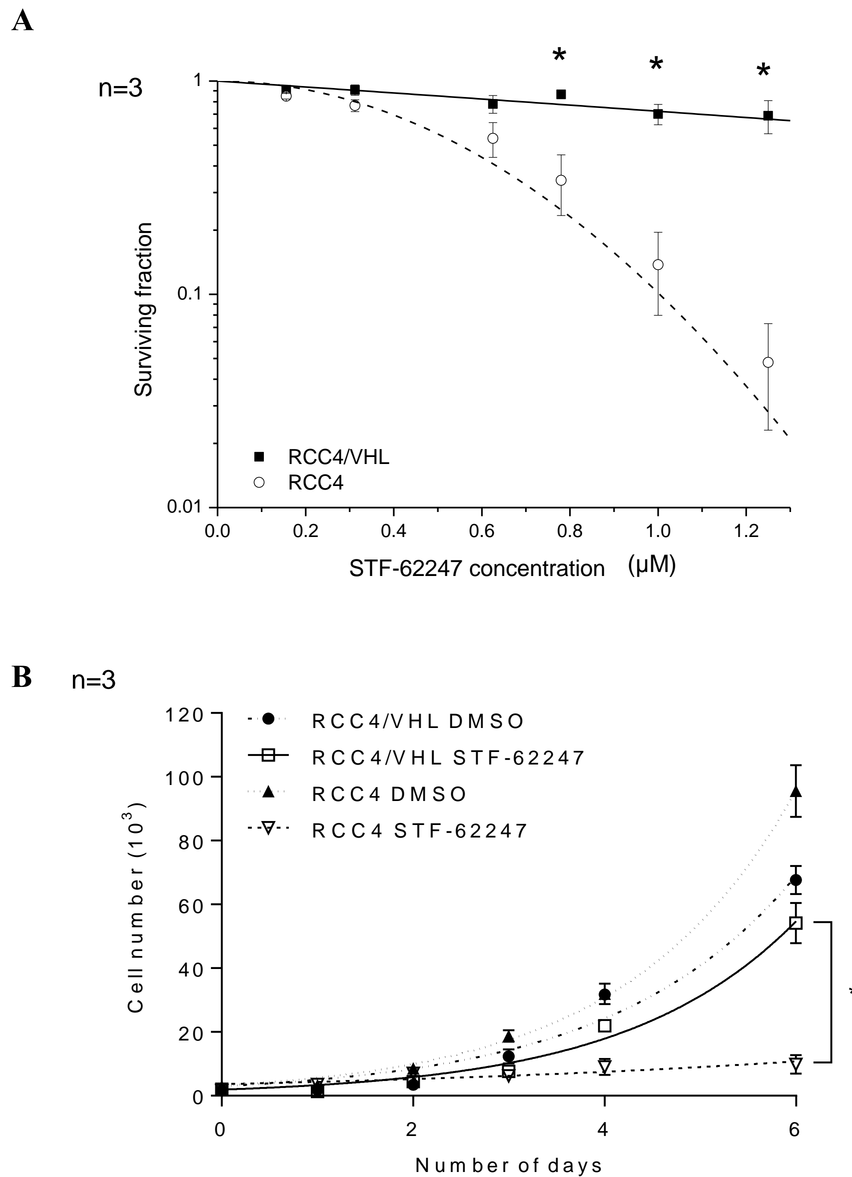

Figure 4.6 STF-62247 treated VHL-deficient RCC4 cells have decreased cell viability (**A**) Clonogenic survival for RCC4 and RCC4/VHL cells treated with the indicated doses of STF-62247 for 10 days at 3% O_2 and the surviving fractions were calculated. (**B**) Cell proliferation of RCC4 and RCC4/VHL cells exposed to STF-62247 (0.62 μM) for the indicated periods of time.'*' p-Value <0.05.

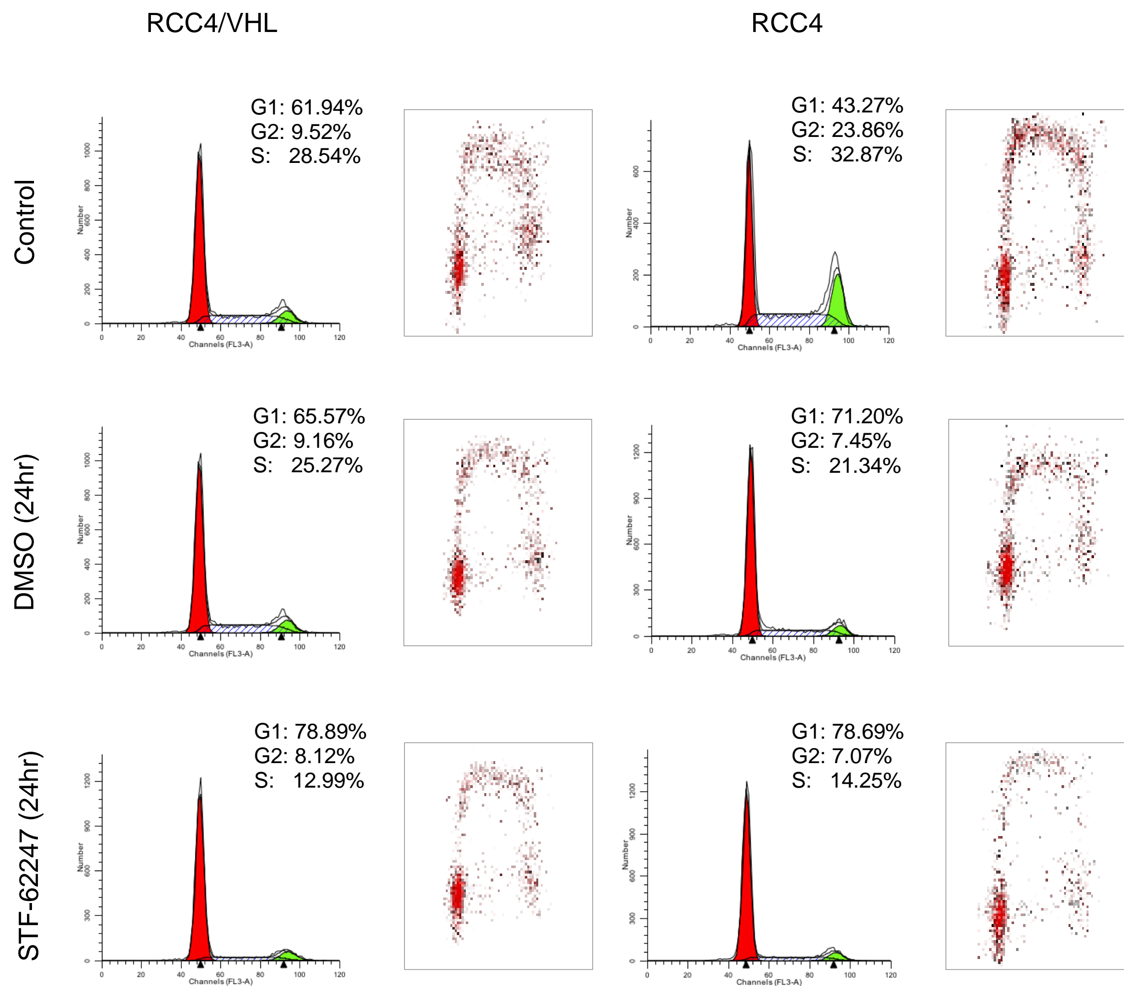

Figure 4.7 Flow cytometric analysis of RCC4/VHL and RCC4 cells treated with STF-62247

RCC4 and RCC4/VHL cells were treated with STF-62247 (0.62 µM) or DMSO analysed 24 h later by FACS, stained for both propidium iodide and BrdU incorporation. The untreated control cells were processed for FACS analysis along with STF-62247 and DMSO treated samples.

4.2.7 RCC4 cells are radiation resistant and do not undergo significant levels of radiation-induced autophagy

As the effects of STF-62247 are VHL-dependent, we predicted that any effect on radiosensitivity would also be VHL-dependent. We first examined the relative radiosensitivity of the RCC cell lines and whether VHL status affected radiosensitivity. A panel of cell lines including RCC4 and RCC4/VHL were plated for colony survival and then exposed to irradiation (0 - 5.3 Gy) (Figure 4.8). The survival fraction is shown in each case. As expected due to the previously reported radioresistant nature of RCC, the RCC4 cell lines were among the most radioresistant of the cell lines tested (Onufrey & Mohiuddin, 1985). The data indicate that the VHL proficient cells (RCC4/VHL) were slightly more radioresistant than the VHL deficient cells (RCC4). Radiation has been demonstrated to induce autophagy in some cell types therefore raising the hypothesis that radiation-induced autophagy in the VHL deficient cells was contributing to radiosensitivity (Kuwahara et al, 2011). However, there was no detectable accumulation of LC3 in RCC4 cells irradiated (5.3 Gy) and harvested over a 24 h period (Figure 4.9A). Radiation-induced autophagy was also not observed in RCC4 cells treated with a range of radiation doses (0.5 - 10 Gy) (Figure 4.9B). Furthermore, RCC4 cells were processed for electron microscopy (EM) 16 h post irradiation, which revealed no obvious increase in autophagic vacuoles compared to controls. However, certain degenerate changes were noted in the irradiated cells including mitochondria with unusually dilated cristae not seen in controls (Figure 4.10). These experiments demonstrate that in this case radioresistance does not correlate with radiation-induced autophagy.

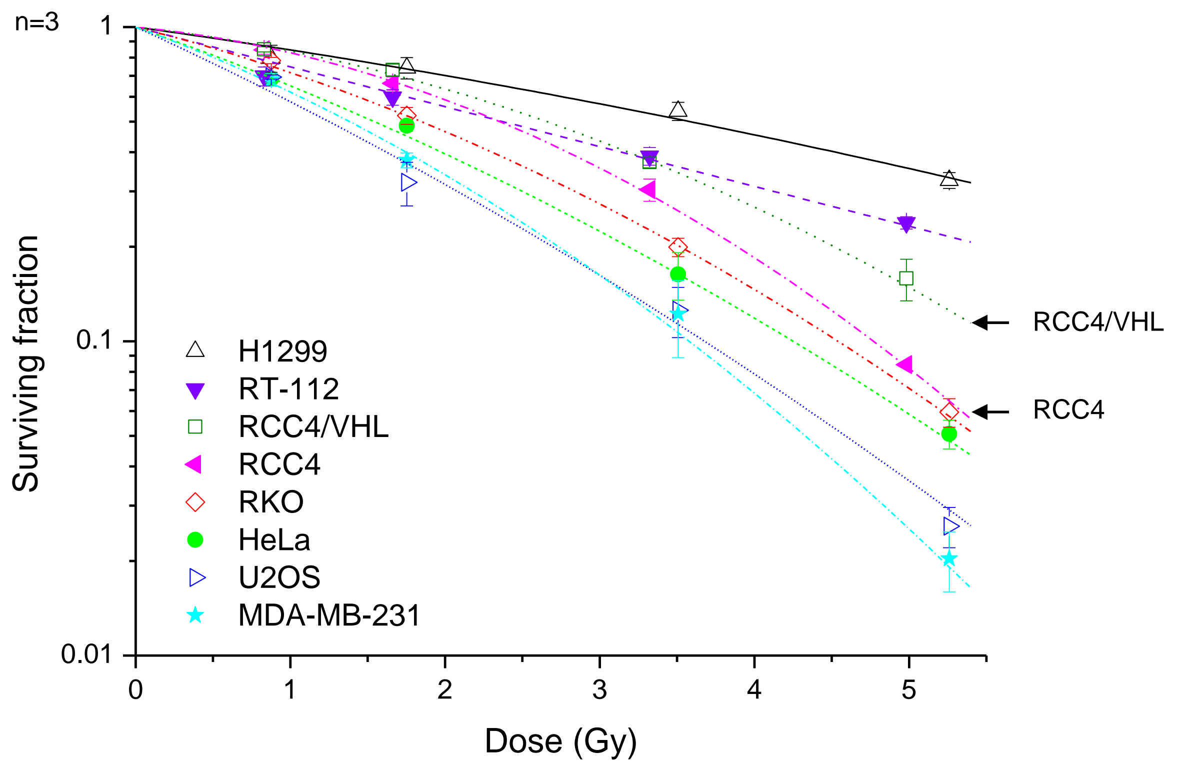

Figure 4.8 Comparison of radiosensitivity in various mammalian tumour cell lines in response to irradiation

Clonogenic survival of the indicated cell lines exposed to irradiation (0 - 5.3 Gy). Post irradiation cells were allowed to form colonies and the surviving fractions were calculated.

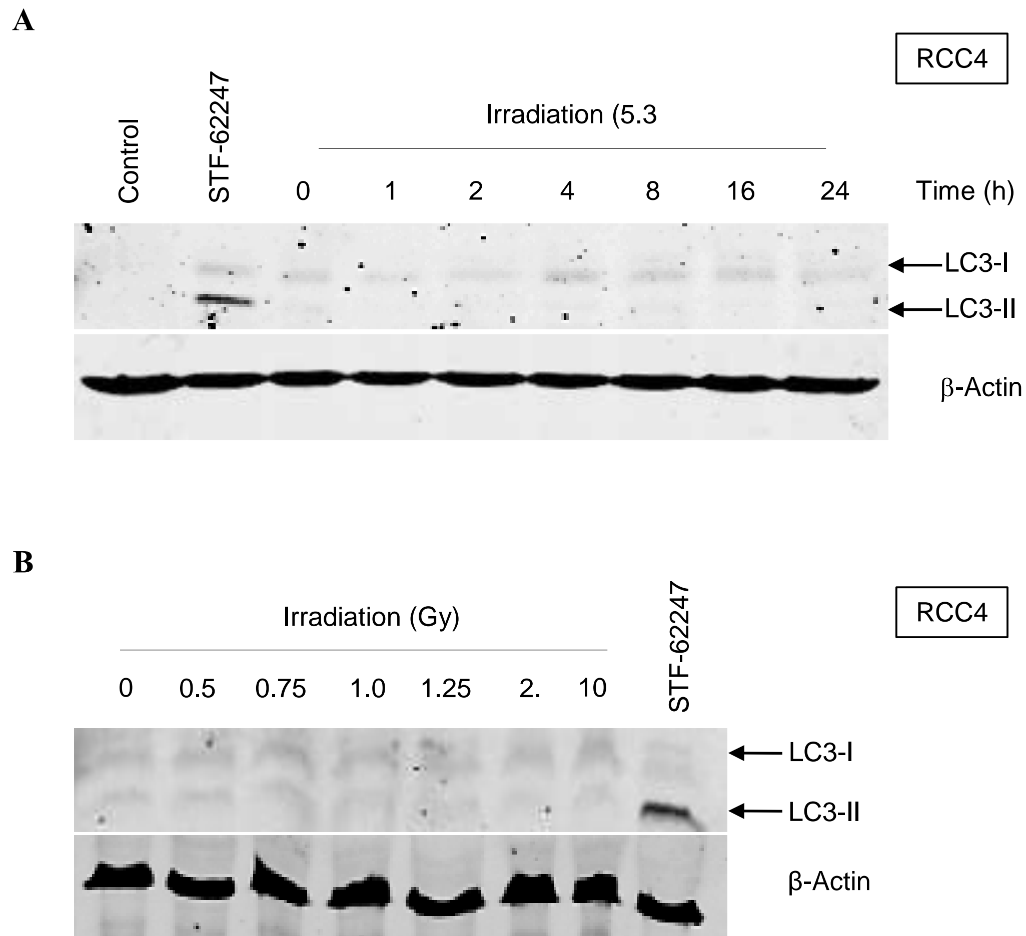

Figure 4.9 Ionising irradiation does not induce autophagy in RCC4 cells
RCC4 cells were exposed either to (**A**) 5.3 Gy radiation and harvested over the indicated periods of time or (**B**) to the indicated doses of radiation (0 - 10 Gy) and 24 h post radiation, irradiated cells were harvested. Western blot analysis was carried out for LC3 and β-Actin (loading control). The STF-62247 (0.62 µM, 16 h) pre-treated RCC4 cells were used as a positive control for LC3 cleavage.

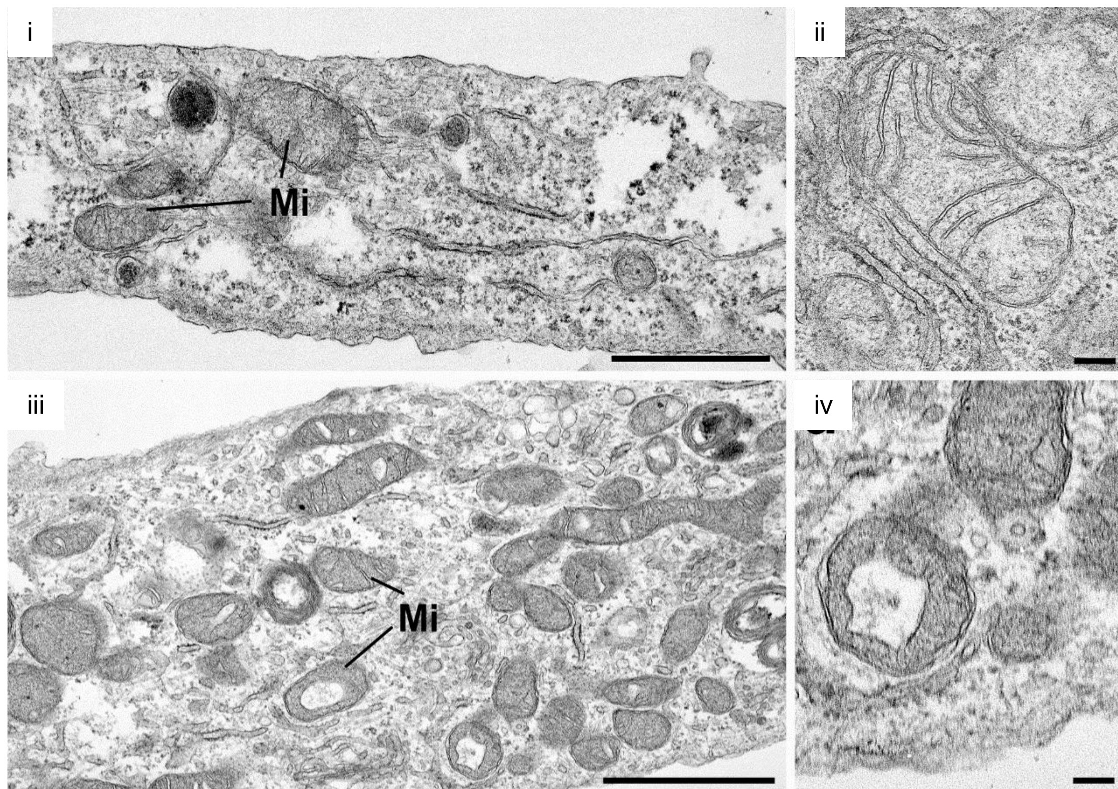

Figure 4.10 Morphological changes in RCC4 cells mitochondria exposed to ionising irradiation
Electron microscopy of RCC4 treated cells exposed to 3.5 Gy irradiation. (i) Low power showing the cytoplasm of a control cell with normal cytoplasm containing mitochondria (Mi). Bar = 1 μm. (ii) Detail showing the normal mitochondrial structure in a control cell. Bar =100 nm. (iii) Low power of the cytoplasm of an irradiated cell showing a number of abnormal mitochondria (Mi). Bar = 1 μm. (iv) Detail of the mitochondria from an irradiated cell showing dilatation of the cristae. Bar = 100 nm. EM carried out by Dr. David J. P. Fergusson.

4.2.8 STF-62247 increases radiosensitivity in a VHL-dependent manner

To test our hypothesis that the induction of autophagic cell death in combination with irradiation increases cell killing of VHL-deficient RCCs, we added STF-62247 to RCC4 and 786-O cells and compared their response with the associated VHL-proficient RCC4/VHL and 786-O/VHL cells. To address our hypothesis we determined initially the pre-treatment time required for the induction of autophagy. Increased induction of LC3 cleavage was observed from 8 h onwards in RCC4 cells exposed to STF-62247 (Figure 4.11). Thus, indicating that the induction of autophagy by STF-62247 pre-treatment is time dependent. We further investigated if the combination of STF-62247 pre-treatment with irradiation shall increase the cell killing. Increased loss of cell survival was observed in RCC4 cells pre-treated with STF-62247 for 24 h and irradiated (Figure 4.12).

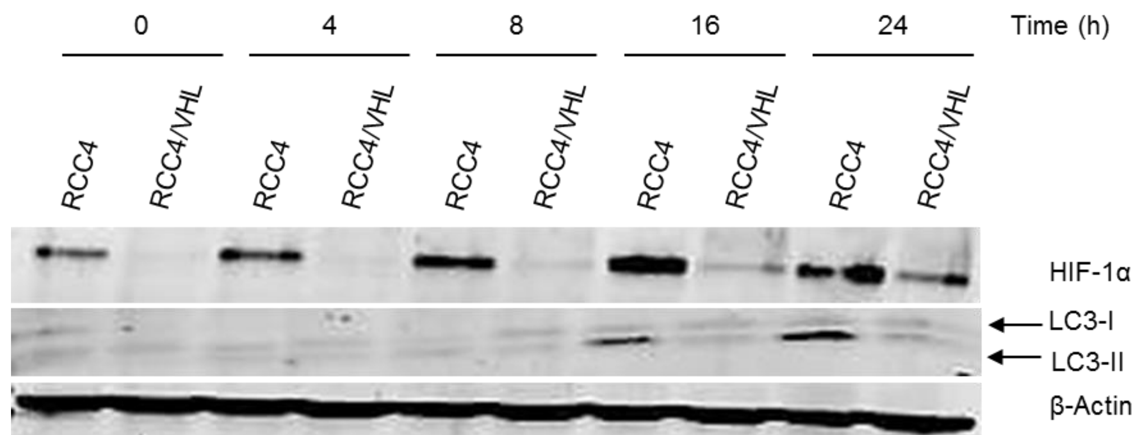

Figure 4.11 Induction of autophagy by STF-62247 is time-dependent
The RCC4 and RCC4/VHL cells were treated with 1.25 μM STF-62247 and harvested at the indicated times. Western blotting was carried out for LC3 and β-Actin (loading control).

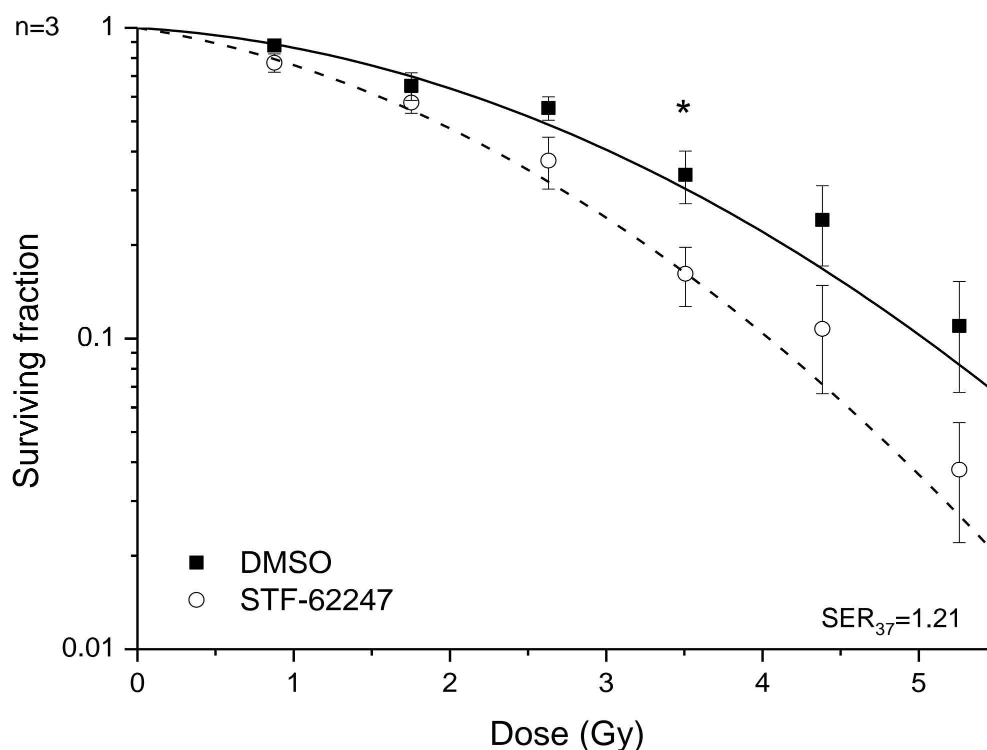

Figure 4.12 Pre-treatment with STF-62247 increases radiosensitivity in RCC4 cells
Colony survival of RCC4 cells pre-treated with STF-62247 (0.62 μM, 24 h) and exposed to the indicated doses of irradiation. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. '*' p-Value <0.05.

In order to verify that this loss of cell viability in RCC4 was due to the combined effect of STF-62247 and irradiation, a colony survival assay was performed. Increasing concentrations of STF-62247 in conjunction with 3.5 Gy resulted in increased cell kill in RCC4 cells (Figure 4.13). This significant loss of cell survival observed indicates that the combined effect of irradiation with STF-62247 pre-treatment in RCC4 cells is more toxic than the STF-62247 pre-treatment alone.

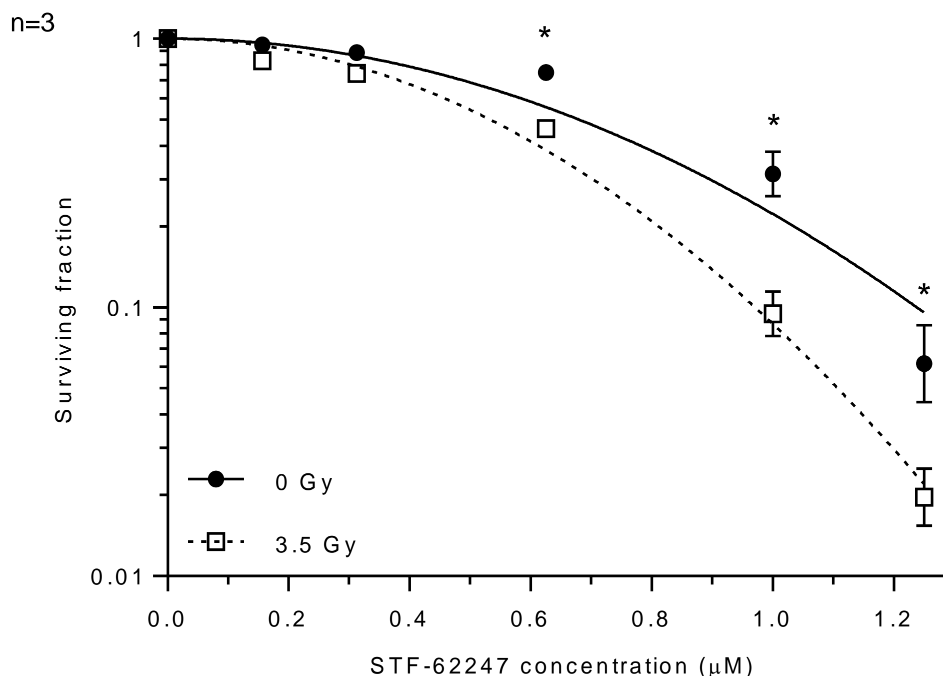

Figure 4.13 Increased doses of STF-62247 exposure in combination with irradiation decreases survival in RCC4 cells
RCC4 cells were pre-treated with the indicated doses of STF-62247 (16 h) and were then irradiated (3.5 Gy) or mock irradiated. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. '*' p-Value <0.05.

4.2.9 RCC4 cells are radiosensitised by STF-62247 through the induction of autophagy

In order to determine whether the effect of STF-62247 was due to autophagy induction, we used the drug in combination with irradiation in the RCC4/VHL cell line, which is resistant to STF-62247-induced autophagic death. Addition of STF-62247 made no difference to radiation-induced killing in RCC4/VHL cells (Figure 4.14). Significantly, the increased radiosensitivity with STF-62247 treatment was not restricted to RCC4s as we also observed this in 786-O cells (Figure 4.15A). As predicted, STF-62247 treatment had no effect on the radiosensitivity of 786-O/VHL cells (Figure 4.15B). To further confirm

our results that the radiosensitisation effect of STF-62247 was due to the induction of autophagy, RCC4 cells were exposed to irradiation in combination with STF-62247 and 3-MA. 3-MA is a class III PI3K inhibitor that inhibits the initial phase of autophagy process by blocking the autophagosome formation. The cells treated in combination with STF-62247 and 3-MA, when exposed to irradiation had an increased survival compared to the cells irradiated with STF-62247 alone. Therefore, suggesting that the effect of radiosensitisation is due to the induction of autophagy with STF-62247 treatment (Figure 4.16). These data strongly support our hypothesis that the induction of pro-death autophagy can increase radiosensitivity in VHL-deficient RCC cells.

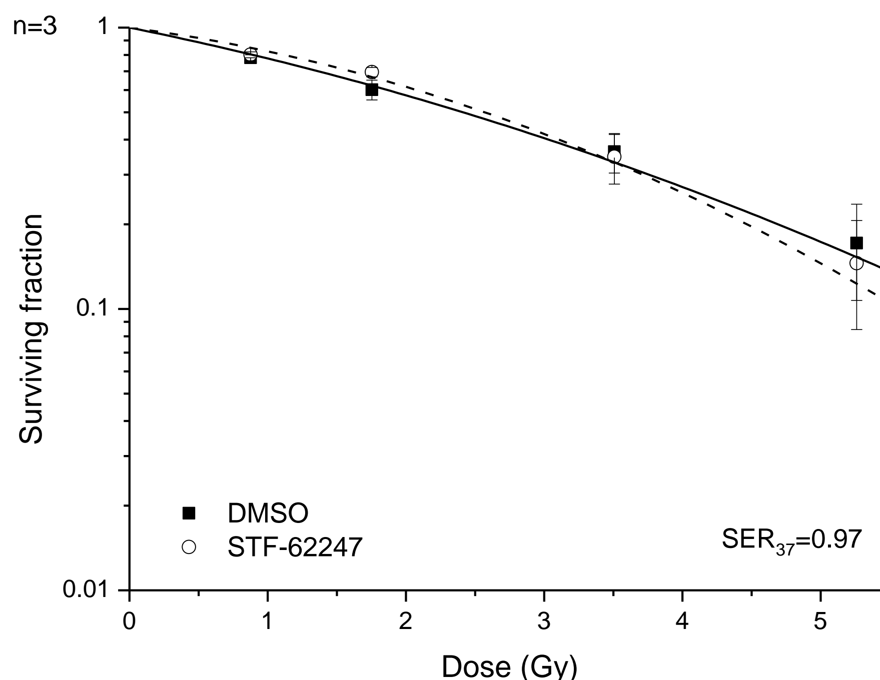

Figure 4.14 RCC4/VHL cells are radiation resistant to STF-62247 pre-treatment
RCC4/VHL cells were pre-treated with STF-62247 (0.62 μM) for 24 h and then irradiated for the indicated doses. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated.

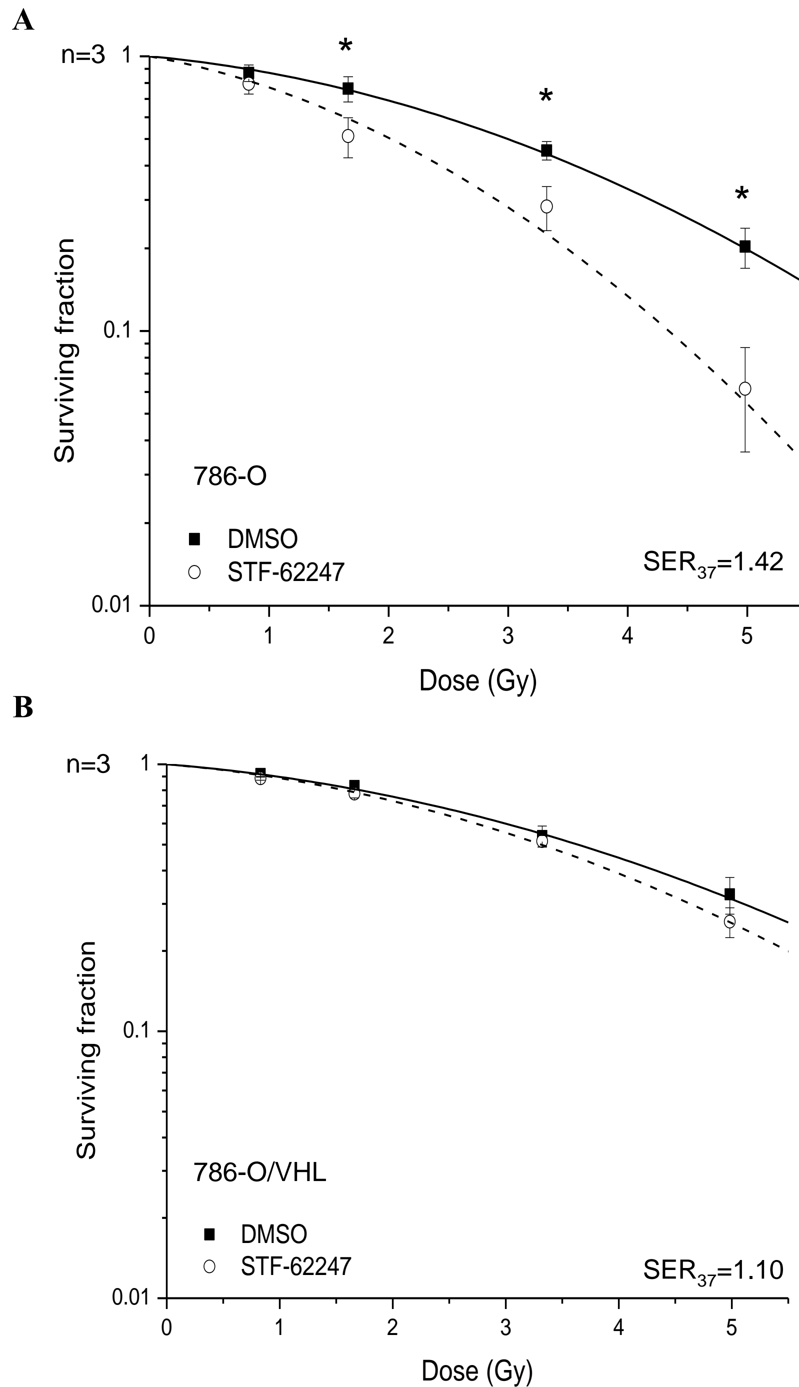

Figure 4.15 STF-62247 pre-treatment selectively radiosensitises VHL-deficient 786-O cells

(**A**) 786-O cells were pre-treated with STF-62247 (0.62 μM) for 24 h and then irradiated as indicated (**B**) 786-O/VHL cells were pre-treated with STF-62247 (0.62 μM) for 24 h and then irradiated as indicated. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. '*' p-Value <0.05.

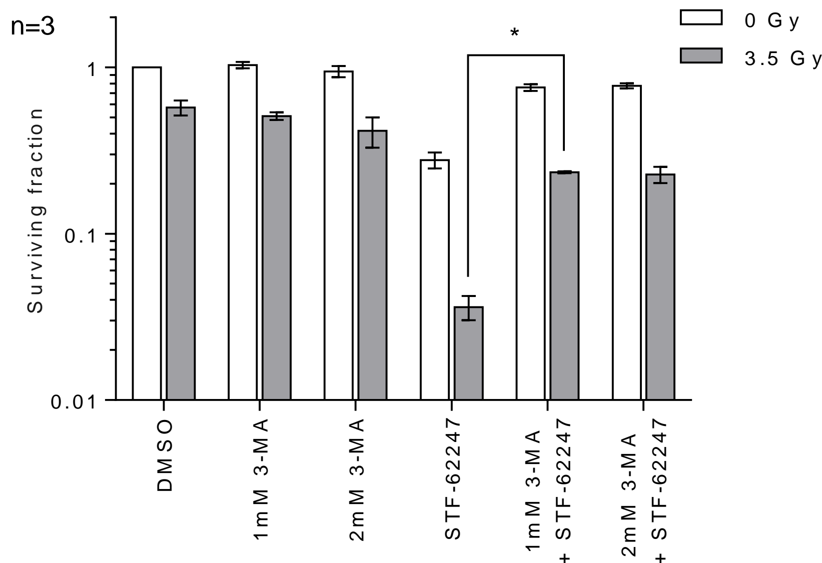

Figure 4.16 RCC4 cells are radiosensitised by STF-62247 through the induction of autophagy
Colony survival of RCC4 cells pre-treated either with STF-62247 (0.62 µM) alone or in combination with the indicated doses of 3-MA for 16 h, and then irradiated as indicated. Post treatment cells were allowed to form colonies and the surviving fractions were calculated. '*' p-Value <0.05.

4.2.10 STF-62247 radiosensitises RCC4 cells under physiologically relevant concentrations of oxygen

It is important to verify that exposure to STF-62247 increased radiosensitivity in conditions, which more accurately mimic physiological conditions. To achieve this cells were exposed to 3% O_2, which more accurately reflects oxygen concentrations found within the body as opposed to those routinely used in tissue culture (Hammond et al, 2014). RCC4 cells were exposed to 3% O_2 for the entire STF-62247 pre-treatment period (16 h), irradiated at 3% O_2 and then allowed colonies to form in normal tissue culture conditions . STF-62247 significantly increased radiation-induced killing in hypoxic conditions suggesting that this effect would occur in physiologically relevant conditions (Figure 4.17).

Finally, electron microscopy of RCC4 cells treated with both STF-62247 and irradiation (3.5 Gy) revealed increased numbers and size of electron lucent vacuoles compared to either treatment alone (Figure 4.18). In addition, certain cells showed mitochondrial changes similar to those seen after radiation. Thus, induction of autophagy can increase sensitivity of RCC under hypoxic conditions to radiation.

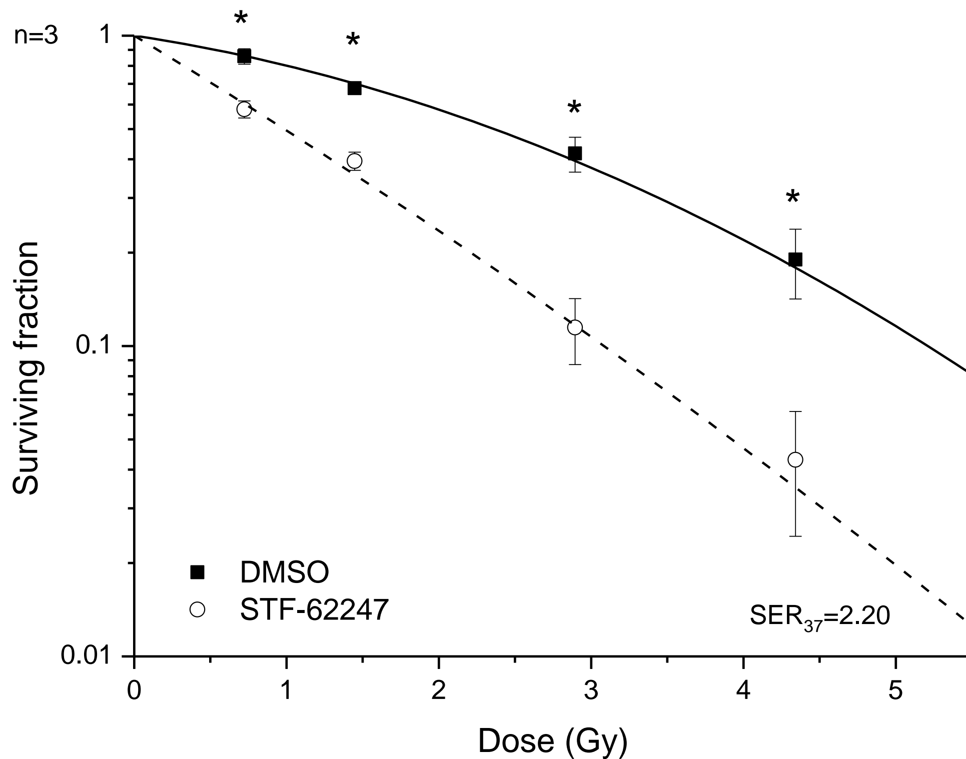

Figure 4.17 STF-62247 radiosensitises RCC4 cells, under physiologically relevant concentrations of oxygen
RCC4 cells were pre-treated with STF-62247 (0.62 μM) for 24 h at 3% O_2 and exposed to irradiation under 3% O_2 at the indicated doses. Post irradiation cells were allowed to form colonies under normal tissue culture conditions and the surviving fractions were calculated. '*' p-Value <0.05.

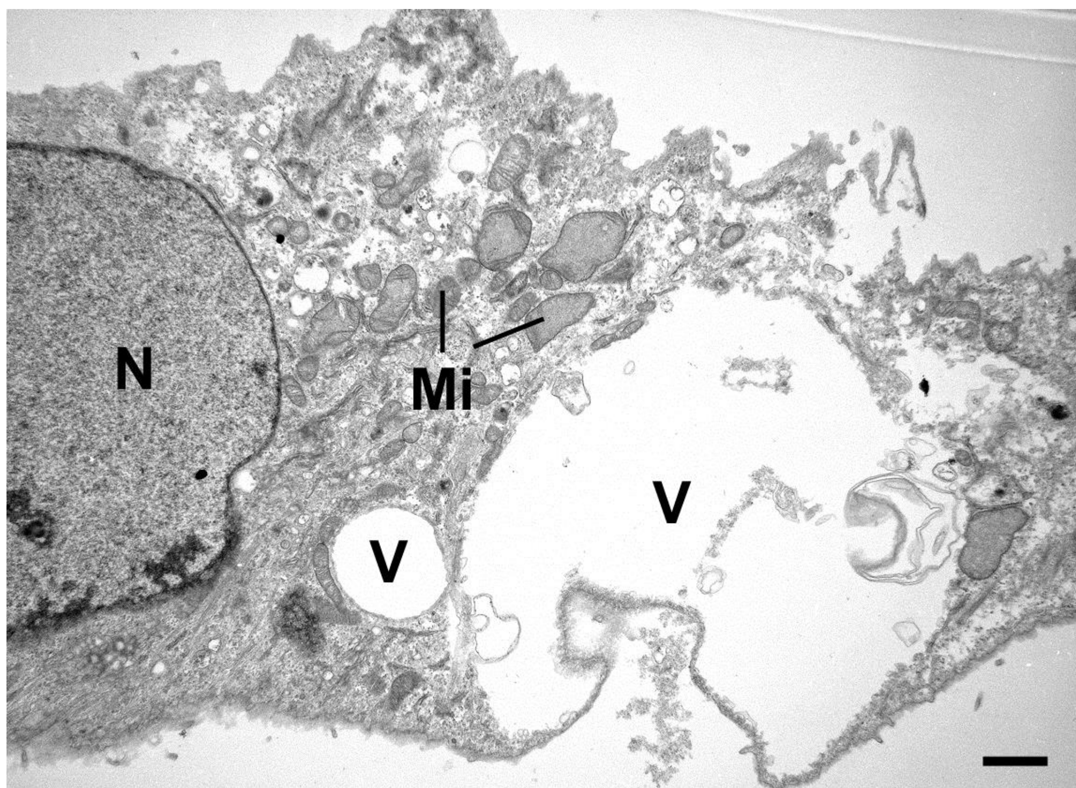

Figure 4.18 Morphological appearance of RCC4 cells pre-treated with STF-62247 and exposed to irradiation
Electron microscopy of RCC4 cells treated with STF-62247 and 3.5 Gy irradiation, showing the cytoplasm containing a number of vacuoles (V) of various sizes most of which were enclosed by a single membrane and mitochondria (Mi) with disrupted cristae. N = nucleus. Bar is 1 μm.

4.2.11 The rapamycin analogue temsirolimus increases radiation sensitivity of RCC4 cells

Temsirolimus can increase the effects of radiation in glioblastomas, therefore we investigated the combination of temsirolimus and radiation in VHL-deficient RCCs (Sarkaria et al, 2010). We first verified that temsirolimus is more toxic to RCC4 cells when compared to RCC4/VHL cells (Figure 4.19). Pre-treatment of RCC4 cells with temsirolimus increased the effects of radiation (0-5.3 Gy), suggesting that the combination of these therapies together would be beneficial (Figure 4.20A). Importantly, the reciprocal

experiment of adding radiation (one dose of either 1.75 or 3.5 Gy) increased the effects of temsirolimus (Figure 4.20B).

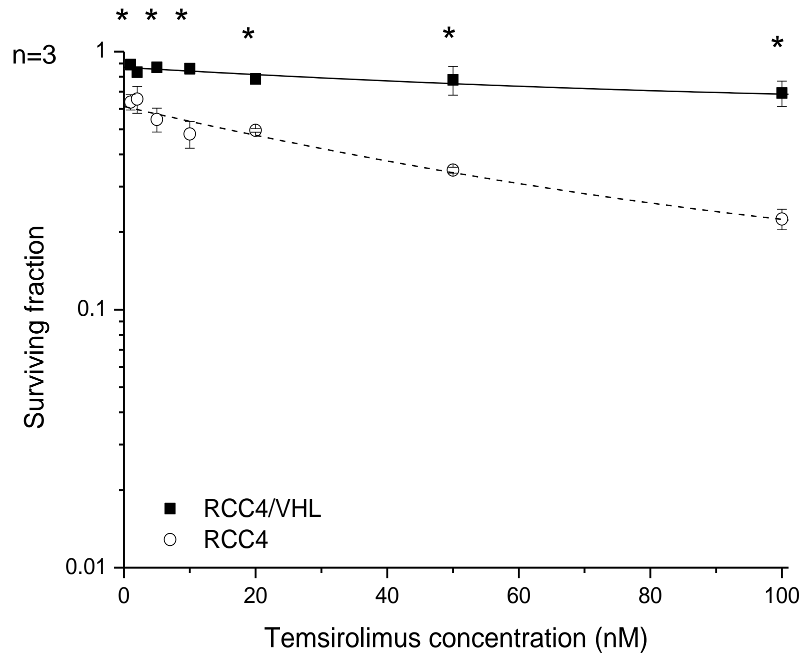

Figure 4.19 Dose dependent cell survival kinetics of RCC4 and RCC4/VHL cells treated with temsirolimus
Clonogenic survival of RCC4 and RCC4/VHL cells treated with temsirolimus (0-100 nM). Post treatment cells were allowed to form colonies and the surviving fractions were calculated. '*' p-Value <0.05.

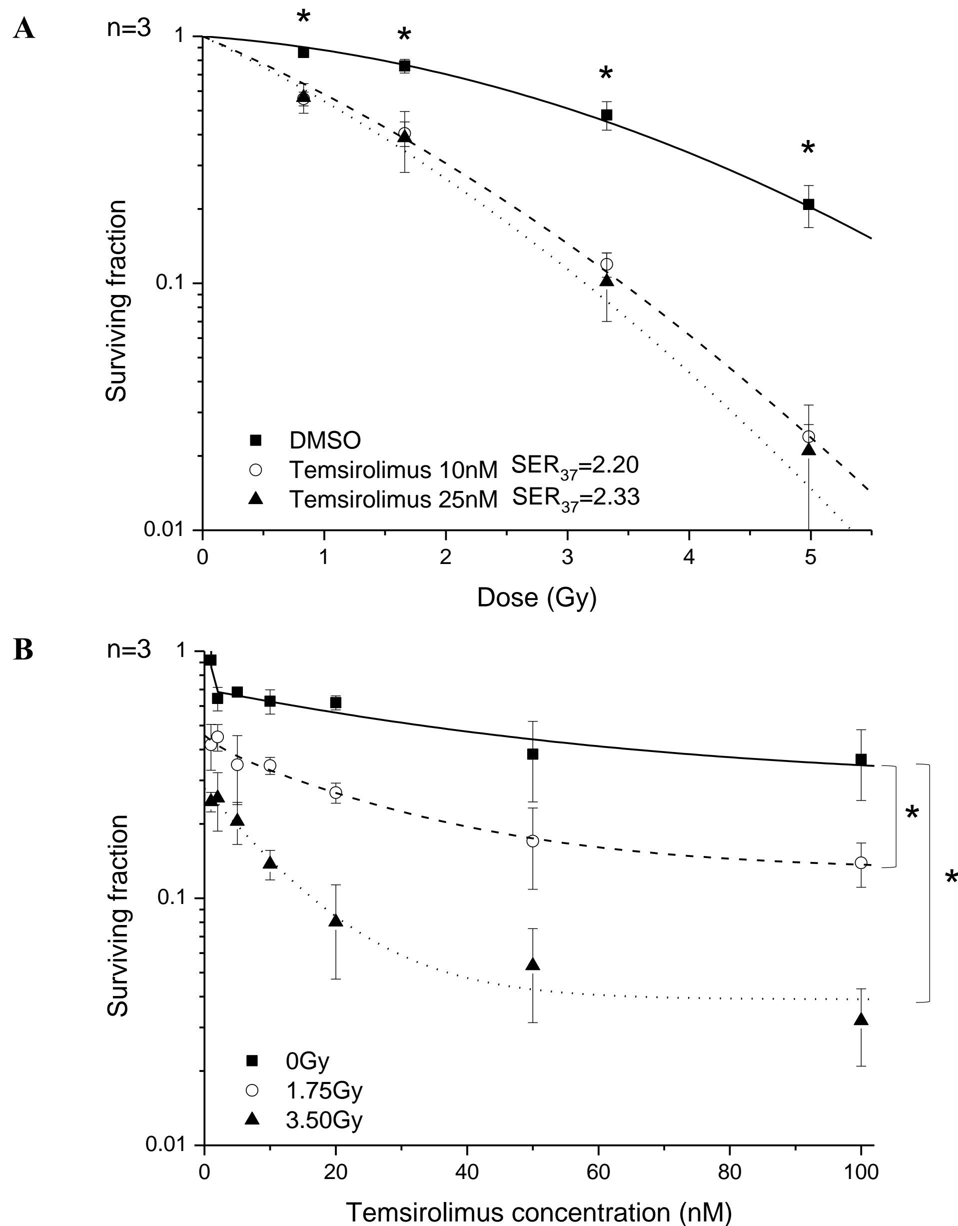

Figure 4.20 Temsirolimus increases radiation sensitivity of RCC4 cells
(**A**) Clonogenic survival of RCC4 cells pre-treated with temsirolimus (10 or 25 nM) for 24 h and then exposed to radiation (0 - 5 Gy). (**B**) RCC4 cells were pre-treated with the indicated doses of temsirolimus and then either mock-irradiated or irradiated (1.75 or 3.5 Gy). Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. '*' p-Value <0.05.

4.3 Discussion

This study highlights the possibility of combining targeted therapeutics such as STF-62247 or temsirolimus with radiation to reduce the reliance on partial or full nephrectomy and improve patient prognosis (Anbalagan et al, 2012).

We have shown that the radioresistance of RCC cells can be modulated by combination with targeted chemotherapeutics. Pre-treatment with STF-62247, a compound shown to induce pro-death autophagy in cells lacking VHL, significantly increased the response to radiation in both the RCC4 and 786-O cell lines. Given that this compound appeared well tolerated in previous xenograft studies, it has real potential as a radiosensitiser (Turcotte et al, 2008a). Radiotherapy is not generally considered for the treatment of RCC for a number of reasons including the relative radioresistance of RCC, the radiosensitivity of the surrounding tissue and the toleration of nephrectomy. However, surgical removal is not always possible, for example if there is vascular involvement or reduced kidney function. The identification of compounds which radiosensitise RCC is essential for radiotherapy to be truly useful in the treatment of kidney cancer. In addition to using radiotherapy to treat primary disease, increasing radiosensitivity could increase the efficacy of palliative radiotherapy for metastases in bone or the brain. It is also conceivable that radiotherapy could be used to palliate symptoms such as pain or haematuria, which can be associated with unresectable primary disease. It is however unlikely that STF-62247, a new therapy, will be tested clinically with radiation without supporting preclinical studies. Therefore, we also tested radiation in combination with compounds already used to treat RCC. Temsirolimus and other rapamycin analogues were of particular interest, as they, like STF-62247, have been shown to specifically kill VHL-deficient cells, and most importantly, also induce autophagy (Thomas et al, 2006; Yazbeck et al, 2008; Zhang et al, 2013). The mechanism of temsirolimus-induced autophagy is

distinct from that described for STF-62247 and in some ways is much more clearly understood. Temsirolimus inhibits mTOR, while STF-62247 interferes with golgi-endoplasmic reticulum transport (Turcotte et al, 2008a; Turcotte et al, 2008b). Our data demonstrate that adding radiation increases the effects of clinically relevant doses of temsirolimus in RCC cells. Together, our data demonstrate that combining radiotherapy for RCC with molecularly targeted therapies is a valid approach that should be evaluated further in pre-clinical models.

Chapter 5

Identification of autophagy promoting compounds which can increase radiation sensitivity

5.1 Introduction

We have demonstrated that renal carcinoma cells, RCC4 and 786-O deficient in VHL gene can be radiosensitised through the induction of autophagy (Chapter 4, (Anbalagan et al, 2012). This led us to hypothesise that additional autophagy inducing compounds combined with radiation could increase the radiation sensitivity of cancer cell lines. To investigate this we identified, from the literature, a number of compounds which have been demonstrated to induce autophagy, albeit in a wide range of cell lines and contexts. Important criteria for selection of the compounds included the use of the compound in clinical settings although not necessarily cancer related (Table 5.1). The advantage of selecting drugs this way includes insight into the side effects and tolerability of the drugs.

As our studies became focused on CHR-2797 (also known as Tosedostat) this drug is described further here. CHR-2797 targets the M1 family of Zn^{2+} metalloenzymes and selectively inhibits aminopeptidase N. Aminopeptidase N (also known as CD13 or alanine aminopeptidase) is an exopeptidase which catalyses the cleavage of an amino acid residue on the free N-terminus of polypeptide chains. It is also involved in the regulation of biologically active peptides, in protein recycling, antigen presentation and also as a receptor for human corona viruses (Chen et al, 2012; Saric et al, 2004; Taylor, 1993; Yeager et al, 1992). CHR-2797 is functionally related to bestatin, which is a naturally occurring aminopeptidase inhibitor. CHR-2797 has been reported to have more specificity in targeting the aminopeptidase N than bestatin (Krige et al, 2008). Although bestatin has been shown to improve overall survival in patients with leukaemia and solid tumours including malignant melanoma, carcinoma of lung, stomach, bladder, head and neck and oesophagus, it is not in widespread clinical use outside Japan (Ichinose et al, 2003; Ota & Uzuka, 1992; Wakita et al, 2012). CHR-2797 is a hydroxymate-containing ester prodrug

that undergoes intracellular de-esterification to form the acid metabolite CHR-79888. CHR-79888 is a charged acid metabolite and has extremely low membrane permeability leading to intracellular accumulation. CHR-2797 not only inhibits the aminopeptidase N but also LTA4 hydrolase, LAP and PuSA due to overlapping substrate preferences. CHR-2797 is less cytotoxic in non-transformed cells (U-937, NCI-H23, MRC5, and NRK) compared to transformed cells (Jenkins et al, 2011; Krige et al, 2008).

Krige and colleagues demonstrated that CHR-2797 has an antiproliferative effect and induces apoptosis in multiple myeloma, myeloid leukaemia and lymphoma cancer cell lines. The antiproliferative effect was proposed to be due to depletion of free intracellular amino acids and the accumulation of smaller peptides that would normally act as substrates for aminopeptidases (Krige et al, 2008). Autophagy has been reported in response to CHR-2797, which was proposed to be due to the decreased protein turnover (Moore et al, 2009). Protein recycling is important for cellular homeostasis and of utmost importance for cancer cells, which are dependent on amino acids for their rapid growth and survival (Scott et al, 2000).

Inhibition of aminopeptidase N by CHR-2797 leads to intracellular accumulation of small peptides which act as substrates for aminopeptidases. The accumulation of smaller peptides results in the depletion of free amino acids that are required for cellular metabolism. This depletion of free amino acids triggers a transcriptional response involved in the up regulation of amino acid synthetic genes, transporters, and tRNA synthetases. This transcriptional response is called the amino acid deprivation response (AADR). Furthermore, CHR-2797 mediated aminopeptidase inhibition decreases the phosphorylation levels of mTOR substrates, which have a role in cellular metabolism (Krige et al, 2008).

AIM: To test the hypothesis that autophagy inducing candidate drugs combined with radiation could radiosensitise cancer cell lines.

5.2 Results

5.2.1 Autophagy induction by the candidate radiosensitisers

As autophagy is known to be both cell line and context dependent we investigated whether the chosen drugs (valproic acid, clonidine, minoxidil, CHR-2797 and niclosamide) (Table 5.1) induced autophagy in our *in vitro* systems. The doses of the drugs used for our studies were selected from the literature (Fu et al, 2010; Gies et al, 2010; Krige et al, 2008; Moore et al, 2009; Williams et al, 2008a). Using a panel of cells lines (U87-MG, OE21 and HCT116) we determined if the treatment with candidate drugs led to LC3 cleavage (Figure 5.1). Of the tested drugs only niclosamide showed any evidence of autophagy induction. Glucose uptake has been demonstrated to either induce or suppress autophagy (Kobayashi et al, 2012; Maruyama et al, 2008; Ravikumar et al, 2003; Sarkar et al, 2007). Routinely our experiments were performed in cell lines cultured using cell culture media supplemented with 25 mM glucose. Therefore, we investigated if autophagy can be induced by the candidate drugs in cancer cell lines cultured in media with physiological levels of glucose. HeLa, HCT116, U87-MG cancer cell lines cultured in media containing physiologically relevant levels of glucose (5 mM) were treated with the selected candidate drugs. The drugs valproic acid, clonidine, minoxidil and CHR-2797 did not induce significant LC3 cleavage in the treated cell lines (Figure 5.2). Irrespective of the glucose concentration (5 or 25 mM) in the culture media only treatment with niclosamide showed evidence of LC3 cleavage.

Drugs	Current clinical use	Working concentration used in the referenced studies	Mechanism of autophagy induction	Reference
Valproic acid	Anti-convulsant	1 mM	Increased oxidative stress	Fu et al, 2010
Clonidine	Anti-hypersensitive	1 µM	L-type Ca^{2+} channel antagonist, K^{+} ATP channel opener	Williams et al, 2008a
Minoxidil	Vasodilator and slows hair loss	1 µM	G protein coupled receptor signalling activator	Williams et al, 2008a
Tosedostat (CHR-2797)	AML and solid tumours (under clinical trials)	100 nM to > 10 µM	Aminopeptidase inhibitor	Cortes et al, 2013; Krige et al, 2008
Niclosamide	Antihelmintic & in clinical trials for AML patients	10 µM	Proteosomal inhibitor	Gies et al, 2010

Table 5.1 List of candidate drugs clinically either under use or in trials that are known to induce autophagy. A table describing a list of candidate drugs, their clinical use, working concentrations used in the referenced studies and its mode of autophagy induction.

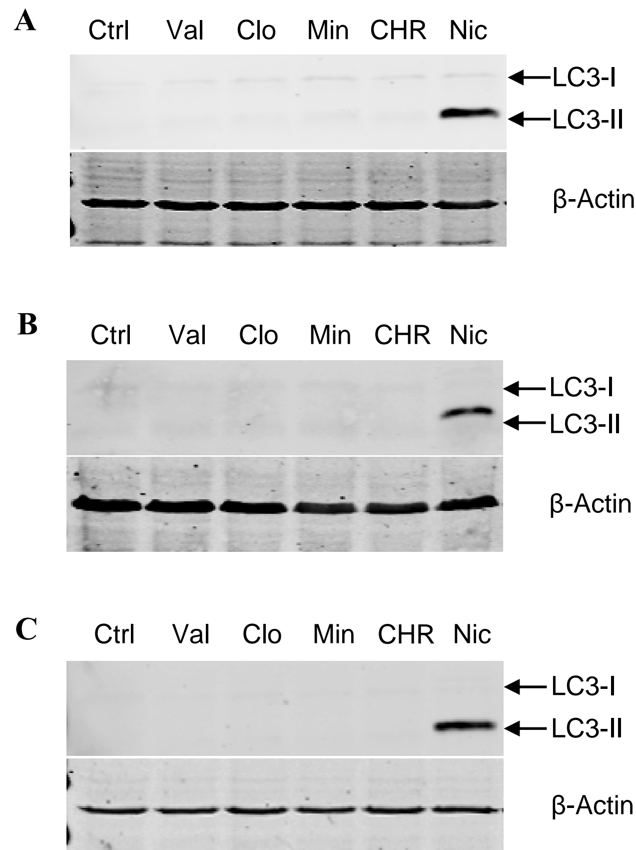

Figure 5.1 Expression levels of LC3 cleavage in a panel of cancer cell lines exposed to candidate drugs

Cell lines (**A**) U87-MG (**B**) OE21 (**C**) HCT116 were treated for 16 h with valproic acid (Val, 1 mM), clonidine (Clo, 1 μM), minoxidil (Min, 1 μM), CHR-2797 (CHR, 0.5 μM), or niclosamide (Nic, 10 μM), with the untreated acting as control (Ctrl). Western blotting was carried out for LC3 cleavage and β-Actin (loading control).

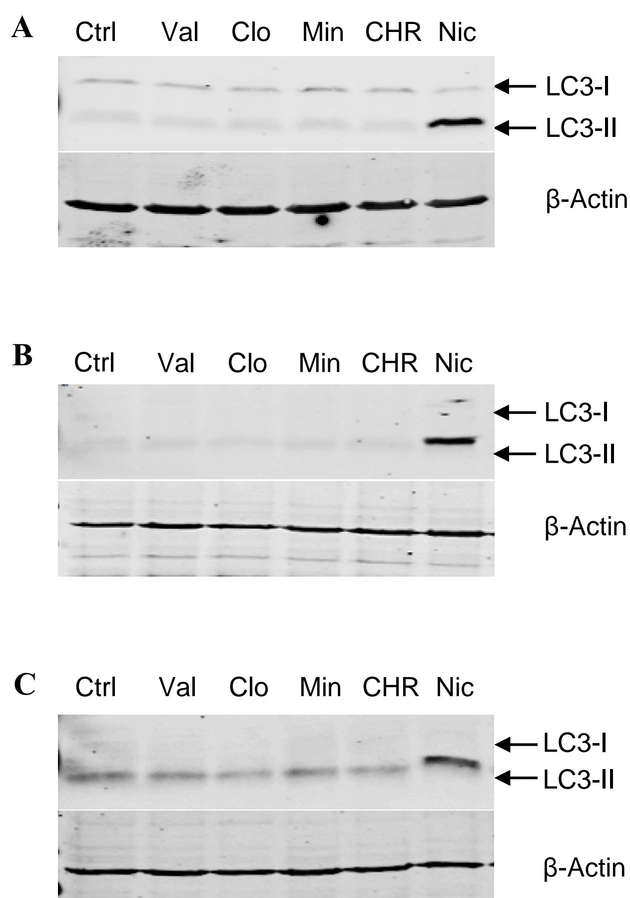

Figure 5.2 Expression levels of LC3 cleavage in a panel of cancer cell lines cultured in media supplemented with 5 mM glucose and exposed to candidate drugs
Cell lines (**A**) U87-MG (**B**) HCT116 (**C**) HeLa cultured in media supplemented with 5 mM glucose were treated for 16 h with valproic acid (Val, 1 mM), clonidine (Clo, 1 μM), minoxidil (Min, 1 μM), CHR-2797 (CHR, 0.5 μM), or niclosamide (Nic, 10 μM), with the untreated acting as control (Ctrl). Western blotting was carried out for LC3 cleavage and β-Actin (loading control).

The lack of apparent autophagy in the selected cell lines was disappointing. We decided to research the literature for examples of cellular contexts, which could suggest cell lines that might respond to the drugs. We identified a report which described the use of bestatin in oesophageal cancer (Oka, 1980). The administration of bestatin has already been reported to radiosensitise human cervical cancer models (Tsukamoto et al, 2008). CHR-2797 one of our candidate drugs has been reported to be functionally related to

bestatin (Krige et al, 2008). Therefore, we decided to investigate the effect of CHR-2797 on oesophageal cell lines. The oesophageal cell line FLO-1 (adenocarcinoma) was pre-treated with CHR-2797 (0.5 µM) over a period of time (up to 8 h) and assessed for LC3 cleavage by western blot analysis (Figure 5.3A). We observed LC3 cleavage in FLO-1 cells pre-treated with CHR-2797 from 2 h onwards. In addition, we determined the levels of p62, another autophagic marker, in response to CHR-2797 pre-treatment. FLO-1 and OE21 cells were pre-treated with CHR-2797 (0.5 µM) over a period of time (up to 4 h) and assessed for p62 by western blot (Figure 5.3B). We observed a decrease in the levels of p62 in response to CHR-2797 pre-treatment at 4 h in both FLO-1 and OE21 cells. As a result of this analysis we decided to pursue Niclosamide and CHR-2797 for our studies.

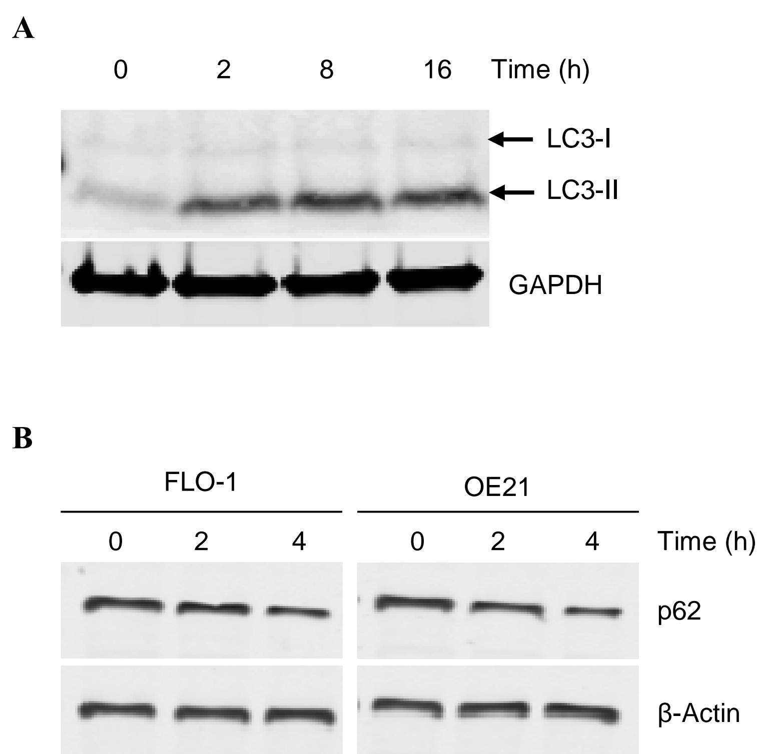

Figure 5.3 CHR-2797 induces autophagy in FLO-1 and OE21 cells.
(**A**) FLO-1 (**B**) FLO-1 and OE21 cells were pre-treated for the times indicated in the presence of CHR-2797 (0.5 µM). Western blotting was carried out for LC3 cleavage, p62, GAPDH (loading control) and β-Actin (loading control). Part B was performed by Deborah Biasoli.

5.2.2 Toxicity of Niclosamide and CHR-2797

As our goal was to identify a radiosensitiser it is important that the drug alone has little or no effect on cell viability. To address this we carried out colony survival assays on oesophageal cell lines FLO-1 and OE21 using niclosamide and CHR-2797.

Initially, the cell viability of FLO-1 and OE21 cells treated with niclosamide (10 μM) was assessed by colony survival assay (Figure 5.4). Treatment with niclosamide was significantly toxic in both FLO-1 and OE21 cells. Moreover, recently it has already been reported that niclosamide radiosensitises lung carcinoma cells and therefore we did not perform any further experiments with niclosamide (You et al, 2014). In order to test the toxicity of CHR-2797 we investigated the effect of CHR-2797 in the oesophageal cell lines FLO-1 and OE21 by colony survival assay (Figure 5.5). CHR-2797 treatment led to a significant decrease in FLO-1 and OE21 cell viability. Next we investigated the effects of cell proliferation in response to CHR-2797 in FLO-1 and OE21 and observed a significant effect on cell proliferation from 6 days onwards for FLO-1 and on the 4th day for OE21 (Figure 5.6).

Figure 5.4 Niclosamide treatment decreases cell viability in oesophageal cell lines FLO-1 and OE21

(**A**) Scheme of colony survival assay in oesophageal cell lines FLO-1 and OE21 treated continuously with niclosamide. (**B**) FLO-1 (**C**) OE21 were treated either with DMSO or niclosamide (10 μM) continuously throughout the assay and allowed to form colonies. Colonies formed were stained and the surviving fractions were calculated.

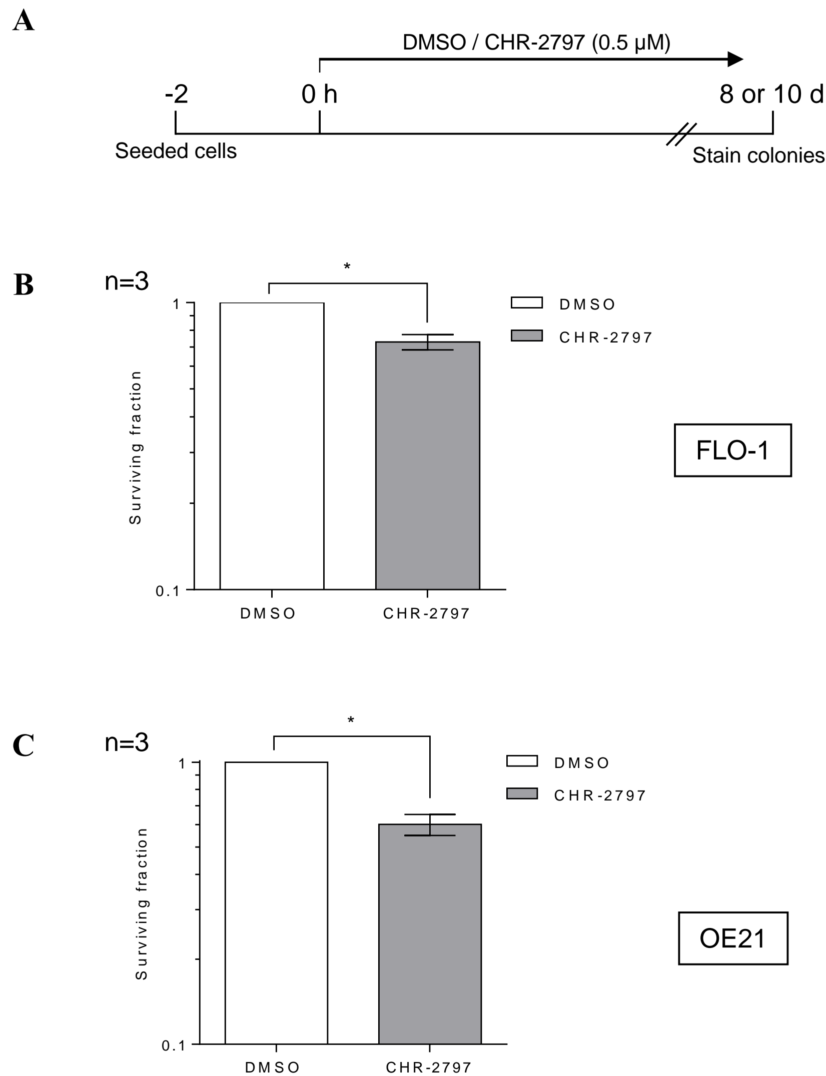

Figure 5.5 Effect of CHR-2797 treatment in oesophageal cell lines FLO-1 and OE21
(**A**) Scheme of colony survival assay in oesophageal cell lines FLO-1 and OE21 treated continuously with CHR-2797. (**B**) FLO-1 (**C**) OE21were treated either with DMSO or CHR-2797 (0.5 µM) continuously throughout the assay and allowed to form colonies. Colonies formed were stained and the surviving fractions were calculated. Significance value: '*' p-Value < 0.05.

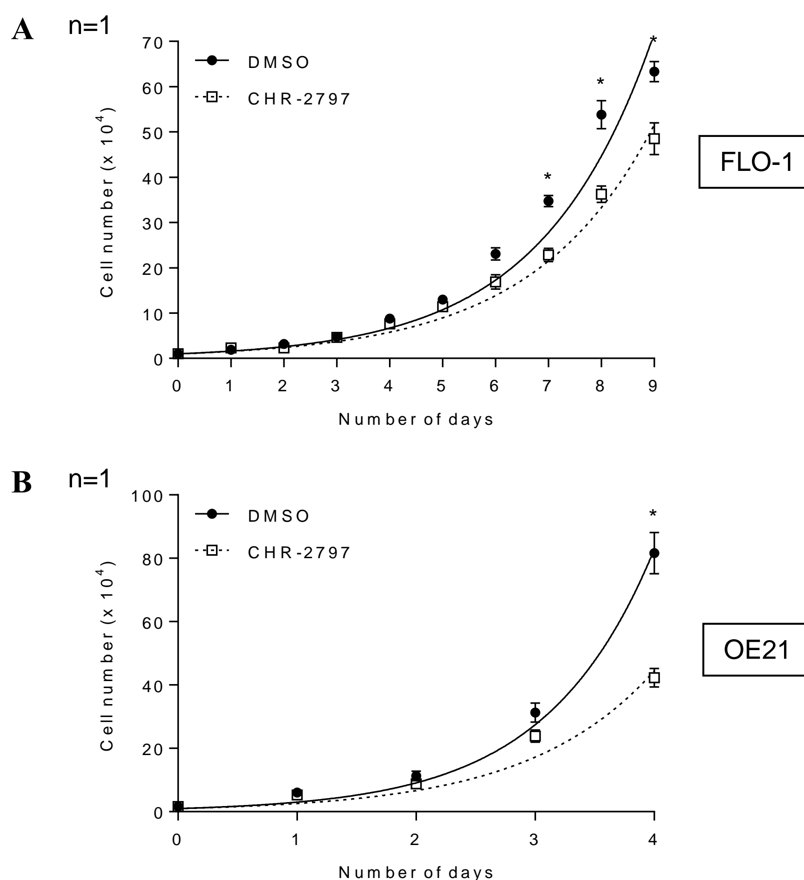

Figure 5.6 CHR-2797 treated FLO-1 and OE21 cells have decreased cell proliferation (**A**) FLO-1 (**B**) OE21 cells (1 x 10^4) were treated with CHR-2797 (0.5 µM) and their cell number was determined over the time period indicated. Significance value: '*' p-Value <0.05.

5.2.3 Oesophageal cancer cell lines FLO-1 and OE21 are radiosensitised by CHR-2797

We pre-treated the OE21 and FLO-1 oesophageal cell lines for 16 h with 0.5 µM CHR-2797 and then irradiated with 0-8 Gy. A colony survival assay was carried out (Figure 5.7). CHR-2797 pre-treatment radiosensitised both the oesophageal cell lines OE21 (SER_{37}=1.30) and FLO-1 (SER_{37}=1.60). Next, we carried out a colony survival assay after pre-treating FLO-1 and OE21 cells with increasing doses of CHR-2797 for 16 h followed by irradiation with 4 Gy or mock irradiated (Figure 5.8). Loss of cell viability

was observed in both FLO-1 and OE21 cells when pre-treated with increasing doses of CHR-2797 in combination with radiation.

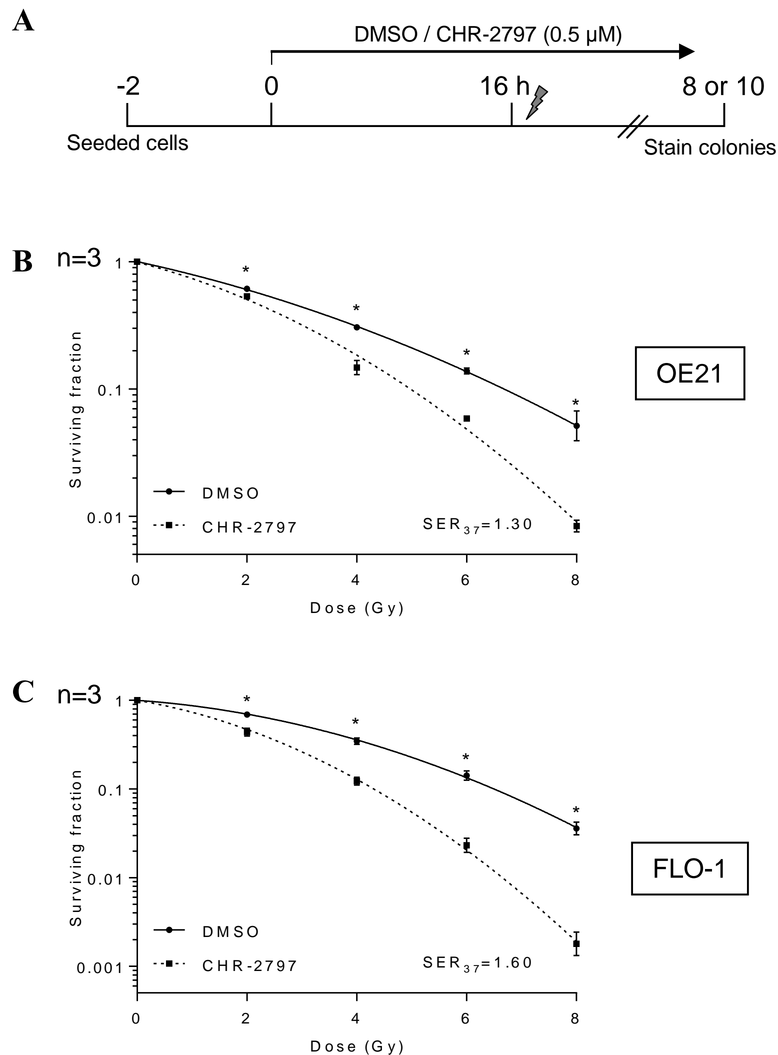

Figure 5.7 Oesophageal cancer cell lines FLO-1 and OE21 are radiosensitised by CHR-2797
(**A**) Scheme of colony survival assay in oesophageal cells pre-treated with CHR-2797 and exposed to radiation. (**B**) OE21 and (**C**) FLO-1 cells were treated either with DMSO or CHR-2797 (0.5 μM) for 16 h and then exposed to a range of radiation doses (0 – 8 Gy). Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05.

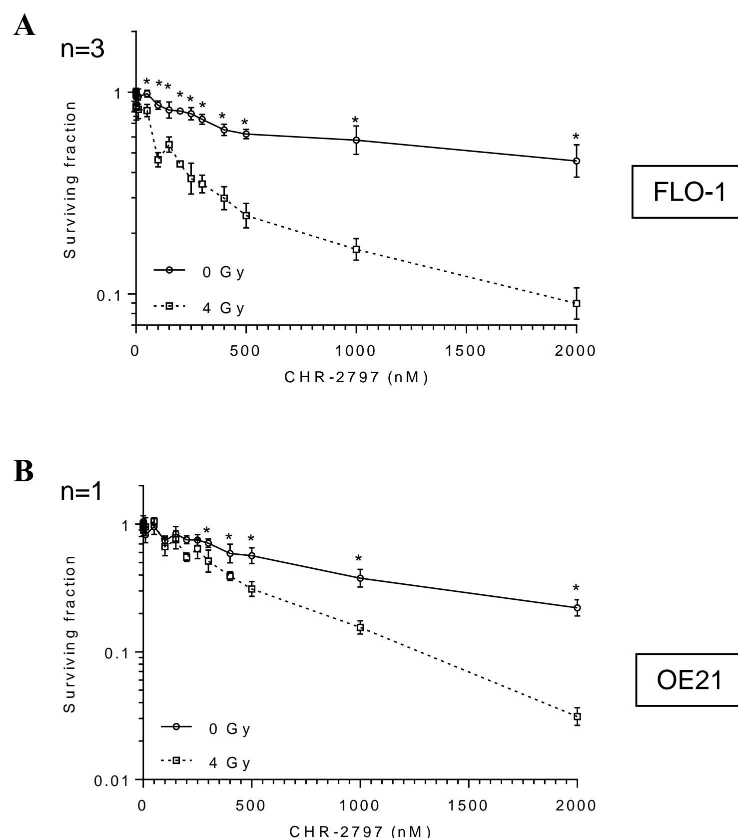

Figure 5.8 Radiosensitivity of FLO-1 and OE21 in response to CHR-2797 treatment is dose dependent

(**A**) FLO-1 (**B**) OE21 cells were treated with CHR-2797 for 16 h with the indicated doses and exposed to either 4 Gy or mock irradiated. Post irradiation the cells were allowed to form colonies and the surviving fraction were calculated. Significance value: '*' p-Value <0.05.

Since CHR-2797 pre-treatment of the oesophageal cell lines lead to an increase in radiosensitivity, we asked if its effects were restricted to oesophageal cell lines. HeLa and RKO cells were pre-treated for 16 h with CHR-2797 (0.5 μM) and then irradiated (0 – 6 Gy) and assayed by colony survival assay (Figure 5.9). Interestingly, although the trend in loss of cell viability was apparent, the effect on radiosensitivity with RKO (SER_{37}=1.14) and HeLa (SER_{37}=1.16) cells pre-treated with CHR-2797 was less than observed in the oesophageal cell lines. The reason for the differences in the radiosensitivity with CHR-2797 pre-treatment among FLO-1, OE21, RKO and HeLa cells needs further investigation. Unfortunately, primary oesophageal cell lines were not available and therefore, we pre-

treated the lung fibroblast cells MRC-5 for 18 h with CHR-2797 (0.1, 0.5 or 1 μM) and irradiated (0 – 6 Gy) (Figure 5.10). We observed decreased cell viability in MRC-5 cells pre-treated with CHR-2797 in response to radiation. The sensitisation enhancement ratio due to combined treatment of CHR-2797 and radiation was observed to be similar in MRC-5 and OE21 (SER_{37}=1.30), but less than observed in FLO-1 (SER_{37}=1.60).

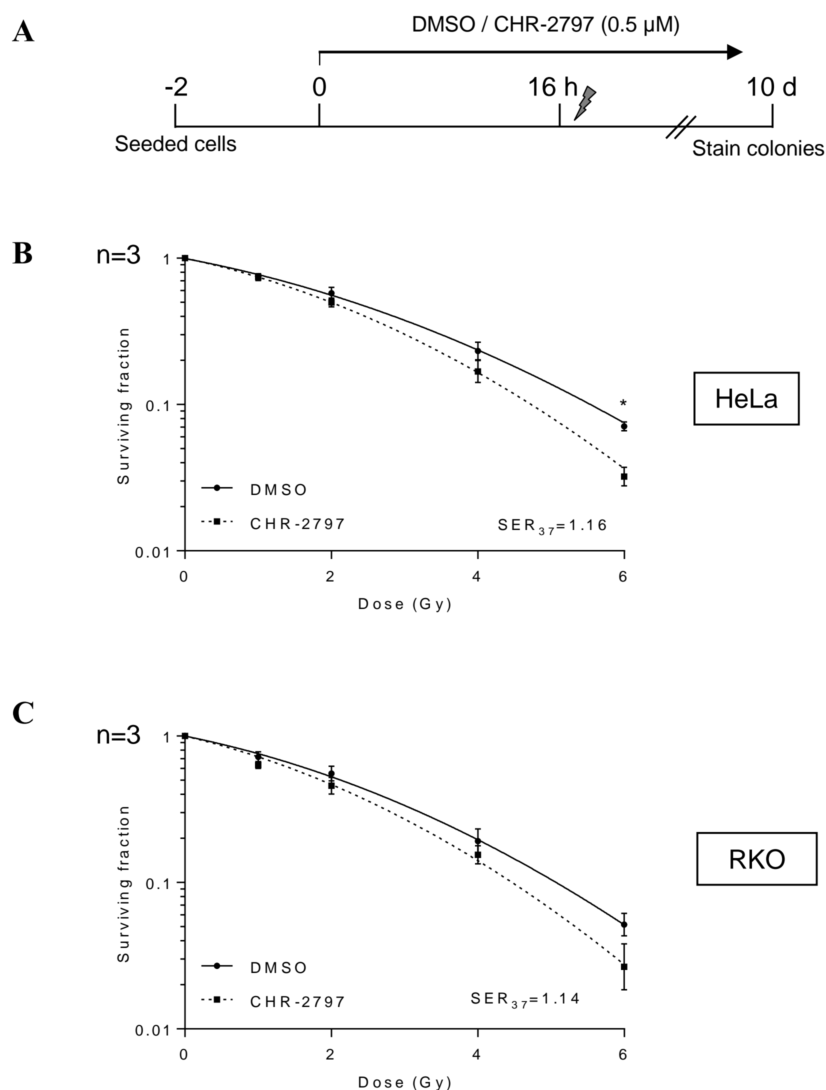

Figure 5.9 CHR-2797 pre-treatment does not radiosensitise HeLa and RKO cell lines (**A**) Scheme of colony survival assay in HeLa and RKO cells pre-treated with CHR-2797 and exposed to radiation. (**B**) HeLa (**C**) RKO cells were treated either with DMSO or CHR-2797 (0.5 μM) for 16 h and then exposed to a range of radiation doses (0 – 6 Gy). Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05.

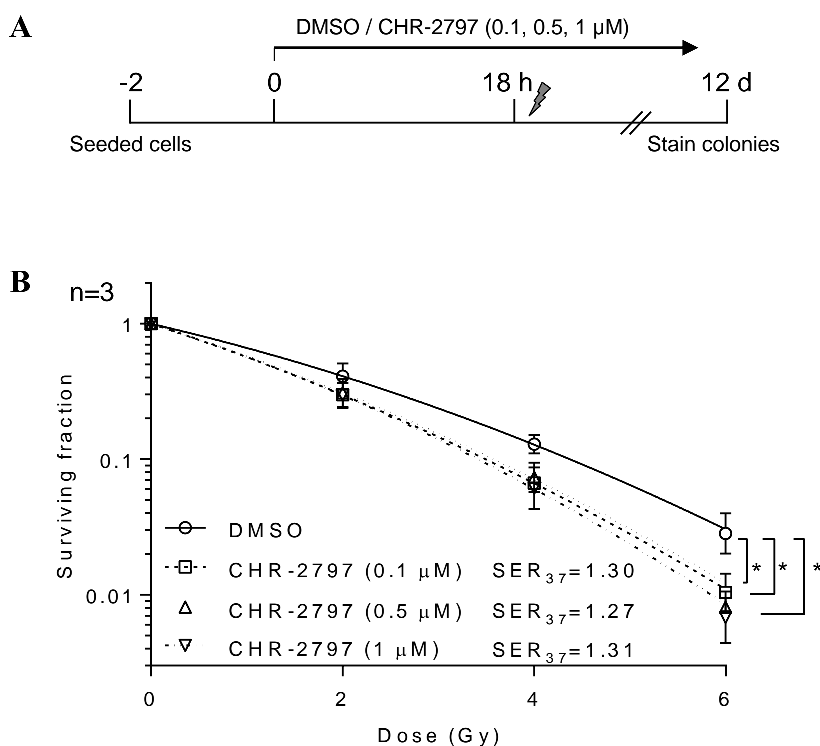

Figure 5.10 Radiosensitivity of MRC-5 cells exposed to CHR-2797 pre-treatment
(**A**) Scheme of colony survival assay in MRC-5 cells pre-treated with CHR-2797 and irradiated. (**B**) MRC-5 cells were pre-treated either with DMSO or CHR-2797 (0.1, 0.5 or 1 µM) for 18 h and then exposed to a range of radiation doses. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05. Experiments performed by Dr. Remko Prevo.

Our results so far suggest that CHR-2797 may be a potential radiosensitiser under normoxia (Figure 5.7, 5.8 & 5.10). In order to test if CHR-2797 pre-treatment can radiosensitise the FLO-1 and OE21 cells in physiologically relevant oxygen levels a colony survival assay was carried out. FLO-1 and OE21 cells were pre-treated for 16 h with CHR-2797 (0.5 µM) in physiologically relevant levels of hypoxia (1% O_2) and exposed to irradiation (4 Gy) under the same conditions (Hammond et al, 2014). Following irradiation, treated cells were allowed to form colonies under normal tissue culture conditions (Figure 5.11). Increased radiosensitivity was observed in the FLO-1 (SF_{4Gy}=3.26) and OE21 (SF_{4Gy}=2.28) cells treated with CHR-2797 in hypoxia. This

suggests that CHR-2797 increased radiation-induced loss of viability would occur under physiologically relevant oxygen tensions.

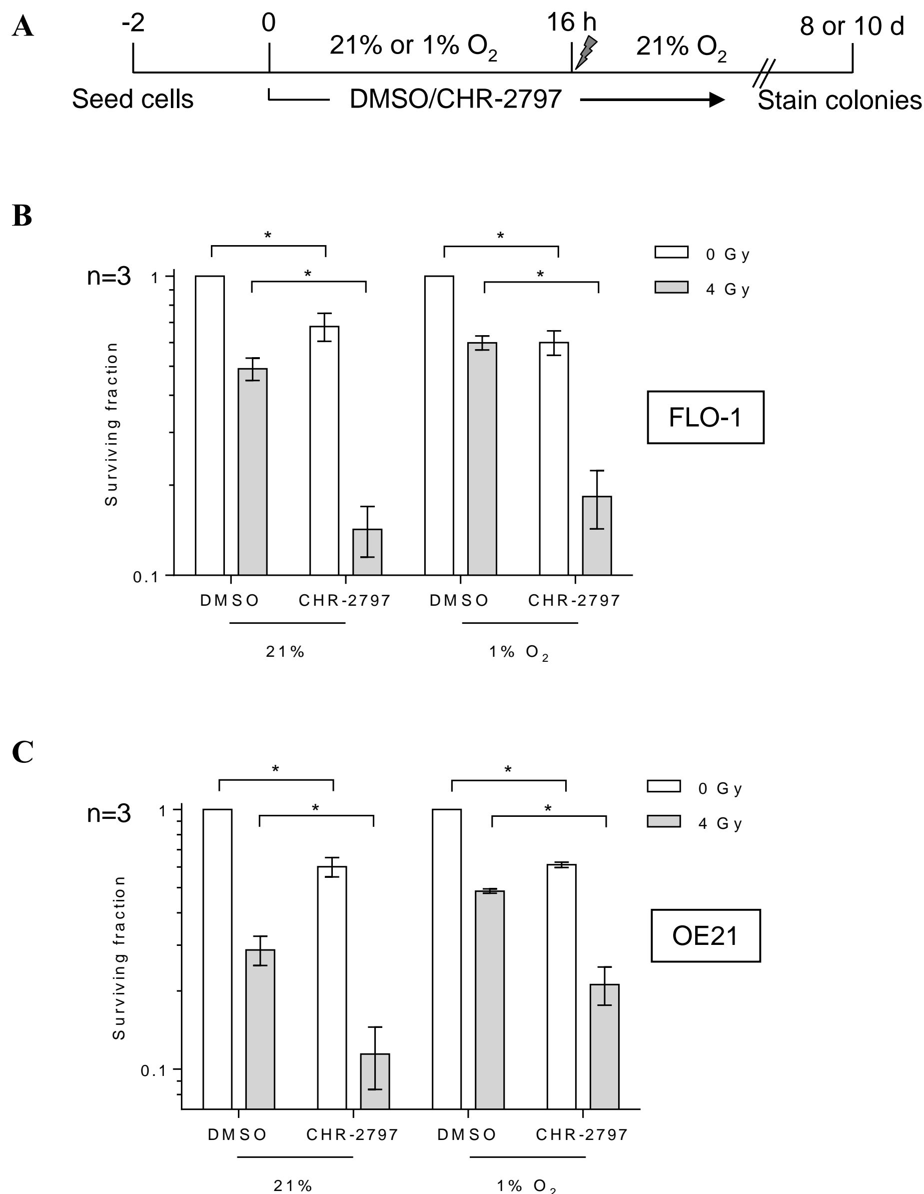

Figure 5.11 CHR-2797 radiosensitises FLO-1 and OE21 cells in hypoxia
(**A**) Scheme of oesophageal cell lines treated with CHR-2797 in hypoxia and exposed to irradiation. The oesophageal cell lines (**B**) FLO-1 (**C**) OE21 cells were treated for 16 h with CHR-2797 (0.5 μM) either under normoxia (21% O_2) or hypoxia (1% O_2) and were exposed to 0 or 4 Gy radiation under the same conditions. Post irradiation cells were allowed to form colonies under normal tissue culture conditions and the surviving fractions were calculated. Significance value: '*' p-Value <0.05.

5.2.4 Scheduling of CHR-2797 treatment and radiation

Our original hypothesis was that CHR-2797-induced autophagy could increase radiation sensitivity. In order to determine this mechanism we first investigated the scheduling of CHR-2797 treatment. Up to this point we pre-treated the cells with CHR-2797 for 16 h as we assumed that the drug would need sufficient time to induce autophagy, as observed for STF-62247. We altered the CHR-2797 pre-treatment time to between 30 mins and 24 h and then irradiated followed by colony survival (Figure 5.12). Surprisingly, the decreased cell viability in the FLO-1 cells was detected irrespective of the pre-treatment time i.e. the effect after 30 mins pre-treatment was not statistically significant from 16 h treatment. Its unlikely 30 mins was sufficient for a biological effect of the drug to enter the cell and undergo conversion to the active metabolite. This raised the possibility that the important time period for CHR-2797 mediated radiosensitisation could be after radiation. Therefore, we irradiated FLO-1 cells and added the CHR-2797 afterwards (Figure 5.13). As suspected the effect on radiosensitivity was apparent even when the drug was not present until after radiation.

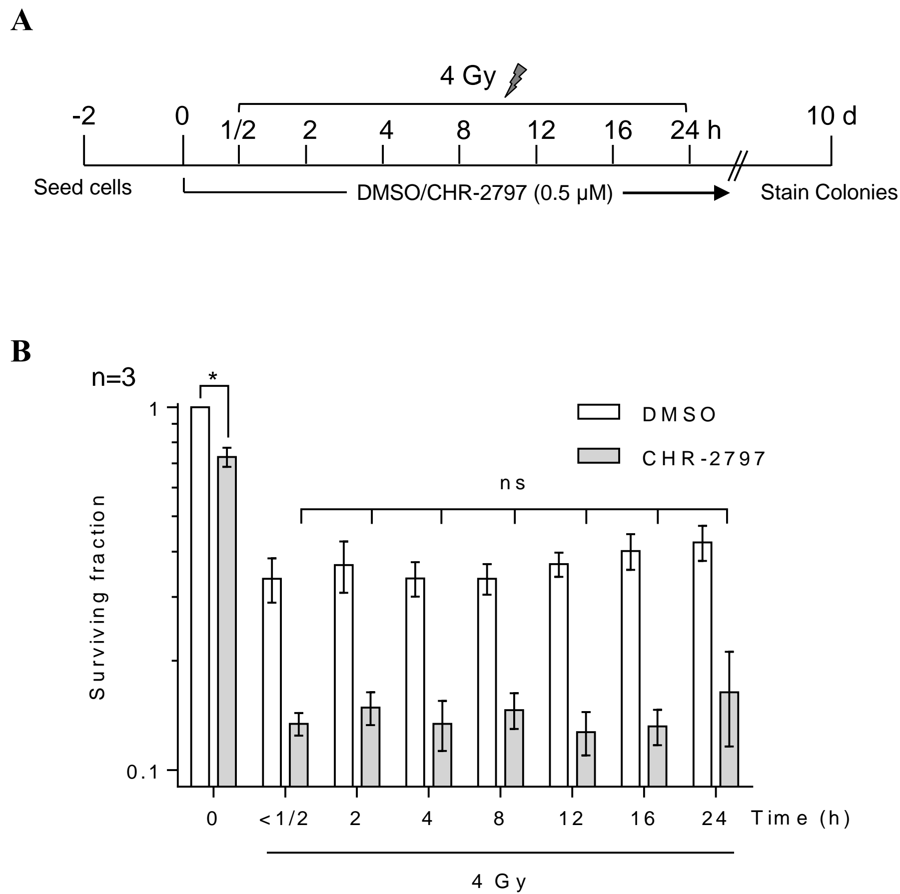

Figure 5.12 Increased radiosensitivity due to CHR-2797 is independent of pre-treatment time

(**A**) Scheme of CHR-2797 treated FLO-1 cells exposed to irradiation at different periods of time. (**B**) FLO-1 cells were pre-treated with CHR-2797 (0.5 µM) and exposed to 4 Gy irradiation after the indicated period of time. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. There was no significant difference between the CHR-2797 treated samples with 4 Gy. Significance value: '*' p-Value <0.05, ns non-significant.

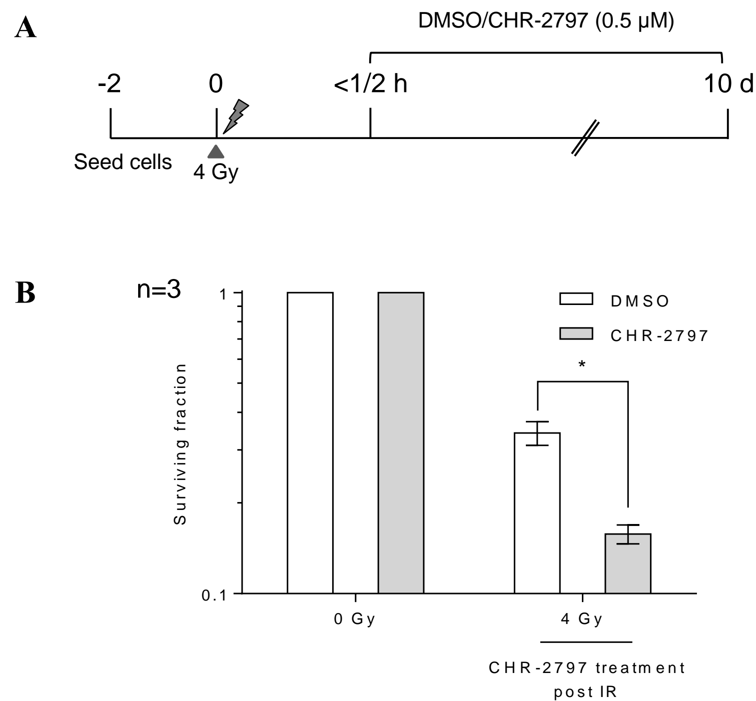

Figure 5.13 Loss of cell viability post irradiation is increased further with CHR-2797 treatment
(**A**) Scheme of FLO-1 cells treated with CHR-2797 post irradiation (**B**) 30 mins post irradiation (4 Gy), FLO-1 cells were treated with media containing either DMSO or CHR-2797 (0.5 μM). Post treatment FLO-1 cells were allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05.

5.2.5 The effect of CHR-2797 on the cell cycle

The effect of CHR-2797 on the cell cycle has not been described in oesophageal cell lines. We carried out FACS analysis to investigate the effect of CHR-2797 on cell cycle progression, which could in turn affect radiosensitivity. As radiosensitivity is in part dependent on cell cycle phase, agents which arrest cells in more sensitive phases can radiosensitise (Seiwert et al, 2007). FLO-1 cells were pre-treated with CHR-2797 (0.5 μM) over a period of time (0 - 16 h) and their cell cycle profile assessed by PI staining and FACS analysis (Figure 5.14). CHR-2797 treatment in FLO-1 cells did not significantly change the distribution of cells throughout the cell cycle compared to the DMSO control.

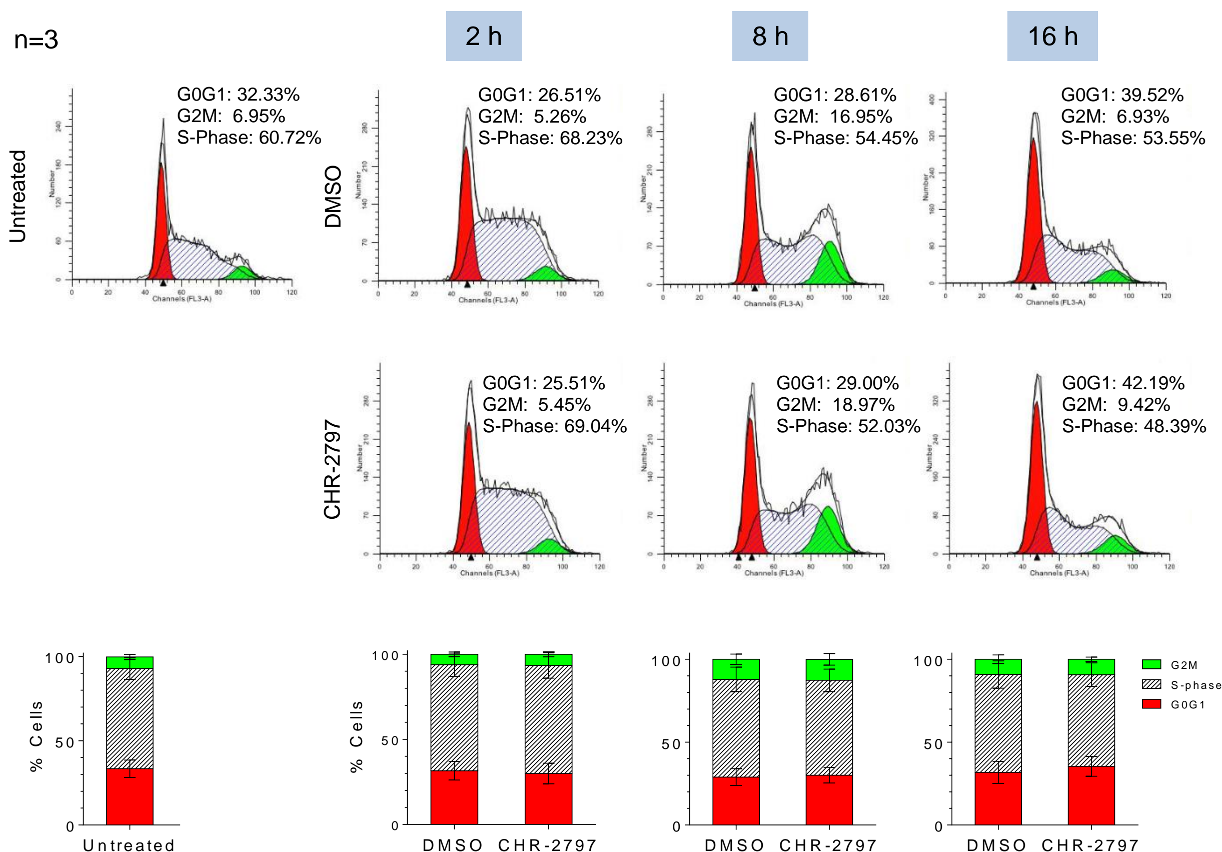

Figure 5.14 Treatment with CHR-2797 has no apparent effect on cell cycle
Plots represent the FACS analysis carried out with FLO-1 cells (0.5 x 10^6) treated with CHR-2797 (0.5 µM) or DMSO with the untreated acting as the initial time point. The treated cells were fixed at the indicated times and stained with propidium iodide. One representative FACS plot out of three individual experiment performed is illustrated, whereas the bar graph represents the average of three individual experiments.

5.2.6 CHR-2797 does not induce apoptosis in FLO-1 cells

The FACS analysis on FLO-1 cells treated with CHR-2797 did not show any apparent increase in the sub G_1 population, which would be indicative of apoptosis (Figure 5.14). As CHR-2797 has been described to induce apoptosis we further investigated by assessing the level of PARP cleavage in response to CHR-2797 alone (Figure 5.15). The combination of no increase in sub G_1 population and an apparent lack of PARP cleavage strongly suggest CHR-2797 does not induce apoptosis in FLO-1 cells.

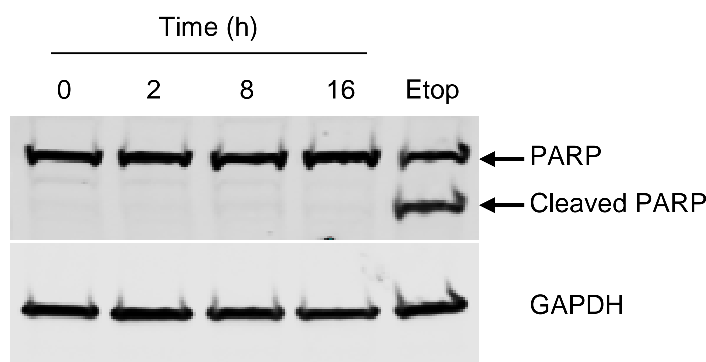

Figure 5.15 CHR-2797 treatment does not induces apoptosis in FLO-1 cells
FLO-1 cells were pre-treated for the times indicated in the presence of CHR-2797 (0.5 μM). Western blotting was carried out for PARP and GAPDH (loading control). As a positive control for PARP cleavage FLO-1 cells were grown in media containing 1% FBS and exposed to etoposide (Etop, 50 μM) for 16 h.

5.2.7 CHR-2797 treatment alone does not induce DNA damage

Next, we asked if CHR-2797 treatment induces DNA damage which could then add to radiation-induced levels of damage to decrease cell viability. We used three complementary assays to investigate the level of damage induced by CHR-2797 treatment alone in FLO-1 cells.

Firstly, FLO-1 cells were pre-treated (0 – 16 h) with CHR-2797 (0.5 μM) and assessed for the expression levels of γH2AX by western blotting (Figure 5.16). There was a moderate increase in the γH2AX levels. Secondly, FLO-1 cells were pre-treated for 16 h with CHR-2797 (0.5 μM) and analysed for the level of DNA damage by alkaline comet assay (Figure 5.17). In contrast to the western blot analysis, no significant levels of DNA damage was observed in FLO-1 cells treated with CHR-2797 by alkaline comet assay. As these two results were somewhat contradictory, we further assessed the levels of DNA damage by measuring the number of nuclear 53BP1 foci formed in response to 8 h treatment with CHR-2797 (0.5 μM) by immunofluorescence assay (Figure 5.18). No significant difference in induction of 53BP1 focus formation was detected between the

DMSO and CHR-2797 treated cells. On the basis of these results we concluded that CHR-2797 treatment alone does not induce DNA damage.

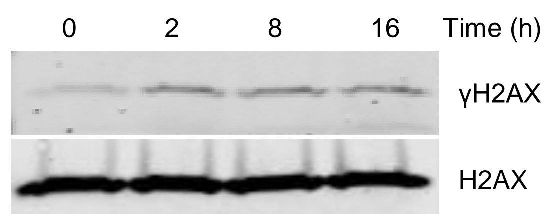

Figure 5.16 CHR-2797 treatment induces a modest increase in γH2AX
FLO-1 cells were pre-treated for the times indicated in the presence of CHR-2797 (0.5 μM). Western blotting was carried out for γH2AX and H2AX (loading control).

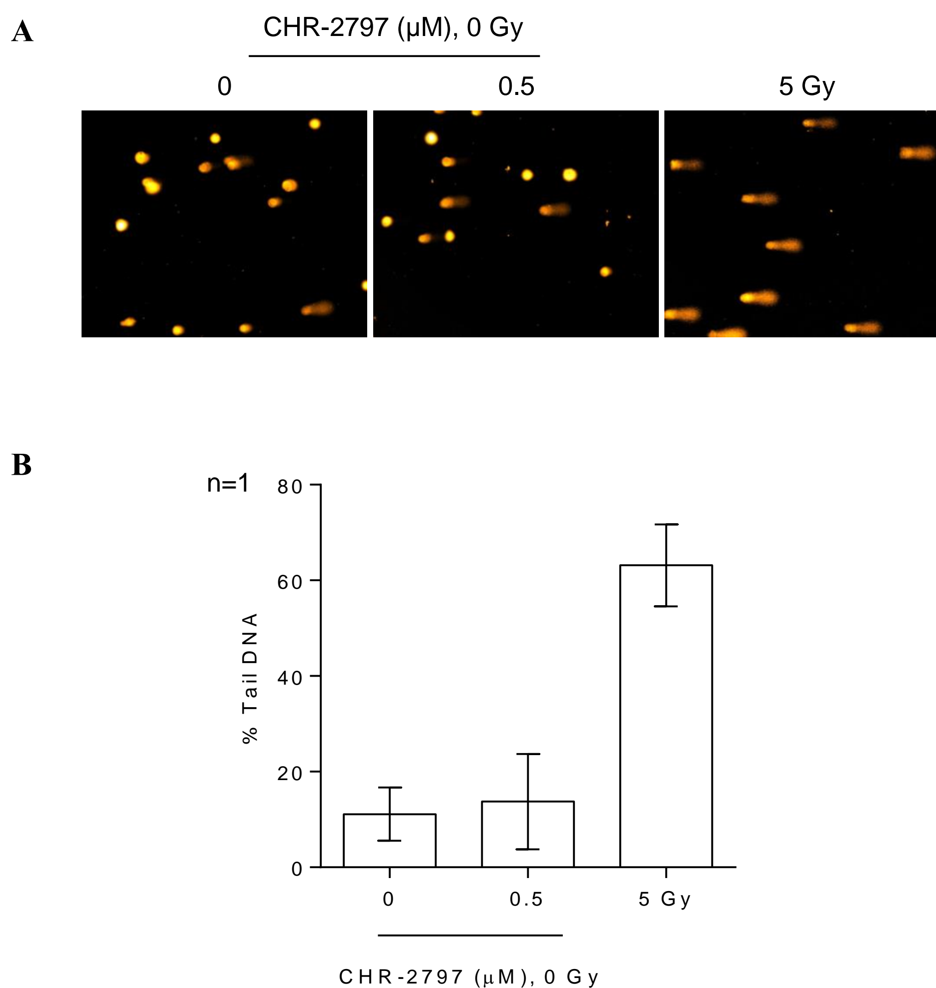

Figure 5.17 CHR-2797 treatment does not induce damage in FLO-1 cells as detected by alkaline comet assay

FLO-1 cells were pre-treated for 16 h with CHR-2797 (0.5 μM) and processed for alkaline comet assay. (**A**) Images of FLO-1 cells treated with either DMSO or CHR-2797 or exposed to radiation (5 Gy). (**B**) % tail DNA for the indicated conditions.

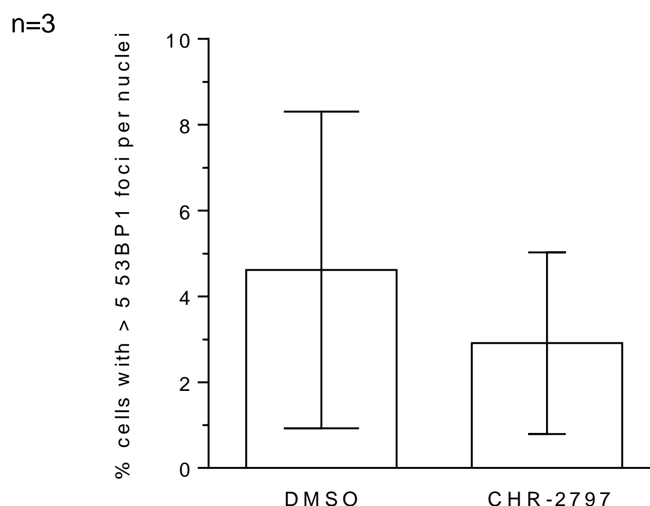

Figure 5.18 CHR-2797 treatment does not induce DNA damage as detected by 53BP1 focus formation assay
FLO-1 cells were pre-treated for 8 h with CHR-2797 (0.5 µM) and stained for 53BP1 by immunofluorescence. Plot shows the percent number of FLO-1 cells in response to the above indicated treatment containing more than 5, 53BP1 foci.

5.2.8 DNA repair kinetics following a combined treatment with CHR-2797 and radiation

So far we have demonstrated in FLO-1 cells that the treatment with CHR-2797 alone does not induce detectable DNA damage (Figure. 5.17, 5.18). We asked if the repair of radiation-induced damage was effected by CHR-2797 treatment. To determine the repair kinetics in FLO-1 cells treated with CHR-2797 and irradiation, we again quantified 53BP1 focus formation. FLO-1 cells were pre-treated for 8 h either with DMSO or CHR-2797 (0.5 µM) and exposed to radiation (2 Gy). Post irradiation, treated FLO-1 cells were fixed over a period of time (1 – 24 h) and immunofluorescence for 53BP1 staining was carried out (Figure. 5.19). The kinetics of 53BP1 focus quantified over the period of time showed no apparent change in the rates of repair between DMSO and CHR-2797 treated FLO-1 cells post irradiation.

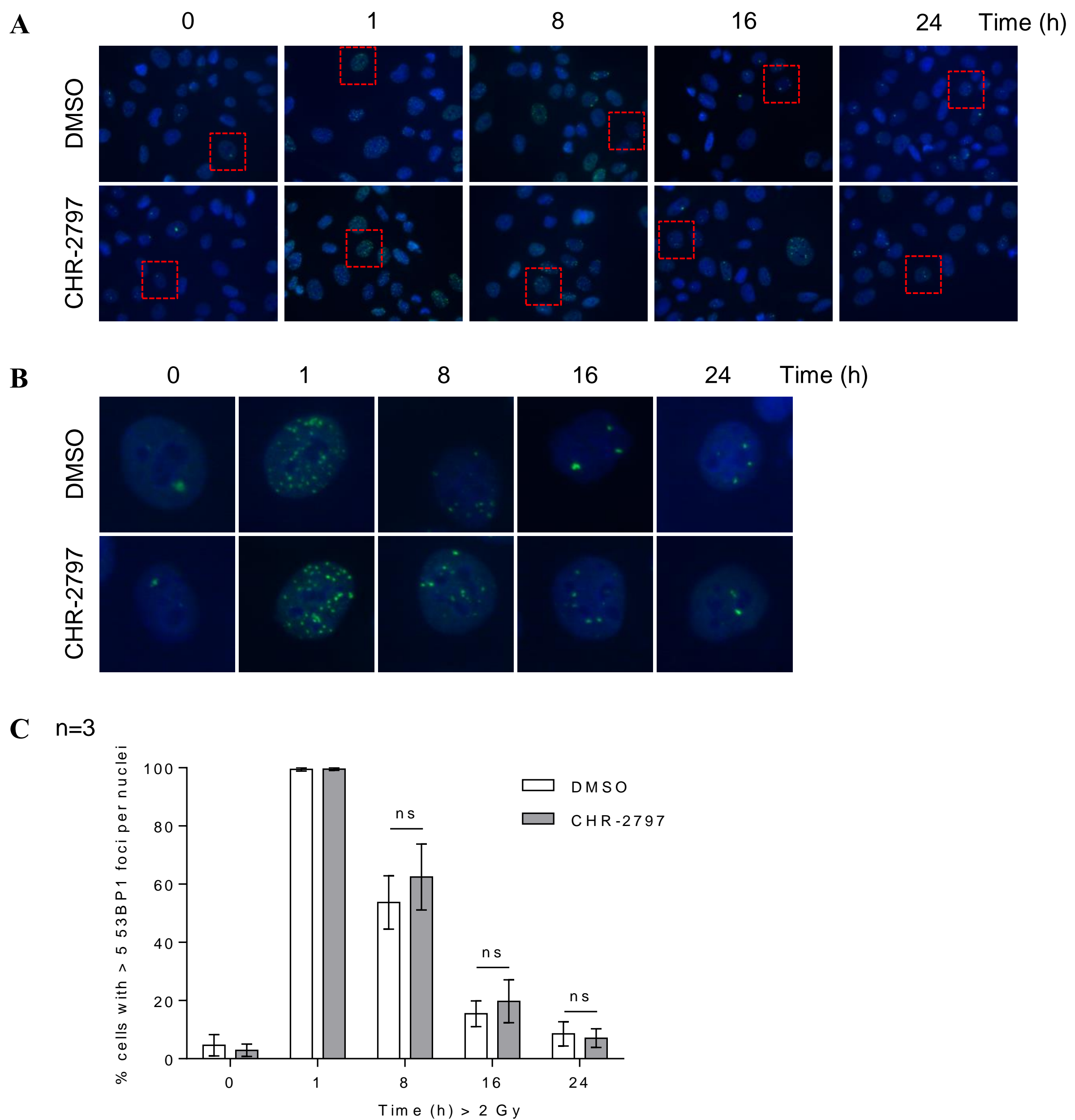

Figure 5.19 There is no effect on the repair kinetics in FLO-1 cells in response to combined treatment with CHR-2797 and radiation

FLO-1 cells were pre-treated for 8 h either with DMSO or CHR-2797 (0.5 µM) and were exposed to either 0 or 2 Gy irradiation. Cells were fixed and processed for 53BP1 foci at the indicated time periods. (**A**) Immunofluorescence images of FLO-1, with (**B**) showing the individual cells from inset, magnification = 40x. Images in (A) and (B) are representative images from one of the 2 experiments performed individually. (**C**) Percentage of FLO-1 cells in response to the above indicated treatment containing more than 5, 53BP1 foci per nuclei. Significance value: 'ns' non-significant.

5.2.9 Cell viability of FLO-1 cells in response to combined treatment with aminopeptidase inhibitors and radiation

CHR-2797 is an aminopeptidase N inhibitor, and we have shown that it radiosensitises FLO-1 and OE21 cells (Figure 5.7, 5.8). We hypothesised that other aminopeptidase inhibitors would also radiosensitise oesophageal cell lines. We selected the aminopeptidase N inhibitors actinonin, bestatin, and leuhistin for our studies. In addition, we also selected SC-57461A, a LTA4 hydrolase inhibitor for our studies. SC-57461A was included in our studies as one of the targets of CHR-2797 apart from aminopeptidase N is LTA4 hydrolase (Krige et al, 2008). FLO-1 cells were pre-treated for 16 h with the selected inhibitors actinonin (0 – 100 µM), bestatin (0.5 – 5 µM), SC-57461A (0 – 50 nM) and leuhistin (0 – 1 µM) and exposed to radiation (4 Gy) and assessed for cell viability by colony survival assay (Figure 5.20). Increased radiosensitivity was observed in FLO-1 cells which were pre-treated with actinonin (5 µM, SF_{4Gy}=2.71), SC-5471A (2.5 nM, SF_4=2.32) and bestatin (5 µM, SF_4=2.55). However, we did not observe any significant increase in radiosensitivity in FLO-1 cells that had been pre-treated with leuhistin.

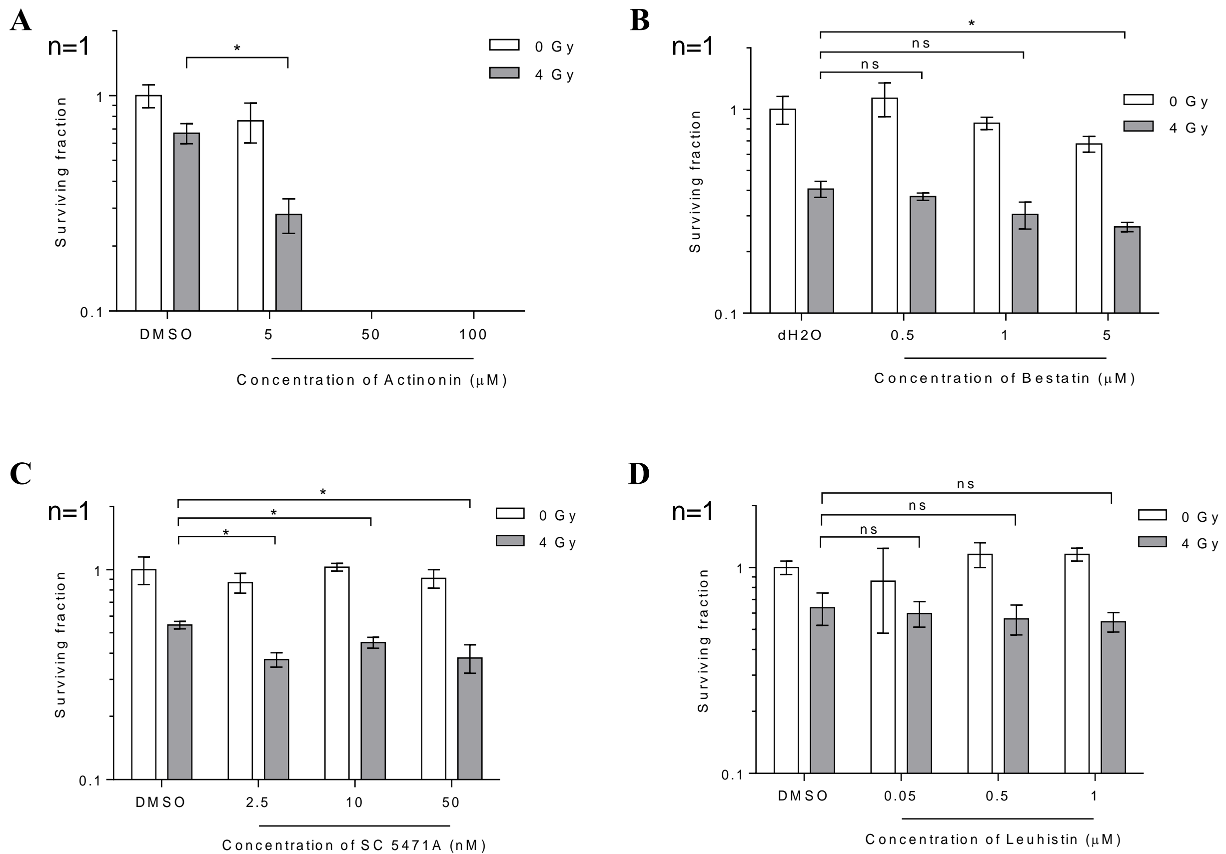

Figure 5.20 Radiosensitivity of FLO-1 cells pre-treated with a range of aminopeptidase inhibitors

FLO-1 cells were pre-treated for 16 h with (**A**) actinonin (**B**) bestatin (**C**) SC-57461A (**D**) leuhistin for the indicated dose concentrations and exposed to radiation either with 4 Gy or mock irradiated. Post irradiation cells were allowed to form colonies and the surviving fractions were calculated. Significance value: '*' p-Value <0.05, 'ns' non-significant.

5.3 Discussion

Our previous study demonstrated that the VHL-deficient RCC cells can be radiosensitised through the induction of autophagy by STF-62247 (Chapter 4). This led us to hypothesise that other autophagy inducing compounds could radiosensitise cancer cells. We investigated this by screening five different autophagy inducing drugs across a panel of cell lines. Our results indicated that among the five candidate drugs niclosamide induced autophagy in all the cell lines screened. Interestingly, we observed CHR-2797 induced autophagy only in the oesophageal cell line FLO-1 and OE21 but not in any other cancer cell lines used. The pro-drug CHR-2797 is an aminopeptidase inhibitor which had been shown to induce apoptosis, autophagy and antiproliferative effects on cancer cell lines irrespective of the p53, PTEN and K-Ras status (Krige et al, 2008). In this study we have demonstrated that the oesophageal cells lines FLO-1 and OE21 can be radiosensitised by CHR-2797 *in vitro* in normoxia as well as under physiologically relevant oxygen levels. CHR-2797 has been in clinical trials for solid tumours (renal cell, colorectal, lung, prostate, breast, bladder, sarcoma, liver, lower oesophageal, melanoma, and ovarian tumours), myelodysplastic syndrome and leukaemia (Cortes et al, 2013; Lowenberg et al, 2010; Reid et al, 2009). The dose concentrations at which we see an increased radiosensitivity in FLO-1 (> 50 nM) and OE21 (> 300 nM) is about 20 – 120 fold lower than the clinically reported peak plasma levels of 6 µM CHR-2797 (Krige et al, 2008).

Our hypothesis was that any potential CHR-2797-mediated radiosensitivity would be autophagy dependent. However, we were unable to detect LC3 cleavage in CHR-2797-induced autophagy in OE21 cells. This could be either a failure to detect autophagy or suggest an alternative mechanism. Interestingly, we observed that CHR-2797 did not radiosensitise RKO and HeLa cells indicating that the radiosensitisation effect of CHR-2797 is cell line dependent. We have begun to explore the mechanism of increased

radiosensitivity. CHR-2797 does not appear to alter the cell cycle, induce apoptosis or effect the rate of DNA damage repair.

CHR-2797 depletes intracellular levels of free amino acids pool leading to an up regulation of AADR. In response to CHR-2797 mediated AADR, genes involved in DNA damage (REDD1, CHOP, GADD45A, ATF4, ATF5), amino acid transport (SLC7A11, SLC38A2) or metabolism (ASNS, ASS, CBS, SARS, MARS) were highly up regulated (Krige et al, 2008). Of particular interest we would focus on REDD1 (regulates mTORC), CHOP (transcriptional factor involved in ER stress response) and GADD45A (involved in genomic stability, DNA repair and in cell cycle). It will be interesting to investigate further if these expression changes might have a role in the radiosensitisation of oesophageal cells FLO-1 and OE21 reported here.

Cancer Research UK has recently deemed oesophageal cancer a cancer of unmet need. Unfortunately, the incidence of oesophageal cancer is increasing globally. Therefore, new strategies are required. We have identified a class of inhibitors (aminopeptidase) which may prove efficacious in combination with radiotherapy. Further, *in vitro* testing of the agents actinonin, SC 5471A, bestatin and leuhistin are required as well as detailed investigation of the mechanism of radiosensitisation.

Chapter 6

Discussion

6.0 Discussion

The aim of this thesis was to identify novel methods to increase radiosensitivity. Radiotherapy is one of the most commonly used and effective forms of cancer treatment, being used in at least 50% of cases. Despite this, there is an urgent need to improve the response to radiotherapy and particularly in tumours which are radioresistant due to the presence of hypoxic regions for example. The three chapters presented focused on firstly the use of inhibitors to the DDR, secondly the use of a relatively uncharacterised compound STF-62247 and finally screening for novel radiosensitisers from candidate autophagy-inducing drugs. Each of these approaches will be discussed in turn.

Until recently inhibition of the ATR kinase has been technically challenging due to a lack of specific inhibitors. This was exacerbated by the large size of ATR, which makes over-expression of kinase dead mutants challenging and the finding that ATR is an essential gene, meaning that long term knock down or knock out is problematic. However, recently a number of ATR inhibitors have been described (Charrier et al, 2011; Foote et al, 2013; Toledo et al, 2011). Using the ATR inhibitor, VE-821 from Vertex we demonstrated that ATR-inhibition is an effective means of improving radiation response to a wide variety of cell lines and that this also occurred in physiologically relevant oxygen levels (Pires et al, 2012). Since these studies were completed the clinical version of this compound, VE-822, has been used *in vivo* to demonstrate increased radiosensitisation of a pancreatic xenograft tumour (Fokas et al, 2012). More recently, VE-822 (known as VE-970 in the clinic) has entered phase I clinical trial.

Wee 1 inhibitors have been described as useful in combination with radiation but to our knowledge had not previously been investigated in hypoxic conditions. Our findings were surprising in that although we observed the reported increase in radiosensitivity in normoxic conditions this was not apparent at levels of oxygen, which occur in the most

radioresistant fraction of a tumour. Our hypothesis is that the effect is lost in severe hypoxia as Wee1 inhibition is unable to alter the cell cycle in this context and that this is essential for the increase in radiosensitivity (O'Brien Eleanor et al, 2013). Furthermore, it is highly likely that in response to severe hypoxia the role of Wee1 in replication is more significant (Figure 6.1). Cells exposed to severe hypoxia undergo replication re-start if oxygen is returned within an acute time period and during this time it is likely that Wee1 activity is critical (Pires et al, 2010a). This study highlights the need to carry out preclinical testing in conditions, which actually mimic the tumour microenvironment. Our prediction is that whilst Wee 1 inhibitors may increase the efficacy of radiotherapy in the most sensitive fractions they will not influence hypoxia-mediated radiation resistance.

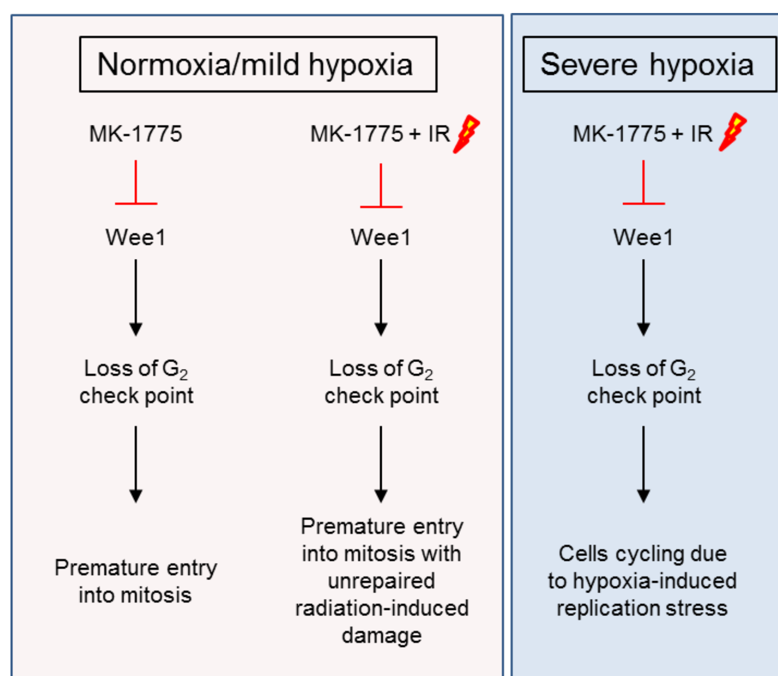

Figure 6.1 A schematic representation of Wee1 inhibitor MK-1775 treatment either alone or in combination with irradiation under different oxygenation status.

Our finding that inhibition of ATM increases radiosensitivity in hypoxic conditions was perhaps not surprising given the role of ATM in the radiation response and the extreme radiosensitivity of AT patients. However, again it is important to verify these effects in hypoxia. Surprisingly, we observed a significant increase in sensitivity to

hypoxia/reoxygenation in the presence of ATM inhibitors. Whilst this was expected at the severe levels of hypoxia (0.1% O_2) this was not foreseen in mild hypoxia (2% O_2) (Figure 3.4) (Olcina et al, 2013). The mechanism behind this is unclear and warrants further investigation. Recently, Cam et al have reported that hypoxia (<0.2% O_2) induces ATM-mediated phosphorylation of HIF-1α at serine 696. This phosphorylation then contributes to hypoxia-induced HIF-1 stability and gene transcription. Therefore, it is possible that inhibition of ATM during hypoxia decreases the stabilisation/activity of HIF-1 which may in turn effect viability (Cam et al, 2010).

The biological response to STF-62247 is very specific in that it induces pro-death autophagy in a VHL dependent manner. This attribute makes STF-62247 an extremely attractive compound for the treatment of RCC, which is associated with loss of VHL. It should be noted that while many drugs induce autophagy this is often pro-survival and that autophagy is no longer generally considered a pro-death pathway (Denton et al, 2012; O'Donovan et al, 2011; Yu et al, 2014). Instead the context of autophagy induction appears to play a major role in the biological response. Although radiotherapy is rarely used in the treatment of RCC this is largely due to historical reasons. Here we have demonstrated that STF-62247 significantly increases radiation-induced loss of viability in RCC cells suggesting that this could be an effective treatment strategy in the future. STF-62247 has been used in mouse models of RCC although we were not able to carry out these studies. Our prediction would be that STF-62247 would increase radiosensitivity *in vivo* also.

There are major challenges in the development of novel drugs with the vast majority not making it to wide spread clinical use. This knowledge led us to thinking about those drugs which have already undergone clinical testing as opposed to the development of novel drugs. With this in mind we combined radiation with temsirolimus in our RCC study. The rationale behind this was that temsirolimus is already used clinically in the

treatment of RCC and that it has been shown to induce autophagy through mTOR inhibition (Kwitkowski et al, 2010; Thomas et al, 2006; Yazbeck et al, 2008). Our data suggest that this is an efficacious combination and this is supported by the use of the same strategy in the treatment of glioblastoma (Sarkaria et al, 2010). It is far more likely that clinical practise could be altered by the inclusion of radiotherapy with an agent already indicated for the disease type (temsirolimus) as opposed to the introduction of a totally novel combination (STF-62247 and radiotherapy). However, it is unlikely that radiotherapy will be used in the treatment of RCC and so we expanded this approach to alternative disease sites where radiotherapy is already indicated and there is a major need to improve treatment response.

Cancer Research UK recently deemed oesophageal as a cancer of unmet need. This is due to an increasing incidence of disease and relatively poor survival rates. Radiotherapy is part of the standard of care for patients with oesophageal cancer and these cancers have be shown to have significant levels of hypoxia (Matsuyama et al, 2005). Therefore, for the last chapter of this thesis we predominantly made use of oesophageal cell lines. Our approach to identifying a novel means of improving the radiation response of oesophageal cancer cells lines was to search the literature for drugs which were in clinical use and had been described to induce autophagy. It is worth noting that although this chapter focused on a list of six drugs there are many more that fulfil these criteria. The possibility of using a larger panel of these drugs in the future in a chemical library screen is an attractive approach to identifying yet more radiosensitisers. Of the drugs initially selected we quickly became interested in CHR-2797, which appears to increase radiation-induced loss of viability. One of the intriguing things we noted regarding this drug is that it does appear to work best in the oesophageal cell lines compared to the others we tested. The reasons behind this are unclear. Further work is required to determine how CHR-2797

increases radiation response and if indeed this is due to its activity as an aminopeptidase inhibitor. Before investigating this mechanism we plan to carry out an experiment to verify that CHR-2797 can radiosensitise oesophageal xenograft tumours *in vivo*.

As previously mentioned there is a clear need for agents which can be used to improve the response to radiotherapy and one of the reasons for this is the relative resistance of hypoxic cells to radiation. Our studies demonstrate that whilst some agents have almost predictable effects on radiosensitivity, ATM inhibitors for example, others absolutely require testing in hypoxic conditions (Wee 1 inhibitors). Given the huge amount of time, effort and money required to bring a new drug to market we suggest that using drugs which have already been through clinical testing for novel applications is a promising and efficient approach.

Appendix

Published papers from this thesis

Olcina MM, Foskolou IP, Anbalagan S, Senra JM, Pires IM, Jiang Y, Ryan AJ, Hammond EM. Replication stress and chromatin context link ATM activation to a role in DNA replication. Mol Cell. 2013 Dec 12;52(5):758-66. doi: 10.1016/j.molcel.2013.10.019. Epub 2013 Nov 21. PubMed PMID: 24268576; PubMed Central PMCID: PMC3898930.

O'Brien Eleanor, M., Senra Joana, M., Anbalagan, S., Hill Mark, A., and Hammond Ester, M. (2013). Impact of Wee1 inhibition on the hypoxia-induced DNA damage response. Tumor Microenvironment and Therapy. 2013 May, Volume 1, Pages 37–45, ISSN (Online) 2299-1123, DOI: 10.2478/tumor-2013-0002.

Pires IM, Olcina MM, Anbalagan S, Pollard JR, Reaper PM, Charlton PA, McKenna WG, Hammond EM. Targeting radiation-resistant hypoxic tumour cells through ATR inhibition. Br J Cancer. 2012 Jul 10;107(2):291-9. doi: 10.1038/bjc.2012.265. Epub 2012 Jun 19. PubMed PMID: 22713662; PubMed Central PMCID: PMC3394988.

Anbalagan S, Pires IM, Blick C, Hill MA, Ferguson DJ, Chan DA, Hammond EM. Radiosensitization of renal cell carcinoma in vitro through the induction of autophagy. Radiother Oncol. 2012 Jun;103(3):388-93. doi: 10.1016/j.radonc.2012.04.001. Epub 2012 Apr 30. PubMed PMID: 22551566.

Phadwal K, Alegre-Abarrategui J, Watson AS, Pike L, Anbalagan S, Hammond EM, Wade-Martins R, McMichael A, Klenerman P, Simon AK. A novel method for autophagy detection in primary cells: impaired levels of macroautophagy in immunosenescent T cells. Autophagy. 2012 Apr;8(4):677-89. doi: 10.4161/auto.18935. Epub 2012 Apr 1. PubMed PMID: 22302009; PubMed Central PMCID: PMC3405842.

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Address: Old Road Campus Research Building, Off Roosevelt Drive, Oxford, OX3 9DQ, United Kingdom.

Contact Person: Selvakumar Anbalagan

Telephone / Fax: +44 01865 617377

E-Mail: selvakumar.anbalagan@oncology.ox.ac.uk

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References

Abbaszadeh F, Clingen PH, Arlett CF, Plowman PN, Bourton EC, Themis M, Makarov EM, Newbold RF, Green MH, Parris CN (2010) A novel splice variant of the DNA-PKcs gene is associated with clinical and cellular radiosensitivity in a patient with xeroderma pigmentosum. *Journal of Medical Genetics* **47:** 176-181

Ahn GO, Seita J, Hong BJ, Kim YE, Bok S, Lee CJ, Kim KS, Lee JC, Leeper NJ, Cooke JP, Kim HJ, Kim IH, Weissman IL, Brown JM (2014) Transcriptional activation of hypoxia-inducible factor-1 (HIF-1) in myeloid cells promotes angiogenesis through VEGF and S100A8. *P Natl Acad Sci USA* **111:** 2698-2703

Alexander A, Kim J, Walker CL (2010) ATM engages the TSC2/mTORC1 signaling node to regulate autophagy. *Autophagy* **6:** 672-673

Altmeyer A, Jung AC, Ignat M, Benzina S, Denis JM, Gueulette J, Noel G, Mutter D, Bischoff P (2010) Pharmacological enhancement of autophagy induced in a hepatocellular carcinoma cell line by high-LET radiation. *Anticancer Res* **30:** 303-310

Alvarez-Quilon A, Serrano-Benitez A, Lieberman JA, Quintero C, Sanchez-Gutierrez D, Escudero LM, Cortes-Ledesma F (2014) ATM specifically mediates repair of double-strand breaks with blocked DNA ends. *Nat Commun* **5:** 3347

Anbalagan S, Pires IM, Blick C, Hill MA, Ferguson DJ, Chan DA, Hammond EM (2012) Radiosensitization of renal cell carcinoma in vitro through the induction of autophagy. *Radiother Oncol* **103:** 388-393

Andegeko Y, Moyal L, Mittelman L, Tsarfaty I, Shiloh Y, Rotman G (2001) Nuclear retention of ATM at sites of DNA double strand breaks. *Journal of Biological Chemistry* **276:** 38224-38230

Anderson RM, Papworth DG, Stevens DL, Sumption ND, Goodhead DT (2006) Increased complexity of radiation-induced chromosome aberrations consistent with a mechanism of sequential formation. *Cytogenet Genome Res* **112:** 35-44

Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A (2008) Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. *Cancer Res* **68:** 1485-1494

Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, Griffiths G, Ktistakis NT (2008) Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol* **182:** 685-701

Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421:** 499-506

Bakry D, Aronson M, Durno C, Rimawi H, Farah R, Alharbi QK, Alharbi M, Shamvil A, Ben-Shachar S, Mistry M, Constantini S, Dvir R, Qaddoumi I, Gallinger S, Lerner-Ellis J, Pollett A, Stephens D, Kelies S, Chao E, Malkin D, Bouffet E, Hawkins C, Tabori U (2014) Genetic and clinical determinants of constitutional mismatch repair deficiency syndrome: Report from the constitutional mismatch repair deficiency consortium. *Eur J Cancer* **50:** 987-996

Balducci L, Ershler WB (2005) Science & society - Cancer and ageing: a nexus at several levels. *Nat Rev Cancer* **5:** 655-662

Balkwill FR, Capasso M, Hagemann T (2012) The tumor microenvironment at a glance. *J Cell Sci* **125:** 5591-5596

Barnett GC, West CML, Dunning AM, Elliott RM, Coles CE, Pharoah PDP, Burnet NG (2009) Normal tissue reactions to radiotherapy: towards tailoring treatment dose by genotype. *Nat Rev Cancer* **9:** 134-142

Beamish H, Kedar P, Kaneko H, Chen P, Fukao T, Peng C, Beresten S, Gueven N, Purdie D, Lees-Miller S, Ellis N, Kondo N, Lavin MF (2002) Functional link between BLM defective in Bloom's syndrome and the ataxia-telangiectasia-mutated protein, ATM. *J Biol Chem* **277:** 30515-30523

Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K, Millard P, Fuggle S, Harris AL (2002) Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. *Cancer Res* **62:** 2493-2497

Begg AC, Stewart FA, Vens C (2011) GENOMIC INSTABILITY IN CANCER Strategies to improve radiotherapy with targeted drugs. *Nat Rev Cancer* **11:** 239-253

Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, Mazure NM (2009) Hypoxia-Induced Autophagy Is Mediated through Hypoxia-Inducible Factor Induction of BNIP3 and BNIP3L via Their BH3 Domains. *Mol Cell Biol* **29:** 2570-2581

Bencokova Z, Kaufmann MR, Pires IM, Lecane PS, Giaccia AJ, Hammond EM (2009) ATM activation and signaling under hypoxic conditions. *Mol Cell Biol* **29:** 526-537

Bernstein JL, Thomas DC, Shore RE, Robson M, Boice JD, Stovall M, Andersson M, Bernstein L, Malone KE, Reiner AS, Lynch CF, Capanu M, Smith SA, Tellhed L, Teraoka SN, Begg CB, Olsen JH, Mellemkjaer L, Liang XL, Diep AT, Borg A, Concannon P, Haile RW, Grp WSC (2013) Contralateral breast cancer after radiotherapy among BRCA1 and BRCA2 mutation carriers: A WECARE Study Report. *Eur J Cancer* **49:** 2979-2985

Bertog M, Smith DJ, Bielfeld-Ackermann A, Bassett J, Ferguson DJ, Korbmacher C, Harris A (2000) Ovine male genital duct epithelial cells differentiate in vitro and express functional CFTR and ENaC. *Am J Physiol Cell Physiol* **278:** C885-894

Bertout JA, Patel SA, Simon MC (2008) The impact of O2 availability on human cancer. *Nat Rev Cancer* **8:** 967-975

Beveridge RD, Aparicio J, Tormo A, Estevan R, Artes J, Gimenez A, Segura A, Roldan S, Palasi R, Ramos D (2012) Long-term results with oral fluoropyrimidines and oxaliplatin-based preoperative chemoradiotherapy in patients with resectable rectal cancer. A single-institution experience. *Clin Transl Oncol* **14:** 471-480

Bindra RS, Gibson SL, Meng A, Westermark U, Jasin M, Pierce AJ, Bristow RG, Classon MK, Glazer PM (2005) Hypoxia-induced down-regulation of BRCA1 expression by E2Fs. *Cancer Res* **65:** 11597-11604

Birgisdottir AB, Lamark T, Johansen T (2013) The LIR motif - crucial for selective autophagy. *J Cell Sci* **126:** 3237-3247

Bissell MJ, Radisky D (2001) Putting tumours in context. *Nat Rev Cancer* **1:** 46-54

Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* **171:** 603-614

Black A, Morris D (2012) Personalized medicine in metastatic non-small-cell lung cancer: promising targets and current clinical trials. *Curr Oncol* **19:** S73-S85

Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, Ove R, Kies MS, Baselga J, Youssoufian H, Amellal N, Rowinsky EK, Ang KK (2006) Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *New Engl J Med* **354:** 567-578

Borges HL, Chao C, Xu Y, Linden R, Wang JYJ (2004) Radiation-induced apoptosis in developing mouse retina exhibits dose-dependent requirement for ATM phosphorylation of p53. *Cell Death Differ* **11:** 494-502

Bradley KA, Zhou T, McNall-Knapp RY, Jakacki RI, Levy AS, Vezina G, Pollack IF (2013) Motexafin-Gadolinium and Involved Field Radiation Therapy for Intrinsic Pontine Glioma of Childhood: A Children's Oncology Group Phase 2 Study. *Int J Radiat Oncol* **85:** E55-E60

Bridges KA, Hirai H, Buser CA, Brooks C, Liu H, Buchholz TA, Molkentine JM, Mason KA, Meyn RE (2011) MK-1775, a novel Wee1 kinase inhibitor, radiosensitizes p53-defective human tumor cells. *Clin Cancer Res* **17:** 5638-5648

Bristol ML, Di X, Beckman MJ, Wilson EN, Henderson SC, Maiti A, Fan Z, Gewirtz DA (2012) Dual functions of autophagy in the response of breast tumor cells to radiation: cytoprotective autophagy with radiation alone and cytotoxic autophagy in radiosensitization by vitamin D 3. *Autophagy* **8:** 739-753

Bristol ML, Emery SM, Maycotte P, Thorburn A, Chakradeo S, Gewirtz DA (2013) Autophagy Inhibition for Chemosensitization and Radiosensitization in Cancer: Do the Preclinical Data Support This Therapeutic Strategy? *J Pharmacol Exp Ther* **344:** 544-552

Brunsvig PF, Hatlevoll R, Berg R, Lauvvang G, Owre K, Wang M, Aamdal S (2005) Weekly docetaxel with concurrent radiotherapy in locally advanced non-small cell lung cancer: A Phase I/II study with 5 years' follow-up. *Lung Cancer-J Iaslc* **50:** 97-105

Butcher DT, Alliston T, Weaver VM (2009) A tense situation: forcing tumour progression. *Nat Rev Cancer* **9:** 108-122

Cai SL, Tee AR, Short JD, Bergeron JM, Kim J, Shen JJ, Guo RF, Johnson CL, Kiguchi K, Walker CL (2006) Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. *J Cell Biol* **173:** 279-289

Cam H, Easton JB, High A, Houghton PJ (2010) mTORC1 Signaling under Hypoxic Conditions Is Controlled by ATM-Dependent Phosphorylation of HIF-1 alpha. *Mol Cell* **40:** 509-520

Carroll VA, Ashcroft M (2006) Role of hypoxia-inducible factor (HIF)-1 alpha-versus HIF-2 alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-1, or loss of von Hippel-Lindau function: Implications for targeting the HIF pathway. *Cancer Res* **66:** 6264-6270

Castillo K, Valenzuela V, Matus S, Nassif M, Onate M, Fuentealba Y, Encina G, Irrazabal T, Parsons G, Court FA, Schneider BL, Armentano D, Hetz C (2013) Measurement of autophagy flux in the nervous system in vivo. *Cell Death Dis* **4:** e917

Cazares-Korner C, Pires IM, Swallow ID, Grayer SC, O'Connor LJ, Olcina MM, Christlieb M, Conway SJ, Hammond EM (2013) CH-01 is a hypoxia-activated prodrug that sensitizes cells to hypoxia/reoxygenation through inhibition of Chk1 and Aurora A. *ACS Chem Biol* **8:** 1451-1459

Cengel KA, Voong KR, Chandrasekaran S, Maggiorella L, Brunner TB, Stanbridge E, Kao GD, McKenna WG, Bernhard EJ (2007) Oncogenic K-Ras signals through epidermal growth factor receptor and wild-type H-Ras to promote radiation survival in pancreatic and colorectal carcinoma cells. *Neoplasia* **9:** 341-348

Ch'ang HJ, Maj JG, Paris F, Xing HR, Zhang JJ, Truman JP, Cardon-Cardo C, Haimovitz-Friedman A, Kolesnick R, Fuks Z (2005) ATM regulates target switching to escalating doses of radiation in the intestines. *Nat Med* **11:** 484-490

Chaachouay H, Ohneseit P, Toulany M, Kehlbach R, Multhoff G, Rodemann HP (2011) Autophagy contributes to resistance of tumor cells to ionizing radiation. *Radiother Oncol* **99:** 287-292

Chan DA, Sutphin PD, Nguyen P, Turcotte S, Lai EW, Banh A, Reynolds GE, Chi JT, Wu J, Solow-Cordero DE, Bonnet M, Flanagan JU, Bouley DM, Graves EE, Denny WA, Hay MP, Giaccia AJ (2011) Targeting GLUT1 and the Warburg effect in renal cell carcinoma by chemical synthetic lethality. *Sci Transl Med* **3:** 94ra70

Chan N, Bristow RG (2010) "Contextual" synthetic lethality and/or loss of heterozygosity: tumor hypoxia and modification of DNA repair. *Clin Cancer Res* **16:** 4553-4560

Charrier JD, Durrant SJ, Golec JMC, Kay DP, Knegtel RMA, MacCormick S, Mortimore M, O'Donnell ME, Pinder JL, Reaper PM, Rutherford AP, Wang PSH, Young SC, Pollard JR (2011) Discovery of Potent and Selective Inhibitors of Ataxia Telangiectasia Mutated and Rad3 Related (ATR) Protein Kinase as Potential Anticancer Agents. *J Med Chem* **54:** 2320-2330

Chaudhry MA (2007) Base excision repair of ionizing radiation-induced DNA damage in G1 and G2 cell cycle phases. *Cancer Cell Int* **7:** 15

Chen L, Lin YL, Peng G, Li F (2012) Structural basis for multifunctional roles of mammalian aminopeptidase N. *Proc Natl Acad Sci U S A* **109:** 17966-17971

Chen LS, Huang SR, Lee L, Davalos A, Schiestl RH, Campisi J, Oshima J (2003) WRN, the protein deficient in Werner syndrome, plays a critical structural role in optimizing DNA repair. *Aging Cell* **2:** 191-199

Chen Y, Dodd SJ, Tangrea MA, Emmert-Buck MR, Koretsky AP (2013) Measuring collective cell movement and extracellular matrix interactions using magnetic resonance imaging. *Sci Rep-Uk* **3:** 1879

Chiu HW, Lin JH, Chen YA, Ho SY, Wang YJ (2010) Combination treatment with arsenic trioxide and irradiation enhances cell-killing effects in human fibrosarcoma cells in vitro and in vivo through induction of both autophagy and apoptosis. *Autophagy* **6:** 353-365

Christensen M, Najy AJ, Snyder M, Movilla LS, Kim H-RC (2014) A critical role of the PTEN/PDGF signaling network for the regulation of radiosensitivity in adenocarcinoma of the prostate. *International journal of radiation oncology, biology, physics* **88:** 151-158

Chung YM, Park SH, Tsai WB, Wang SY, Ikeda MA, Berek JS, Chen DJ, Hu MCT (2012) FOXO3 signalling links ATM to the p53 apoptotic pathway following DNA damage. *Nat Commun* **3:** 1000

Clark AJ, Chan DC, Chen MY, Fillmore H, Dos Santos WG, Van Meter TE, Graf MR, Broaddus WC (2007) Down-regulation of Wilms' tumor 1 expression in glioblastoma cells increases radiosensitivity independently of p53. *J Neuro-Oncol* **83:** 163-172

Clutton SM, Townsend KMS, Walker C, Ansell JD, Wright EG (1996) Radiation-induced genomic instability and persisting oxidative stress in primary bone marrow cultures. *Carcinogenesis* **17:** 1633-1639

Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ, Maxwell PH (2000) Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *Journal of Biological Chemistry* **275:** 25733-25741

Cole AJ, Hanna GG, Jain S, O'Sullivan JM (2014) Motion management for radical radiotherapy in non-small cell lung cancer. *Clin Oncol (R Coll Radiol)* **26:** 67-80

Cortes J, Feldman E, Yee K, Rizzieri D, Advani AS, Charman A, Spruyt R, Toal M, Kantarjian H (2013) Two dosing regimens of tosedostat in elderly patients with relapsed or refractory acute myeloid leukaemia (OPAL): a randomised open-label phase 2 study. *Lancet Oncol* **14:** 354-362

Coussens LM, Werb Z (2002) Inflammation and cancer. *Nature* **420:** 860-867

Cox R, Masson WK, Weichselbaum RR, Nove J, Little JB (1981) The repair of potentially lethal damage in x-irradiated cultures of normal and ataxia telangiectasia human fibroblasts. *Int J Radiat Biol Relat Stud Phys Chem Med* **39:** 357-365

Crighton D, Wilkinson S, Ryan KM (2007) DRAM links autophagy to p53 and programmed cell death. *Autophagy* **3:** 72-74

Crosby ME, Kulshreshtha R, Ivan M, Glazer PM (2009) MicroRNA regulation of DNA repair gene expression in hypoxic stress. *Cancer Res* **69:** 1221-1229

CRUK (2014) All Cancers combined. Available from: http://publications.cancerresearchuk.org/downloads/Product/CS_KF_ALLCANCERS.pdf

Cui J, Mao XZ, Olman V, Hastings PJ, Xu Y (2012) Hypoxia and miscoupling between reduced energy efficiency and signaling to cell proliferation drive cancer to grow increasingly faster. *J Mol Cell Biol* **4:** 174-176

d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP (2003) A DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426:** 194-198

Daley JM, Gaines WA, Kwon Y, Sung P (2014) Regulation of DNA Pairing in Homologous Recombination. *Cold Spring Harb Perspect Biol***:** doi: 10.1101/cshperspect.a017954

Dang CV (2012) Links between metabolism and cancer. *Gene Dev* **26:** 877-890

Darby SC, Ewertz M, McGale P, Bennet AM, Blom-Goldman U, Bronnum D, Correa C, Cutter D, Gagliardi G, Gigante B, Jensen MB, Nisbet A, Peto R, Rahimi K, Taylor C, Hall P (2013) Risk of ischemic heart disease in women after radiotherapy for breast cancer. *N Engl J Med* **368:** 987-998

De Witt Hamer PC, Mir SE, Noske D, Van Noorden CJ, Wurdinger T (2011) WEE1 kinase targeting combined with DNA-damaging cancer therapy catalyzes mitotic catastrophe. *Clin Cancer Res* **17:** 4200-4207

Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X, Abbott D, Cuny GD, Yuan C, Wagner G, Hedrick SM, Gerber SA, Lugovskoy A, Yuan J (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol* **4:** 313-321

Delia D, Fontanella E, Ferrario C, Chessa L, Mizutani S (2003) DNA damage-induced cell-cycle phase regulation of p53 and p21(waf1) in normal and ATM-defective cells. *Oncogene* **22:** 7866-7869

Denton D, Nicolson S, Kumar S (2012) Cell death by autophagy: facts and apparent artefacts. *Cell Death Differ* **19:** 87-95

Depping R, Steinhoff A, Schindler SG, Friedrich B, Fagerlund R, Metzen E, Hartmann E, Kohler M (2008) Nuclear translocation of hypoxia-inducible factors (HIFs): involvement of the classical importin alpha/beta pathway. *Biochim Biophys Acta* **1783:** 394-404

Deriano L, Roth DB (2013) Modernizing the nonhomologous end-joining repertoire: alternative and classical NHEJ share the stage. *Annu Rev Genet* **47:** 433-455

Deschavanne PJ, Fertil B (1996) A review of human cell radiosensitivity in vitro. *Int J Radiat Oncol* **34:** 251-266

Devereaux K, Dall'Armi C, Alcazar-Roman A, Ogasawara Y, Zhou X, Wang F, Yamamoto A, De Camilli P, Di Paolo G (2013) Regulation of mammalian autophagy by class II and III PI 3-kinases through PI3P synthesis. *Plos One* **8:** e76405

di Fagagna FD, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, von Zglinicki T, Saretzki G, Carter NP, Jackson SP (2003) A DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426:** 194-198

Dixon H, Norbury CJ (2002) Therapeutic exploitation of checkpoint defects in cancer cells lacking p53 function. *Cell Cycle* **1:** 362-368

Doedens AL, Phan AT, Stradner MH, Fujimoto JK, Nguyen JV, Yang E, Johnson RS, Goldrath AW (2013) Hypoxia-inducible factors enhance the effector responses of CD8(+) T cells to persistent antigen. *Nat Immunol* **14:** 1173-U1199

Donahue SL, Campbell C (2004) A Rad50-dependent pathway of DNA repair is deficient in Fanconi anemia fibroblasts. *Nucleic Acids Res* **32:** 3248-3257

Drappatz J, Norden AD, Wong ET, Doherty LM, LaFrankie DC, Ciampa A, Kesari S, Sceppa C, Gerard M, Phan P, Schiff D, Batchelor TT, Ligon KL, Young G, Muzikansky A, Weiss SE, Wen PY (2010) Phase I Study of Vandetanib with Radiotherapy and Temozolomide for Newly Diagnosed Glioblastoma. *Int J Radiat Oncol* **78:** 85-90

Edwards BK, Howe HL, Ries LAG, Thun MJ, Rosenberg HM, Yancik R, Wingo PA, Jemal A, Feigal EG (2002) Annual report to the nation on the status of cancer, 1973-1999, featuring implications of age and aging on US cancer burden. *Cancer* **94:** 2766-2792

Egeblad M, Nakasone ES, Werb Z (2010) Tumors as Organs: Complex Tissues that Interface with the Entire Organism. *Dev Cell* **18:** 884-901

Egger M, Schgoer W, Beer AG, Jeschke J, Leierer J, Theurl M, Frauscher S, Tepper OM, Niederwanger A, Ritsch A, Kearney M, Wanschitz J, Gurtner GC, Fischer-Colbrie R,

Weiss G, Piza-Katzer H, Losordo DW, Patsch JR, Schratzberger P, Kirchmair R (2007) Hypoxia up-regulates the angiogenic cytokine secretoneurin via an HIF-1alpha- and basic FGF-dependent pathway in muscle cells. *FASEB J* **21:** 2906-2917

Elinav E, Nowarski R, Thaiss CA, Hu B, Jin CC, Flavell RA (2013) Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nat Rev Cancer* **13:** 759-771

Emerling BM, Weinberg F, Liu JL, Mak TW, Chandel NS (2008) PTEN regulates p300-dependent hypoxia-inducible factor 1 transcriptional activity through Forkhead transcription factor 3a (FOXO3a). *P Natl Acad Sci USA* **105:** 2622-2627

Ernestos B, Nikolaos P, Koulis G, Eleni R, Konstantinos B, Alexandra G, Michael K (2010) Increased Chromosomal Radiosensitivity in Women Carrying Brca1/Brca2 Mutations Assessed with the G2 Assay. *Int J Radiat Oncol* **76:** 1199-1205

Errafiy R, Aguado C, Ghislat G, Esteve JM, Gil A, Loutfi M, Knecht E (2013) PTEN increases autophagy and inhibits the ubiquitin-proteasome pathway in glioma cells independently of its lipid phosphatase activity. *Plos One* **8:** e83318

Escribano-Diaz C, Orthwein A, Fradet-Turcotte A, Xing MT, Young JTF, Tkac J, Cook MA, Rosebrock AP, Munro M, Canny MD, Xu DY, Durocher D (2013) A Cell Cycle-Dependent Regulatory Circuit Composed of 53BP1-RIF1 and BRCA1-CtIP Controls DNA Repair Pathway Choice. *Mol Cell* **49:** 872-883

Estrella V, Chen TA, Lloyd M, Wojtkowiak J, Cornnell HH, Ibrahim-Hashim A, Bailey K, Balagurunathan Y, Rothberg JM, Sloane BF, Johnson J, Gatenby RA, Gillies RJ (2013) Acidity Generated by the Tumor Microenvironment Drives Local Invasion. *Cancer Res* **73:** 1524-1535

Fabbro M, Savage K, Hobson K, Deans AJ, Powell SN, McArthur GA, Khanna KK (2004) BRCA1-BARD1 complexes are required for p53Ser-15 phosphorylation and a G1/S arrest following ionizing radiation-induced DNA damage. *J Biol Chem* **279:** 31251-31258

Farber S (1949) Some Observations on the Effect of Folic Acid Antagonists on Acute Leukemia and Other Forms of Incurable Cancer. *Blood* **4:** 160-167

Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, Corazzari M, Fuoco C, Ucar A, Schwartz P, Gruss P, Piacentini M, Chowdhury K, Cecconi F (2007) Ambra1 regulates autophagy and development of the nervous system. *Nature* **447:** 1121-1125

Firat E, Weyerbrock A, Gaedicke S, Grosu AL, Niedermann G (2012) Chloroquine or chloroquine-PI3K/Akt pathway inhibitor combinations strongly promote gamma-irradiation-induced cell death in primary stem-like glioma cells. *Plos One* **7:** e47357

Fokas E, Prevo R, Hammond EM, Brunner TB, McKenna WG, Muschel RJ (2014) Targeting ATR in DNA damage response and cancer therapeutics. *Cancer Treat Rev* **40:** 109-117

Fokas E, Prevo R, Pollard JR, Reaper PM, Charlton PA, Cornelissen B, Vallis KA, Hammond EM, Olcina MM, McKenna WG, Muschel RJ, Brunner TB (2012) Targeting ATR in vivo using the novel inhibitor VE-822 results in selective sensitization of pancreatic tumors to radiation. *Cell Death Dis* **3:** e441

Foote KM, Blades K, Cronin A, Fillery S, Guichard SS, Hassall L, Hickson I, Jacq X, Jewsbury PJ, McGuire TM, Nissink JWM, Odedra R, Page K, Perkins P, Suleman A, Tam K, Thommes P, Broadhurst R, Wood C (2013) Discovery of 4-{4-[(3R)-3-Methylmorpholin-4-yl]-6[1-(methylsulfonyl)cyclopropyl]pyrimidin-2-yl}-1H-indole (AZ20): A Potent and Selective Inhibitor of ATR Protein Kinase with Monotherapy in Vivo Antitumor Activity. *J Med Chem* **56:** 2125-2138

Foray N, Marot D, Gabriel A, Randrianarison V, Carr AM, Perricaudet M, Ashworth A, Jeggo P (2003) A subset of ATM- and ATR-dependent phosphorylation events requires the BRCA1 protein. *Embo Journal* **22:** 2860-2871

Frankenberg-Schwager M, Gebauer A, Koppe C, Wolf H, Pralle E, Frankenberg D (2009) Single-Strand Annealing, Conservative Homologous Recombination, Nonhomologous DNA End Joining, and the Cell Cycle-Dependent Repair of DNA Double-Strand Breaks Induced by Sparsely or Densely Ionizing Radiation. *Radiat Res* **171:** 265-273

Freedman SJ, Sun ZY, Poy F, Kung AL, Livingston DM, Wagner G, Eck MJ (2002) Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1 alpha. *Proc Natl Acad Sci U S A* **99:** 5367-5372

Freiberg RA, Hammond EM, Dorie MJ, Welford SM, Giaccia AJ (2006) DNA damage during reoxygenation elicits a Chk2-dependent checkpoint response. *Mol Cell Biol* **26:** 1598-1609

Fu J, Shao CJ, Chen FR, Ng HK, Chen ZP (2010) Autophagy induced by valproic acid is associated with oxidative stress in glioma cell lines. *Neuro-Oncology* **12:** 328-340

Fujita N, Itoh T, Omori H, Fukuda M, Noda T, Yoshimori T (2008) The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol Biol Cell* **19:** 2092-2100

Fujiwara K, Iwado E, Mills GB, Sawaya R, Kondo S, Kondo Y (2007) Akt inhibitor shows anticancer and radiosensitizing effects in malignant glioma cells by inducing autophagy. *Int J Oncol* **31:** 753-760

Furuya N, Yu F, Byfield M, Pattingre S, Levine B (2005) The evolutionarily conserved domain of Beclin 1 is required for Vps34 binding, autophagy and tumor suppressor function. *Autophagy* **1:** 46-52

Gamper AM, Rofougaran R, Watkins SC, Greenberger JS, Beumer JH, Bakkenist CJ (2013) ATR kinase activation in G1 phase facilitates the repair of ionizing radiation-induced DNA damage. *Nucleic Acids Res* **41:** 10334-10344

Ganley IG, Lam DH, Wang JR, Ding XJ, Chen S, Jiang XJ (2009) ULK1 center dot ATG13 center dot FIP200 Complex Mediates mTOR Signaling and Is Essential for Autophagy. *Journal of Biological Chemistry* **284:** 12297-12305

Gannon HS, Woda BA, Jones SN (2012) ATM Phosphorylation of Mdm2 Ser394 Regulates the Amplitude and Duration of the DNA Damage Response in Mice. *Cancer Cell* **21:** 668-679

Gatei M, Sloper K, Sorensen C, Syljuasen R, Falck J, Hobson K, Savage K, Lukas J, Zhou BB, Bartek J, Khanna KK (2003) Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. *Journal of Biological Chemistry* **278:** 14806-14811

Gewirtz DA, Hilliker ML, Wilson EN (2009) Promotion of autophagy as a mechanism for radiation sensitization of breast tumor cells. *Radiotherapy and Oncology* **92:** 323-328

Gies E, Wilde I, Winget JM, Brack M, Rotblat B, Novoa CA, Balgi AD, Sorensen PH, Roberge M, Mayor T (2010) Niclosamide prevents the formation of large ubiquitin-containing aggregates caused by proteasome inhibition. *Plos One* **5:** e14410

Gilkes DM, Bajpai S, Chaturvedi P, Wirtz D, Semenza GL (2013) Hypoxia-inducible Factor 1 (HIF-1) Promotes Extracellular Matrix Remodeling under Hypoxic Conditions by Inducing P4HA1, P4HA2, and PLOD2 Expression in Fibroblasts. *Journal of Biological Chemistry* **288:** 10819-10829

Golden EB, Frances D, Pellicciotta I, Demaria S, Barcellos-Hoff MH, Formenti SC (2014) Radiation fosters dose-dependent and chemotherapy-induced immunogenic cell death. *Oncoimmunology* **3:** e28518

Good JS, Harrington KJ (2013) The Hallmarks of Cancer and the Radiation Oncologist: Updating the 5Rs of Radiobiology. *Clin Oncol-Uk* **25:** 569-577

Goodhead DT (1989) The Initial Physical Damage Produced by Ionizing-Radiations. *Int J Radiat Biol* **56:** 623-634

Gorgoulis VG, Vassiliou LVF, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, DiTullio RA, Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD (2005) Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* **434:** 907-913

Grasso D, Ropolo A, Lo Re A, Boggio V, Molejon MI, Iovanna JL, Gonzalez CD, Urrutia R, Vaccaro MI (2011) Zymophagy, a novel selective autophagy pathway mediated by VMP1-USP9x-p62, prevents pancreatic cell death. *J Biol Chem* **286:** 8308-8324

Greer SN, Metcalf JL, Wang Y, Ohh M (2012) The updated biology of hypoxia-inducible factor. *EMBO J* **31:** 2448-2460

Greijer AE, van der Groep P, Kemming D, Shvarts A, Semenza GL, Meijer GA, van de Wiel MA, Belien JAM, van Diest PJ, van der Wall E (2005) Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). *J Pathol* **206:** 291-304

Groulx I, Lee S (2002) Oxygen-dependent ubiquitination and degradation of hypoxia-inducible factor requires nuclear-cytoplasmic trafficking of the von Hippel-Lindau tumor suppressor protein. *Mol Cell Biol* **22:** 5319-5336

Haie-Meder C, Siebert FA, Potter R (2011) Image guided, adaptive, accelerated, high dose brachytherapy as model for advanced small volume radiotherapy. *Radiotherapy and Oncology* **100:** 333-343

Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, Lippincott-Schwartz J (2010) Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell* **141:** 656-667

Hall EJ, Giaccia AJ (2006) *Radiobiology for the Radiologist*: Lippincott Williams & Wilkins.

Hall JS, Iype R, Senra J, Taylor J, Armenoult L, Oguejiofor K, Li YY, Stratford I, Stern PL, O'Connor MJ, Miller CJ, West CML (2014) Investigation of Radiosensitivity Gene Signatures in Cancer Cell Lines. *Plos One* **9:** e86329

Hamasaki M, Furuta N, Matsuda A, Nezu A, Yamamoto A, Fujita N, Oomori H, Noda T, Haraguchi T, Hiraoka Y, Amano A, Yoshimori T (2013) Autophagosomes form at ER-mitochondria contact sites. *Nature* **495:** 389-393

Hammond EM, Asselin MC, Forster D, O'Connor JPB, Senra JM, Williams KJ (2014) The Meaning, Measurement and Modification of Hypoxia in the Laboratory and the Clinic. *Clin Oncol-Uk* **26:** 277-288

Hammond EM, Denko NC, Dorie MJ, Abraham RT, Giaccia AJ (2002) Hypoxia links ATR and p53 through replication arrest. *Mol Cell Biol* **22:** 1834-1843

Hammond EM, Dorie MJ, Giaccia AJ (2004) Inhibition of ATR leads to increased sensitivity to hypoxia/reoxygenation. *Cancer Res* **64:** 6556-6562

Hammond EM, Giaccia AJ (2004) The role of ATM and ATR in the cellular response to hypoxia and re-oxygenation. *DNA Repair (Amst)* **3:** 1117-1122

Hammond EM, Kaufmann MR, Giaccia AJ (2007) Oxygen sensing and the DNA-damage response. *Curr Opin Cell Biol* **19:** 680-684

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* **100:** 57-70

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* **144:** 646-674

Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* **441:** 885-889

Hardie DG, Ross FA, Hawley SA (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Bio* **13:** 251-262

Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T, Yamamoto A (2009) A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. *Nat Cell Biol* **11:** 1433-1437

Hebert C, Norris K, Parashar P, Ord RA, Nikitakis NG, Sauk JJ (2006) Hypoxia-inducible factor-1alpha polymorphisms and TSC1/2 mutations are complementary in head and neck cancers. *Mol Cancer* **5:** 3

Hendricksen K, Cornel EB, de Reijke TM, Arentsen HC, Chawla S, Witjes JA (2012) Phase 2 Study of Adjuvant Intravesical Instillations of Apaziquone for High Risk Nonmuscle Invasive Bladder Cancer. *J Urology* **187:** 1195-1199

Henze AT, Acker T (2010) Feedback regulators of hypoxia-inducible factors and their role in cancer biology. *Cell Cycle* **9:** 2749-2763

Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, Ratcliffe PJ, Pugh CW, Schofield CJ (2002) Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *Journal of Biological Chemistry* **277:** 26351-26355

Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NM, Orr AI, Reaper PM, Jackson SP, Curtin NJ, Smith GC (2004) Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res* **64:** 9152-9159

Higgins GS, Prevo R, Lee YF, Helleday T, Muschel RJ, Taylor S, Yoshimura M, Hickson ID, Bernhard EJ, McKenna WG (2010) A Small Interfering RNA Screen of Genes Involved in DNA Repair Identifies Tumor-Specific Radiosensitization by POLQ Knockdown. *Cancer Res* **70:** 2984-2993

Hinz JM, Yamada NA, Salazar EP, Tebbs RS, Thompson LH (2005) Influence of double-strand-break repair pathways on radiosensitivity throughout the cell cycle in CHO cells. *DNA Repair* **4:** 782-792

Hirai H, Arai T, Okada M, Nishibata T, Kobayashi M, Sakai N, Imagaki K, Ohtani J, Sakai T, Yoshizumi T, Mizuarai S, Iwasawa Y, Kotani H (2010) MK-1775, a small molecule Wee1 inhibitor, enhances anti-tumor efficacy of various DNA-damaging agents, including 5-fluorouracil. *Cancer Biol Ther* **9:** 514-522

Hirai H, Iwasawa Y, Okada M, Arai T, Nishibata T, Kobayashi M, Kimura T, Kaneko N, Ohtani J, Yamanaka K, Itadani H, Takahashi-Suzuki I, Fukasawa K, Oki H, Nambu T, Jiang J, Sakai T, Arakawa H, Sakamoto T, Sagara T, Yoshizumi T, Mizuarai S, Kotani H (2009) Small-molecule inhibition of Wee1 kinase by MK-1775 selectively sensitizes p53-deficient tumor cells to DNA-damaging agents. *Mol Cancer Ther* **8:** 2992-3000

Hockel M, Vaupel P (2001) Tumor hypoxia: Definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer I* **93:** 266-276

Hoeijmakers JHJ (2012) The Key Role of DNA Damage on Cancer, Aging and Longevity. *Environ Mol Mutagen* **53:** S13-S13

Hollebecque A, Massard C, De Baere T, Auger N, Lacroix L, Koubi-Pick V, Vielh P, Lazar V, Bahleda R, Ngo-camus M, Angevin E, Varga A, Deschamps F, Gazzah A, Mazoyer C, Richon C, Vassal G, Eggermont AM, Andre F, Soria JC (2013) Molecular screening for cancer treatment optimization (MOSCATO 01): A prospective molecular triage trial-Interim results. *J Clin Oncol* **31:** (Suppl.), a2512.

Holler N, Zaru R, Micheau O, Thome M, Attinger A, Valitutti S, Bodmer JL, Schneider P, Seed B, Tschopp J (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol* **1:** 489-495

Hollingsworth JM, Miller DC, Daignault S, Hollenbeck BK (2006a) Rising incidence of small renal masses: a need to reassess treatment effect. *J Natl Cancer I* **98:** 1331-1334

Hollingsworth JM, Miller DC, Dunn RL, Montgomery JS, Roberts WW, Hafez KS, Wolf JS, Jr. (2006b) Surgical management of low-stage renal cell carcinoma: Technology does not supersede biology. *Urology* **67:** 1175-1180

Horsman MR, Overgaard J (2007) Hyperthermia: a potent enhancer of radiotherapy. *Clin Oncol-Uk* **19:** 418-426

Hotchkiss RS, Strasser A, McDunn JE, Swanson PE (2009) Mechanisms of Disease Cell Death. *New Engl J Med* **361:** 1570-1583

Houldsworth J, Lavin MF (1980) Effect of ionizing radiation on DNA synthesis in ataxia telangiectasia cells. *Nucleic Acids Res* **8:** 3709-3720

Hovinga KE, Stalpers LJA, van Bree C, Donker M, Verhoeff JJC, Rodermond HM, Bosch DA, van Furth WR (2005) Radiation-enhanced vascular endothelial growth factor (VEGF) secretion in glioblastoma multiforme cell lines - a clue to radioresistance? *J Neuro-Oncol* **74:** 99-103

Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC (2003) Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* **23:** 9361-9374

Huang WC, Elkin EB, Levey AS, Jang TL, Russo P (2009) Partial nephrectomy versus radical nephrectomy in patients with small renal tumors--is there a difference in mortality and cardiovascular outcomes? *J Urol* **181:** 55-61; discussion 61-52

Ichinose Y, Genka K, Koike T, Kato H, Watanabe Y, Mori T, Iioka S, Sakuma A, Ohta M (2003) Randomized double-blind placebo-controlled trial of bestatin in patients with resected stage I squamous-cell lung carcinoma. *J Natl Cancer I* **95:** 605-610

Inagaki-Ohara K, Yada S, Takamura N, Reaves M, Yu XH, Liu ER, Rooney I, Nicholas S, Castro A, Ware CF, Green DR, Lin T (2001) p53-dependent radiation-induced crypt intestinal epithelial cells apoptosis is mediated in part through TNF-TNFR1 system. *Oncogene* **20:** 812-818

Inoki K, Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, Yang Q, Bennett C, Harada Y, Stankunas K, Wang CY, He X, MacDougald OA, You M, Williams BO, Guan KL (2006) TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* **126:** 955-968

Inoki K, Zhu T, Guan KL (2003) TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115:** 577-590

Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y (2005) Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. *Int J Oncol* **26:** 1401-1410

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr. (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* **292:** 464-468

Iyer R, Chhatrala R, Shefter T, Yang G, Malhotra U, Tan W, Levea C, Robins M, Khushalani N (2013) Erlotinib and Radiation Therapy for Elderly Patients with Esophageal Cancer - Clinical and Correlative Results from a Prospective Multicenter Phase 2 Trial. *Oncology-Basel* **85:** 53-58

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O-2-regulated prolyl hydroxylation. *Science* **292:** 468-472

Jacobson L, Wachowski TJ (1947) The use of nitrogen mustard in the treatment of neoplastic diseases. *Bull North Shore Branch Chic Med Soc***:** 3-8

Jager S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, Eskelinen EL (2004) Role for Rab7 in maturation of late autophagic vacuoles. *J Cell Sci* **117:** 4837-4848

Janssens GO, Rademakers SE, Terhaard CH, Doornaert PA, Bijl HP, van den Ende P, Chin A, Marres HA, de Bree R, van der Kogel AJ, Hoogsteen IJ, Bussink J, Span PN, Kaanders JH (2012) Accelerated Radiotherapy With Carbogen and Nicotinamide for Laryngeal Cancer: Results of a Phase III Randomized Trial. *J Clin Oncol* **30:** 1777-1783

Jazayeri A, Falck J, Lukas C, Bartek J, Smith GCM, Lukas J, Jackson SP (2006) ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol* **8:** 37-45

Jean JC, Rich CB, Joyce-Brady M (2006) Hypoxia results in an HIF-1-dependent induction of brain-specific aldolase C in lung epithelial cells. *Am J Physiol-Lung C* **291:** L950-L956

Jenkins C, Hewamana S, Krige D, Pepper C, Burnett A (2011) Aminopeptidase inhibition by the novel agent CHR-2797 (tosedostat) for the therapy of acute myeloid leukemia. *Leuk Res* **35:** 677-681

Jiang P, Nishimura T, Sakamaki Y, Itakura E, Hatta T, Natsume T, Mizushima N (2014) The HOPS complex mediates autophagosome-lysosome fusion through interaction with syntaxin 17. *Mol Biol Cell* **25:** 1327-1337

Joensuu G, Joensuu T, Nokisalmi P, Reddy C, Isola J, Ruutu M, Kouri M, Kupelian PA, Collan J, Pesonen S, Hemminki A (2010) A Phase I/Ii Trial of Gefitinib Given Concurrently with Radiotherapy in Patients with Nonmetastatic Prostate Cancer. *Int J Radiat Oncol* **78:** 42-49

Johnson G (1902) Letter. *Phil Med Journal***:** 220-221

Jones JA, Lutz ST, Chow E, Johnstone PA (2014) Palliative radiotherapy at the end of life: A critical review. *CA: A Cancer Journal for Clinicians* **64:** 295-310

Jones KR, Elmore LW, Jackson-Cook C, Demasters G, Povirk LF, Holt SE, Gewirtz DA (2005) p53-dependent accelerated senescence induced by ionizing radiation in breast tumour cells. *Int J Radiat Biol* **81:** 445-458

Junttila MR, de Sauvage FJ (2013) Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature* **501:** 346-354

Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T (2004) LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J Cell Sci* **117:** 2805-2812

Kakarougkas A, Ismail A, Klement K, Goodarzi AA, Conrad S, Freire R, Shibata A, Lobrich M, Jeggo PA (2013) Opposing roles for 53BP1 during homologous recombination. *Nucleic Acids Res* **41:** 9719-9731

Kamada T (2012) Clinical evidence of particle beam therapy (carbon). *International Journal of Clinical Oncology* **17:** 85-88

Kamura T, Koepp DM, Conrad MN, Skowyra D, Moreland RJ, Iliopoulos O, Lane WS, Kaelin WG, Jr., Elledge SJ, Conaway RC, Harper JW, Conaway JW (1999) Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science* **284:** 657-661

Karanam K, Kafri R, Loewer A, Lahav G (2012) Quantitative Live Cell Imaging Reveals a Gradual Shift between DNA Repair Mechanisms and a Maximal Use of HR in Mid S Phase. *Mol Cell* **47:** 320-329

Katz D, Ito E, Liu FF (2009) On the Path to Seeking Novel Radiosensitizers. *Int J Radiat Oncol* **73:** 988-996

Kawata T, Ito H, George K, Wu HL, Uno T, Isobe K, Cucinotta FA (2003) Radiation-induced chromosome aberrations in ataxia telangiectasia cells: High frequency of deletions and misrejoining detected by fluorescence in situ hybridization. *Radiat Res* **159:** 597-603

Keith B, Johnson RS, Simon MC (2012) HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. *Nat Rev Cancer* **12:** 9-22

Kim GJ, Fiskum GM, Morgan WF (2006) A role for mitochondrial dysfunction in perpetuating radiation-induced genomic instability. *Cancer Res* **66:** 10377-10383

Kim H, Bernard ME, Flickinger J, Epperly MW, Wang H, Dixon TM, Shields D, Houghton F, Zhang XC, Greenberger JS (2011a) The autophagy-inducing drug carbamazepine is a radiation protector and mitigator. *Int J Radiat Biol* **87:** 1052-1060

Kim J, Kundu M, Viollet B, Guan KL (2011b) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* **13:** 132-141

Kim KW, Moretti L, Mitchell LR, Jung DK, Lu B (2009) Combined Bcl-2/mammalian target of rapamycin inhibition leads to enhanced radiosensitization via induction of apoptosis and autophagy in non-small cell lung tumor xenograft model. *Clin Cancer Res* **15:** 6096-6105

Kirkin V, Lamark T, Johansen T, Dikic I (2009) NBR1 cooperates with p62 in selective autophagy of ubiquitinated targets. *Autophagy* **5:** 732-733

Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, et. al (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* **8:** 445-544

Ko A, Kanehisa A, Martins I, Senovilla L, Chargari C, Dugue D, Marino G, Kepp O, Michaud M, Perfettini JL, Kroemer G, Deutsch E (2014) Autophagy inhibition radiosensitizes in vitro, yet reduces radioresponses in vivo due to deficient immunogenic signalling. *Cell Death Differ* **21:** 92-99

Kobayashi S, Xu XM, Chen K, Liang QR (2012) Suppression of autophagy is protective in high glucose-induced cardiomyocyte injury. *Autophagy* **8:** 577-592

Koch CJ, Oprysko PR, Shuman AL, Jenkins WT, Brandt G, Evans SM (2002) Radiosensitization of hypoxic tumor cells by dodecafluoropentane: A gas-phase perfluorochemical emulsion. *Cancer Res* **62:** 3626-3629

Koshiji M, To KK, Hammer S, Kumamoto K, Harris AL, Modrich P, Huang LE (2005) HIF-1alpha induces genetic instability by transcriptionally downregulating MutSalpha expression. *Mol Cell* **17:** 793-803

Kraft C, Deplazes A, Sohrmann M, Peter M (2008) Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. *Nat Cell Biol* **10:** 602-610

Krige D, Needham LA, Bawden LJ, Flores N, Farmer H, Miles LE, Stone E, Callaghan J, Chandler S, Clark VL, Kirwin-Jones P, Legris V, Owen J, Patel T, Wood S, Box G, Laber D, Odedra R, Wright A, Wood LM, Eccles SA, Bone EA, Ayscough A, Drummond AH (2008) CHR-2797: an antiproliferative aminopeptidase inhibitor that leads to amino acid deprivation in human leukemic cells. *Cancer Res* **68:** 6669-6679

Kubota K, Hida T, Ishikura S, Mizusawa J, Nishio M, Kawahara M, Yokoyama A, Imamura F, Takeda K, Negoro S, Harada M, Okamoto H, Yamamoto N, Shinkai T, Sakai H, Matsui K, Nakagawa K, Shibata T, Saijo N, Tamura T, Grp JCO (2014) Etoposide and cisplatin versus irinotecan and cisplatin in patients with limited-stage small-cell lung cancer treated with etoposide and cisplatin plus concurrent accelerated hyperfractionated thoracic radiotherapy (JCOG0202): a randomised phase 3 study. *Lancet Oncol* **15:** 106-113

Kuhne M, Riballo E, Rief N, Rothkamm K, Jeggo PA, Lobrich M (2004) A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity. *Cancer Res* **64:** 500-508

Kuribayashi K, Finnberg N, Jeffers JR, Zambetti GP, El-Deiry WS (2011) The relative contribution of pro-apoptotic p53-target genes in the triggering of apoptosis following DNA damage in vitro and in vivo. *Cell Cycle* **10:** 2380-2389

Kuwahara Y, Oikawa T, Ochiai Y, Roudkenar MH, Fukumoto M, Shimura T, Ohtake Y, Ohkubo Y, Mori S, Uchiyama Y (2011) Enhancement of autophagy is a potential modality for tumors refractory to radiotherapy. *Cell Death Dis* **2:** e177

Kwitkowski VE, Prowell TM, Ibrahim A, Farrell AT, Justice R, Mitchell SS, Sridhara R, Pazdur R (2010) FDA Approval Summary: Temsirolimus as Treatment for Advanced Renal Cell Carcinoma. *Oncologist* **15:** 428-435

Lamark T, Kirkin V, Dikic I, Johansen T (2009) NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. *Cell Cycle* **8:** 1986-1990

Lambert CM, Roy M, Robitaille GA, Richard DE, Bonnet S (2010) HIF-1 inhibition decreases systemic vascular remodelling diseases by promoting apoptosis through a hexokinase 2-dependent mechanism. *Cardiovasc Res* **88:** 196-204

Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK (2002a) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Gene Dev* **16:** 1466-1471

Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML (2002b) Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* **295:** 858-861

Langland G, Elliott J, Li YL, Creaney J, Dixon K, Groden J (2002) The BLM helicase is necessary for normal DNA double-strand break repair. *Cancer Res* **62:** 2766-2770

Lee C, Kim JS, Waldman T (2004) PTEN gene targeting reveals a radiation-induced size checkpoint in human cancer cells. *Cancer Res* **64:** 6906-6914

Lee EJ, Tournier C (2011) The requirement of uncoordinated 51-like kinase 1 (ULK1) and ULK2 in the regulation of autophagy. *Autophagy* **7:** 689-695

Lee J, Kumagai A, Dunphy WG (2001) Positive regulation of Wee1 by Chk1 and 14-3-3 proteins. *Mol Biol Cell* **12:** 551-563

Lee JH, Jin Y, He G, Zeng SX, Wang YV, Wahl GM, Lu H (2012) Hypoxia activates tumor suppressor p53 by inducing ATR-Chk1 kinase cascade-mediated phosphorylation and consequent 14-3-3gamma inactivation of MDMX protein. *J Biol Chem* **287:** 20898-20903

Lehmann AR (2003) DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. *Biochimie* **85:** 1101-1111

Levine AJ (1997) p53, the cellular gatekeeper for growth and division. *Cell* **88:** 323-331

Lewis CE, Pollard JW (2006) Distinct role of macrophages in different tumor microenvironments. *Cancer Res* **66:** 605-612

Liang C, Lee JS, Inn KS, Gack MU, Li Q, Roberts EA, Vergne I, Deretic V, Feng P, Akazawa C, Jung JU (2008) Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. *Nat Cell Biol* **10:** 776-787

Liang N, Jia LL, Liu Y, Liang B, Kong DJ, Yan MM, Ma SM, Liu XD (2013) ATM pathway is essential for ionizing radiation-induced autophagy. *Cell Signal* **25:** 2530-2539

Lim YC, Roberts TL, Day BW, Stringer BW, Kozlov S, Fazry S, Bruce ZC, Ensbey KS, Walker DG, Boyd AW, Lavin MF (2014) Increased sensitivity to ionizing radiation by targeting the homologous recombination pathway in glioma initiating cells. *Molecular Oncology***:** S1574-7891(1514)00147-00141

Lin CI, Whang EE, Donner DB, Du J, Lorch J, He F, Jiang X, Price BD, Moore FD, Jr., Ruan DT (2010) Autophagy induction with RAD001 enhances chemosensitivity and radiosensitivity through Met inhibition in papillary thyroid cancer. *Mol Cancer Res* **8:** 1217-1226

Lin L, Yang P, Huang X, Zhang H, Lu Q (2013) The scaffold protein EPG-7 links cargo-receptor complexes with the autophagic assembly machinery. *J Cell Biol* **201:** 113-129

Ling YH, Aracil M, Zou Y, Yuan Z, Lu B, Jimeno J, Cuervo AM, Perez-Soler R (2011) PM02734 (Elisidepsin) Induces Caspase-Independent Cell Death Associated with Features of Autophagy, Inhibition of the Akt/mTOR Signaling Pathway, and Activation of Death-Associated Protein Kinase. *Clin Cancer Res* **17:** 5353-5366

Lingos TI, Recht A, Vicini F, Abner A, Silver B, Harris JR (1991) Radiation Pneumonitis in Breast-Cancer Patients Treated with Conservative Surgery and Radiation-Therapy. *Int J Radiat Oncol* **21:** 355-360

Lizarraga KJ, Allen-Auerbach M, Czernin J, DeSalles AA, Yong WH, Phelps ME, Chen W (2014) (18)F-FDOPA PET for differentiating recurrent or progressive brain metastatic tumors from late or delayed radiation injury after radiation treatment. *Journal of Nuclear Medicine* **55:** 30-36

Loehrer PJ, Feng Y, Cardenes H, Wagner L, Brell JM, Cella D, Flynn P, Ramanathan RK, Crane CH, Alberts SR, Benson A (2011) Gemcitabine Alone Versus Gemcitabine Plus

Radiotherapy in Patients With Locally Advanced Pancreatic Cancer: An Eastern Cooperative Oncology Group Trial. *J Clin Oncol* **29:** 4105-4112

Lomax ME, Folkes LK, O'Neill P (2013) Biological Consequences of Radiation-induced DNA Damage: Relevance to Radiotherapy. *Clin Oncol-Uk* **25:** 578-585

Lomonaco SL, Finniss S, Xiang C, Decarvalho A, Umansky F, Kalkanis SN, Mikkelsen T, Brodie C (2009) The induction of autophagy by gamma-radiation contributes to the radioresistance of glioma stem cells. *Int J Cancer* **125:** 717-722

Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G (2013) The hallmarks of aging. *Cell* **153:** 1194-1217

Lowenberg B, Morgan G, Ossenkoppele GJ, Burnett AK, Zachee P, Duhrsen U, Dierickx D, Muller-Tidow C, Sonneveld P, Krug U, Bone E, Flores N, Richardson AF, Hooftman L, Jenkins C, Zweegman S, Davies F (2010) Phase I/II clinical study of Tosedostat, an inhibitor of aminopeptidases, in patients with acute myeloid leukemia and myelodysplasia. *J Clin Oncol* **28:** 4333-4338

Lu F, Li YQ, Aubert I, Wong CS (2012a) Endothelial cells regulate p53-dependent apoptosis of neural progenitors after irradiation. *Cell Death Dis* **3:** e324

Lu PF, Weaver VM, Werb Z (2012b) The extracellular matrix: A dynamic niche in cancer progression. *J Cell Biol* **196:** 395-406

Luo HM, Wang L, Schulte BA, Yang AM, Tang SS, Wang GY (2013) Resveratrol enhances ionizing radiation-induced premature senescence in lung cancer cells. *Int J Oncol* **43:** 1999-2006

Luo S, Garcia-Arencibia M, Zhao R, Puri C, Toh PP, Sadiq O, Rubinsztein DC (2012) Bim inhibits autophagy by recruiting Beclin 1 to microtubules. *Mol Cell* **47:** 359-370

Mackey MA, Ianzini F (2000) Enhancement of radiation-induced mitotic catastrophe by moderate hyperthermia. *Int J Radiat Biol* **76:** 273-280

Malaise EP, Deschavanne PJ, Fertil B (1992) Intrinsic Radiosensitivity of Human-Cells. *Adv Radiat Biol* **15:** 37-70

Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, Burden SJ, Di Lisi R, Sandri C, Zhao J, Goldberg AL, Schiaffino S, Sandri M (2007) FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab* **6:** 458-471

Mancias JD, Wang XX, Gygi SP, Harper JW, Kimmelman AC (2014) Quantitative proteomics identifies NCOA4 as the cargo receptor mediating ferritinophagy. *Nature* **509:** 105-109

Mangoni M, Bisanzi S, Carozzi F, Sani C, Biti G, Livi L, Barletta E, Costantini AS, Gorini G (2011) Association between Genetic Polymorphisms in the Xrcc1, Xrcc3, Xpd, Gstm1, Gstt1, Msh2, Mlh1, Msh3, and Mgmt Genes and Radiosensitivity in Breast Cancer Patients. *Int J Radiat Oncol* **81:** 52-58

Mao K, Liu X, Feng YC, Klionsky DJ (2014) The progression of peroxisomal degradation through autophagy requires peroxisomal division. *Autophagy* **10:** 652-661

Mao Z, Bozzella M, Seluanov A, Gorbunova V (2008) DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. *Cell Cycle* **7:** 2902-2906

Marteijn JA, Lans H, Vermeulen W, Hoeijmakers JHJ (2014) Understanding nucleotide excision repair and its roles in cancer and ageing. *Nat Rev Mol Cell Bio* **15:** 465-481

Maruyama R, Goto K, Takemura G, Ono K, Nagao K, Horie T, Tsujimoto A, Kanamori H, Miyata S, Ushikoshi H, Nagashima K, Minatoguchi S, Fujiwara T, Fujiwara H (2008) Morphological and biochemical characterization of basal and starvation-induced autophagy in isolated adult rat cardiomyocytes. *Am J Physiol Heart Circ Physiol* **295:** H1599-1607

Matsunaga K, Morita E, Saitoh T, Akira S, Ktistakis NT, Izumi T, Noda T, Yoshimori T (2010) Autophagy requires endoplasmic reticulum targeting of the PI3-kinase complex via Atg14L. *J Cell Biol* **190:** 511-521

Matsunaga K, Saitoh T, Tabata K, Omori H, Satoh T, Kurotori N, Maejima I, Shirahama-Noda K, Ichimura T, Isobe T, Akira S, Noda T, Yoshimori T (2009) Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat Cell Biol* **11:** 385-396

Matsushita M, Suzuki NN, Obara K, Fujioka Y, Ohsumi Y, Inagaki F (2007) Structure of Atg5.Atg16, a complex essential for autophagy. *J Biol Chem* **282:** 6763-6772

Matsuyama T, Nakanishi K, Hayashi T, Yoshizumi Y, Aiko S, Sugiura Y, Tanimoto T, Uenoyama M, Ozeki Y, Maehara T (2005) Expression of hypoxia-inducible factor-1alpha in esophageal squamous cell carcinoma. *Cancer Sci* **96:** 176-182

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* **399:** 271-275

McCullough J, Colf LA, Sundquist WI (2013) Membrane fission reactions of the mammalian ESCRT pathway. *Annu Rev Biochem* **82:** 663-692

McKeage MJ, Jameson MB, Ramanathan RK, Rajendran J, Gu YC, Wilson WR, Melink TJ, Tchekmedyian NS (2012) PR-104 a bioreductive pre-prodrug combined with gemcitabine or docetaxel in a phase Ib study of patients with advanced solid tumours. *Bmc Cancer* **12:** 496

McKeown SR (2014) Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. *Br J Radiol* **87:** 20130676

Melchionna R, Chen XB, Blasina A, McGowan CH (2000) Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. *Nat Cell Biol* **2:** 762-765

Meng AX, Jalali F, Cuddihy A, Chan N, Bindra RS, Glazer PM, Bristow RG (2005) Hypoxia down-regulates DNA double strand break repair gene expression in prostate cancer cells. *Radiotherapy and Oncology* **76:** 168-176

Mesicek J, Lee H, Feldman T, Jiang XJ, Skobeleva A, Berdyshev EV, Haimovitz-Friedman A, Fuks Z, Kolesnick R (2010) Ceramide synthases 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells. *Cell Signal* **22:** 1300-1307

Metwally MAH, Frederiksen KD, Overgaard J (2014) Compliance and toxicity of the hypoxic radiosensitizer nimorazole in the treatment of patients with head and neck squamous cell carcinoma (HNSCC). *Acta Oncol* **53:** 654-661

Miqueli AD, Rolff J, Lemm M, Fichtner I, Perez R, Montero E (2009) Radiosensitisation of U87MG brain tumours by anti-epidermal growth factor receptor monoclonal antibodies. *Brit J Cancer* **100:** 950-958

Mizuarai S, Yamanaka K, Itadani H, Arai T, Nishibata T, Hirai H, Kotani H (2009) Discovery of gene expression-based pharmacodynamic biomarker for a p53 context-specific anti-tumor drug Wee1 inhibitor. *Molecular Cancer* **8:** 34

Mizushima N, Yoshimori T, Levine B (2010) Methods in mammalian autophagy research. *Cell* **140:** 313-326

Mizushima N, Yoshimori T, Ohsumi Y (2011) The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol* **27:** 107-132

Moding EJ, Lee CL, Castle KD, Oh P, Mao L, Zha S, Min HD, Ma Y, Das S, Kirsch DG (2014) Atm deletion with dual recombinase technology preferentially radiosensitizes tumor endothelium. *Journal of Clinical Investigation* **124:** 3325-3338

Momcilovic O, Choi S, Varum S, Bakkenist C, Schatten G, Navara C (2009) Ionizing radiation induces ataxia telangiectasia mutated-dependent checkpoint signaling and G(2) but not G(1) cell cycle arrest in pluripotent human embryonic stem cells. *Stem Cells* **27:** 1822-1835

Moore HE, Davenport EL, Smith EM, Muralikrishnan S, Dunlop AS, Walker BA, Krige D, Drummond AH, Hooftman L, Morgan GJ, Davies FE (2009) Aminopeptidase inhibition as a targeted treatment strategy in myeloma. *Mol Cancer Ther* **8:** 762-770

Moretti L, Li B, Kim KW, Chen HD, Lu B (2010) AT-101, a Pan-Bcl-2 Inhibitor, Leads to Radiosensitization of Non-small Cell Lung Cancer. *J Thorac Oncol* **5:** 680-687

Morgan JL, Holcomb TM, Morrissey RW (1968) Radiation reaction in ataxia telangiectasia. *Am J Dis Child* **116:** 557-558

Moullin MC (1899) Presidential Address ON THE APPLICATION OF THE ROENTGEN RAYS TO MEDICINE AND SURGERY. *The Lancet* **154:** 474-476

Moumen A, Magill C, Dry KL, Jackson SP (2013) ATM-dependent phosphorylation of heterogeneous nuclear ribonucleoprotein K promotes p53 transcriptional activation in response to DNA damage. *Cell Cycle* **12:** 698-704

Mukubou H, Tsujimura T, Sasaki R, Ku Y (2010) The role of autophagy in the treatment of pancreatic cancer with gemcitabine and ionizing radiation. *Int J Oncol* **37:** 821-828

Myers JS, Cortez D (2006) Rapid activation of ATR by ionizing radiation requires ATM and Mre11. *Journal of Biological Chemistry* **281:** 9346-9350

Nagasawa H, Little JB (1983) Comparison of kinetics of X-ray-induced cell killing in normal, ataxia telangiectasia and hereditary retinoblastoma fibroblasts. *Mutat Res* **109:** 297-308

Najafov A, Shpiro N, Alessi DR (2012) Akt is efficiently activated by PIF-pocket- and PtdIns(3,4,5)P-3-dependent mechanisms leading to resistance to PDK1 inhibitors. *Biochem J* **448:** 285-295

Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsumura Y, Asahi M, Nishida K, Hori M, Mizushima N, Otsu K (2007) The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. *Nat Med* **13:** 619-624

Nakatogawa H, Ichimura Y, Ohsumi Y (2007) Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell* **130:** 165-178

Nath S, Dancourt J, Shteyn V, Puente G, Fong WM, Nag S, Bewersdorf J, Yamamoto A, Antonny B, Melia TJ (2014) Lipidation of the LC3/GABARAP family of autophagy proteins relies on a membrane-curvature-sensing domain in Atg3. *Nat Cell Biol* **16:** 415-424

Neubauer S, Arutyunyan R, Stumm M, Dork T, Bendix R, Bremer M, Varon R, Sauer R, Gebhart E (2002) Radiosensitivity of ataxia telangiectasia and Nijmegen breakage syndrome homozygotes and heterozygotes as determined by three-color FISH chromosome painting. *Radiat Res* **157:** 312-321

Nomiya T, Tsuji H, Hirasawa N, Kato H, Kamada T, Mizoe J, Kishi H, Kamura K, Wada H, Nemoto K, Tsujii H (2008) Carbon ion radiation therapy for primary renal cell carcinoma: initial clinical experience. *Int J Radiat Oncol Biol Phys* **72:** 828-833

O'Donovan TR, O'Sullivan GC, McKenna SL (2011) Induction of autophagy by drug-resistant esophageal cancer cells promotes their survival and recovery following treatment with chemotherapeutics. *Autophagy* **7:** 509-524

O’Brien Eleanor M, Senra Joana M, Anbalagan S, Hill Mark A, Hammond Ester M. (2013) Impact of Wee1 inhibition on the hypoxia-induced DNA damage response. *Tumor Microenvironment and Therapy*, Vol. 1, pp. 37-45.

OConnell MJ, Raleigh JM, Verkade HM, Nurse P (1997) Chk1 is a wee1 kinase in the G(2) DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation. *Embo Journal* **16:** 545-554

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288:** 1053-1058

Ogata M, Hino S-i, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K, Shiosaka S, Hammarback JA, Urano F, Imaizumi K (2006) Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol Cell Biol* **26:** 9220-9231

Ogawa K, Ishiuchi S, Inoue O, Yoshii Y, Saito A, Watanabe T, Iraha S, Toita T, Kakinohana Y, Ariga T, Kasuya G, Murayama S (2012) Phase Ii Trial of Radiotherapy after Hyperbaric Oxygenation with Multiagent Chemotherapy (Procarbazine, Nimustine, and Vincristine) for High-Grade Gliomas: Long-Term Results. *Int J Radiat Oncol* **82:** 732-738

Ohh M, Park CW, Ivan N, Hoffman MA, Kim TY, Huang LE, Pavletich N, Chau V, Kaelin WG (2000) Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol* **2:** 423-427

Oka S (1980) A review of clinical studies of bestatin. *Recent Results Cancer Res* **75:** 126-132

Olcina MM, Foskolou IP, Anbalagan S, Senra JM, Pires IM, Jiang YY, Ryan AJ, Hammond EM (2013) Replication Stress and Chromatin Context Link ATM Activation to a Role in DNA Replication. *Mol Cell* **52:** 758-766

Olive PL (1999) DNA damage and repair in individual cells: applications of the comet assay in radiobiology. *Int J Radiat Biol* **75:** 395-405

Olive PL, Banath JP (2006) The comet assay: a method to measure DNA damage in individual cells. *Nat Protoc* **1:** 23-29

Olive PL, Banath JP, Durand RE (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiat Res* **122:** 86-94

On KF, Chen Y, Ma HT, Chow JP, Poon RY (2011) Determinants of mitotic catastrophe on abrogation of the G2 DNA damage checkpoint by UCN-01. *Mol Cancer Ther* **10:** 784-794

Onufrey V, Mohiuddin M (1985) Radiation therapy in the treatment of metastatic renal cell carcinoma. *Int J Radiat Oncol Biol Phys* **11:** 2007-2009

Osborne MP (2007) William Stewart Halsted: his life and contributions to surgery. *Lancet Oncol* **8:** 256-265

Ota K, Uzuka Y (1992) Clinical-Trials of Bestatin for Leukemia and Solid Tumors. *Biotherapy* **4:** 205-214

Overgaard J (2007) Hypoxic radiosensitization: Adored and ignored. *J Clin Oncol* **25:** 4066-4074

Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J (2001) A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* **61:** 439-444

Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, Johansen T (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* **282:** 24131-24145

Papandreou I, Lim AL, Laderoute K, Denko NC (2008) Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. *Cell Death Differ* **15:** 1572-1581

Park HJ, Griffin RJ, Hui S, Levitt SH, Song CW (2012) Radiation-Induced Vascular Damage in Tumors: Implications of Vascular Damage in Ablative Hypofractionated Radiotherapy (SBRT and SRS). *Radiat Res* **177:** 311-327

Parlanti E, Locatelli G, Maga G, Dogliotti E (2007) Human base excision repair complex is physically associated to DNA replication and cell cycle regulatory proteins. *Nucleic Acids Res* **35:** 1569-1577

Parsons JL, Khoronenkova SV, Dianova, II, Ternette N, Kessler BM, Datta PK, Dianov GL (2012) Phosphorylation of PNKP by ATM prevents its proteasomal degradation and enhances resistance to oxidative stress. *Nucleic Acids Res* **40:** 11404-11415

Partch CL, Gardner KH (2011) Coactivators necessary for transcriptional output of the hypoxia inducible factor, HIF, are directly recruited by ARNT PAS-B. *P Natl Acad Sci USA* **108:** 7739-7744

Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **122:** 927-939

Petrovic S, Leskovac A, Joksic I, Filipovic J, Vujic D, Guc-Scekic M, Joksic G (2013) Leukocyte apoptosis as a predictor of radiosensitivity in Fanconi anemia. *Curr Sci India* **105:** 56-60

Pinkel D (1959) Actinomycin D in Childhood Cancer - a Preliminary Report. *Pediatrics* **23:** 342-347

Pires IM, Bencokova Z, McGurk C, Hammond EM (2010a) Exposure to acute hypoxia induces a transient DNA damage response which includes Chk1 and TLK1. *Cell Cycle* **9:** 2502-2507

Pires IM, Bencokova Z, Milani M, Folkes LK, Li JL, Stratford MR, Harris AL, Hammond EM (2010b) Effects of acute versus chronic hypoxia on DNA damage responses and genomic instability. *Cancer Res* **70:** 925-935

Pires IM, Olcina MM, Anbalagan S, Pollard JR, Reaper PM, Charlton PA, McKenna WG, Hammond EM (2012) Targeting radiation-resistant hypoxic tumour cells through ATR inhibition. *Brit J Cancer* **107:** 291-299

Pirrotta MT, Bernardeschi P, Fiorentini G (2011) Targeted-therapy in advanced renal cell carcinoma. *Curr Med Chem* **18:** 1651-1657

Puri C, Renna M, Bento CF, Moreau K, Rubinsztein DC (2014) ATG16L1 meets ATG9 in recycling endosomes: additional roles for the plasma membrane and endocytosis in autophagosome biogenesis. *Autophagy* **10:** 182-184

Rajecki M, af Hallstrom T, Hakkarainen T, Nokisalmi P, Hautaniemi S, Nieminen AI, Tenhunen M, Rantanen V, Desmond RA, Chen DT, Guse K, Stenman UH, Gargini R, Kapanen M, Klefstrom J, Kanerva A, Pesonen S, Ahtiainen L, Hemminki A (2009) Mre11 inhibition by oncolytic adenovirus associates with autophagy and underlies synergy with ionizing radiation. *Int J Cancer* **125:** 2441-2449

Rajeshkumar NV, De Oliveira E, Ottenhof N, Watters J, Brooks D, Demuth T, Shumway SD, Mizuarai S, Hirai H, Maitra A, Hidalgo M (2011) MK-1775, a potent Wee1 inhibitor, synergizes with gemcitabine to achieve tumor regressions, selectively in p53-deficient pancreatic cancer xenografts. *Clin Cancer Res* **17:** 2799-2806

Ravikumar B, Imarisio S, Sarkar S, O'Kane CJ, Rubinsztein DC (2008) Rab5 modulates aggregation and toxicity of mutant huntingtin through macroautophagy in cell and fly models of Huntington disease. *J Cell Sci* **121:** 1649-1660

Ravikumar B, Moreau K, Jahreiss L, Puri C, Rubinsztein DC (2010) Plasma membrane contributes to the formation of pre-autophagosomal structures. *Nat Cell Biol* **12:** 747-757

Ravikumar B, Stewart A, Kita H, Kato K, Duden R, Rubinsztein DC (2003) Raised intracellular glucose concentrations reduce aggregation and cell death caused by mutant huntingtin exon 1 by decreasing mTOR phosphorylation and inducing autophagy. *Hum Mol Genet* **12:** 985-994

Reid AH, Protheroe A, Attard G, Hayward N, Vidal L, Spicer J, Shaw HM, Bone EA, Carter J, Hooftman L, Harris A, De Bono JS (2009) A first-in-man phase i and pharmacokinetic study on CHR-2797 (Tosedostat), an inhibitor of M1 aminopeptidases, in patients with advanced solid tumors. *Clin Cancer Res* **15:** 4978-4985

Reitman ZJ, Duncan CG, Poteet E, Winters A, Yan LJ, Gooden DM, Spasojevic I, Boros LG, Yang SH, Yan H (2014) Cancer-associated Isocitrate Dehydrogenase 1 (IDH1) R132H Mutation and d-2-Hydroxyglutarate Stimulate Glutamine Metabolism under Hypoxia. *J Biol Chem* **289:** 23318-23328

Riballo E, Kuhne M, Rief N, Doherty A, Smith GCM, Recio MJ, Reis C, Dahm K, Fricke A, Krempler A, Parker AR, Jackson SP, Gennery A, Jeggo PA, Lobrich M (2004) A pathway of double-strand break rejoining dependent upon ATM, artemis, and proteins locating to gamma-H2AX foci. *Mol Cell* **16:** 715-724

Rieckmann T, Tribius S, Grob TJ, Meyer F, Busch CJ, Petersen C, Dikomey E, Kriegs M (2013) HNSCC cell lines positive for HPV and p16 possess higher cellular radiosensitivity due to an impaired DSB repair capacity. *Radiotherapy and Oncology* **107:** 242-246

Riesterer O, Matsumoto F, Wang L, Pickett J, Molkentine D, Giri U, Milas L, Raju U (2011) A novel Chk inhibitor, XL-844, increases human cancer cell radiosensitivity through promotion of mitotic catastrophe. *Invest New Drug* **29:** 514-522

Rockwell S, Dobrucki IT, Kim EY, Marrison ST, Vu VT (2009) Hypoxia and Radiation Therapy: Past History, Ongoing Research, and Future Promise. *Curr Mol Med* **9:** 442-458

Romanov J, Walczak M, Ibiricu I, Schuchner S, Ogris E, Kraft C, Martens S (2012) Mechanism and functions of membrane binding by the Atg5-Atg12/Atg16 complex during autophagosome formation. *EMBO J* **31:** 4304-4317

Roslin M, Henriksson R, Bergstrom P, Ungerstedt U, Bergenheim AT (2003) Baseline levels of glucose metabolites, glutamate and glycerol in malignant glioma assessed by stereotactic microdialysis. *J Neurooncol* **61:** 151-160

Rothkamm K, Kruger I, Thompson LH, Lobrich M (2003) Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol Cell Biol* **23:** 5706-5715

Rouschop KMA, van den Beucken T, Dubois L, Niessen H, Bussink J, Savelkouls K, Keulers T, Mujcic H, Landuyt W, Voncken JW, Lambin P, van der Kogel AJ, Koritzinsky M, Wouters BG (2010) The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. *Journal of Clinical Investigation* **120:** 127-141

Ruas JL, Poellinger L, Pereira T (2005) Role of CBP in regulating HIF-1-mediated activation of transcription. *J Cell Sci* **118:** 301-311

Rubinsztein DC, Codogno P, Levine B (2012) Autophagy modulation as a potential therapeutic target for diverse diseases. *Nat Rev Drug Discov* **11:** 709-730

Rubinsztein DC, Cuervo AM, Ravikumar B, Sarkar S, Korolchuk V, Kaushik S, Klionsky DJ (2009) In search of an "autophagomometer". *Autophagy* **5:** 585-589

Russell RC, Tian Y, Yuan H, Park HW, Chang YY, Kim J, Kim H, Neufeld TP, Dillin A, Guan KL (2013) ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat Cell Biol* **15:** 741-750

Sabin RJ, Anderson RM (2011) Cellular Senescence - its role in cancer and the response to ionizing radiation. *Genome Integr* **2:** 7

Saita S, Shirane M, Nakayama KI (2013) Selective escape of proteins from the mitochondria during mitophagy. *Nat Commun* **4:** 1410

Saito S, Goodarzi AA, Higashimoto Y, Noda Y, Lees-Miller SP, Appella E, Anderson CW (2002) ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. *Journal of Biological Chemistry* **277:** 12491-12494

Santin AD, Bellone S, Parrish RS, Coke C, Dunn D, Roman J, Theus JW, Cannon MJ, Parham GP, Pecorelli S (2003) Influence of allogeneic blood transfusion on clinical outcome during radiotherapy for cancer of the uterine cervix. *Gynecol Obstet Inves* **56:** 28-34

Sarcar B, Kahali S, Prabhu AH, Shumway SD, Xu Y, Demuth T, Chinnaiyan P (2011) Targeting radiation-induced G(2) checkpoint activation with the Wee-1 inhibitor MK-1775 in glioblastoma cell lines. *Mol Cancer Ther* **10:** 2405-2414

Saric T, Graef CI, Goldberg AL (2004) Pathway for degradation of peptides generated by proteasomes: a key role for thimet oligopeptidase and other metallopeptidases. *J Biol Chem* **279:** 46723-46732

Sarkar S, Davies AA, Ulrich HD, McHugh PJ (2006) DNA interstrand crosslink repair during G1 involves nucleotide excision repair and DNA polymerase zeta. *Embo Journal* **25:** 1285-1294

Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC (2007) Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. *J Biol Chem* **282:** 5641-5652

Sarkaria JN, Galanis E, Wu W, Dietz AB, Kaufmann TJ, Gustafson MP, Brown PD, Uhm JH, Rao RD, Doyle L, Giannini C, Jaeckle KA, Buckner JC (2010) Combination of temsirolimus (CCI-779) with chemoradiation in newly diagnosed glioblastoma multiforme (GBM) (NCCTG trial N027D) is associated with increased infectious risks. *Clin Cancer Res* **16:** 5573-5580

Sarkaria JN, Galanis E, Wu WT, Peller PJ, Giannini C, Brown PD, Uhm JH, McGraw S, Jaeckle KA, Buckner JC (2011) North Central Cancer Treatment Group Phase I Trial N057k of Everolimus (Rad001) and Temozolomide in Combination with Radiation Therapy in Patients with Newly Diagnosed Glioblastoma Multiforme. *Int J Radiat Oncol* **81:** 468-475

Sawa-Makarska J, Abert C, Romanov J, Zens B, Ibiricu I, Martens S (2014) Cargo binding to Atg19 unmasks additional Atg8 binding sites to mediate membrane-cargo apposition during selective autophagy. *Nat Cell Biol* **16:** 425-433

Schietke R, Warnecke C, Wacker I, Schodel J, Mole DR, Campean V, Amann K, Goppelt-Struebe M, Behrens J, Eckardt KU, Wiesener MS (2010) The lysyl oxidases LOX and LOXL2 are necessary and sufficient to repress E-cadherin in hypoxia: insights into cellular transformation processes mediated by HIF-1. *J Biol Chem* **285:** 6658-6669

Schito L, Rey S, Tafani M, Zhang HF, Wong CCL, Russo A, Russo MA, Semenza GL (2012) Hypoxia-inducible factor 1-dependent expression of platelet-derived growth factor B promotes lymphatic metastasis of hypoxic breast cancer cells. *P Natl Acad Sci USA* **109:** E2707-E2716

Schroeder SA (2013) New Evidence That Cigarette Smoking Remains the Most Important Health Hazard. *New Engl J Med* **368:** 389-390

Schroering AG, Edelbrock MA, Richards TJ, Williams KJ (2007) The cell cycle and DNA mismatch repair. *Exp Cell Res* **313:** 292-304

Scott L, Lamb J, Smith S, Wheatley DN (2000) Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells. *Br J Cancer* **83:** 800-810

Sedoris KC, Thomas SD, Miller DM (2010) Hypoxia induces differential translation of enolase/MBP-1. *Bmc Cancer* **10:** 157

Seiwert TY, Salama JK, Vokes EE (2007) The concurrent chemoradiation paradigm--general principles. *Nat Clin Pract Oncol* **4:** 86-100

Semenza GL (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3:** 721-732

Semenza GL (2010) Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene* **29:** 625-634

Senra JM, Telfer BA, Cherry KE, McCrudden CM, Hirst DG, O'Connor MJ, Wedge SR, Stratford IJ (2011) Inhibition of PARP-1 by olaparib (AZD2281) increases the radiosensitivity of a lung tumor xenograft. *Mol Cancer Ther* **10:** 1949-1958

Simonsen TG, Gaustad JV, Leinaas MN, Rofstad EK (2012) High Interstitial Fluid Pressure Is Associated with Tumor-Line Specific Vascular Abnormalities in Human Melanoma Xenografts. *Plos One* **7:** e40006

Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, Tanaka K, Cuervo AM, Czaja MJ (2009) Autophagy regulates lipid metabolism. *Nature* **458:** 1131-1135

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New Engl J Med* **344:** 783-792

Snell CE, Turley H, McIntyre A, Li D, Masiero M, Schofield CJ, Gatter KC, Harris AL, Pezzella F (2014) Proline-hydroxylated hypoxia-inducible factor 1alpha (HIF-1alpha) upregulation in human tumours. *Plos One* **9:** e88955

Solomon VR, Lee H (2009) Chloroquine and its analogs: a new promise of an old drug for effective and safe cancer therapies. *Eur J Pharmacol* **625:** 220-233

Sorensen CS, Syljuasen RG (2012) Safeguarding genome integrity: the checkpoint kinases ATR, CHK1 and WEE1 restrain CDK activity during normal DNA replication. *Nucleic Acids Res* **40:** 477-486

Sowter HM, Raval RR, Moore JW, Ratcliffe PJ, Harris AL (2003) Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. *Cancer Res* **63:** 6130-6134

Steel GG, Mcmillan TJ, Peacock JH (1989) The 5rs of Radiobiology. *Int J Radiat Biol* **56:** 1045-1048

Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJB, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S, Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG, Mirimanoff RO, Treatment EOR, Grp CBT, Grp RO, Trials NCICC (2009) Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* **10:** 459-466

Suit H, DeLaney T, Goldberg S, Paganetti H, Clasie B, Gerweck L, Niemierko A, Hall E, Flanz J, Hallman J, Trofimov A (2010) Proton vs carbon ion beams in the definitive radiation treatment of cancer patients. *Radiother Oncol* **95:** 3-22

Sun RC, Denko NC (2014) Hypoxic regulation of glutamine metabolism through HIF1 and SIAH2 supports lipid synthesis that is necessary for tumor growth. *Cell Metab* **19:** 285-292

Sutphin PD, Chan DA, Li JM, Turcotte S, Krieg AJ, Giaccia AJ (2007) Targeting the loss of the von Hippel-Lindau tumor suppressor gene in renal cell carcinoma cells. *Cancer Res* **67:** 5896-5905

Szumiel I (2008) Intrinsic radiation sensitivity: Cellular signaling is the key. *Radiat Res* **169:** 249-258

Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, Liang C, Jung JU, Cheng JQ, Mule JJ, Pledger WJ, Wang HG (2007) Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* **9:** 1142-1151

Takakusagi Y, Matsumoto S, Saito K, Matsuo M, Kishimoto S, Wojtkowiak JW, DeGraff W, Kesarwala AH, Choudhuri R, Devasahayam N, Subramanian S, Munasinghe JP, Gillies RJ, Mitchell JB, Hart CP, Krishna MC (2014) Pyruvate Induces Transient Tumor Hypoxia by Enhancing Mitochondrial Oxygen Consumption and Potentiates the Anti-Tumor Effect of a Hypoxia-Activated Prodrug TH-302. *Plos One* **9:** e107995

Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL (2000) The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol* **157:** 411-421

Tamulevicius P, Wang ML, Iliakis G (2007) Homology-directed repair is required for the development of radioresistance during S phase: Interplay between double-strand break repair and checkpoint response. *Radiat Res* **167:** 1-11

Tan CTC, Dargeon HW, Burchenal JH (1959) The Effect of Actinomycin-D on Cancer in Childhood. *Pediatrics* **24:** 544-561

Tasdemir E, Maiuri MC, Galluzzi L, Vitale I, Djavaheri-Mergny M, D'Amelio M, Criollo A, Morselli E, Zhu CL, Harper F, Nannmark U, Samara C, Pinton P, Vicencio JM, Carnuccio R, Moll UM, Madeo F, Paterlini-Brechot P, Rizzuto R, Szabadkai G, Pierron G, Blomgren K, Tavernarakis N, Codogno P, Cecconi F, Kroemer G (2008a) Regulation of autophagy by cytoplasmic p53. *Nat Cell Biol* **10:** 676-687

Tasdemir E, Maiuri MC, Orhon I, Kepp O, Morselli E, Criollo A, Kroemer G (2008b) p53 represses autophagy in a cell cycle-dependent fashion. *Cell Cycle* **7:** 3006-3011

Taylor A (1993) Aminopeptidases: structure and function. *FASEB J* **7:** 290-298

Thangavel C, Boopathi E, Ciment S, Liu Y, O'Neill R, Sharma A, McMahon SB, Mellert H, Addya S, Ertel A, Birbe R, Fortina P, Dicker AP, Knudsen KE, Den R (2014) The retinoblastoma tumor suppressor modulates DNA repair and radioresponsiveness. *Clin Cancer Res*: [Epub ahead of Print] doi: 10.1158/1078-0432.CCR-1114-0326

Thariat J, Hannoun-Levi JM, Myint AS, Vuong T, Gerard JP (2013) Past, present, and future of radiotherapy for the benefit of patients. *Nat Rev Clin Oncol* **10:** 52-60

Thind G, Johal B, Follwell M, Kennecke HF (2014) Chemoradiation with capecitabine and mitomycin-C for stage I-III anal squamous cell carcinoma. *Radiat Oncol* **9:** 124

Thomas GV, Tran C, Mellinghoff IK, Welsbie DS, Chan E, Fueger B, Czernin J, Sawyers CL (2006) Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. *Nat Med* **12:** 122-127

Thomas M, Alegre-Abarrategui J, Wade-Martins R (2013) RNA dysfunction and aggrephagy at the centre of an amyotrophic lateral sclerosis/frontotemporal dementia disease continuum. *Brain* **136:** 1345-1360

Thomson D, Yang H, Baines H, Miles E, Bolton S, West C, Slevin N (2014) NIMRAD - A Phase III Trial to Investigate the Use of Nimorazole Hypoxia Modification with Intensity-modulated Radiotherapy in Head and Neck Cancer. *Clin Oncol-Uk* **26:** 344-347

Toledo LI, Murga M, Zur R, Soria R, Rodriguez A, Martinez S, Oyarzabal J, Pastor J, Bischoff JR, Fernandez-Capetillo O (2011) A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. *Nat Struct Mol Biol* **18:** 721-727

Torres KE, Ravi V, Kin K, Yi M, Guadagnolo BA, May CD, Arun BK, Hunt KK, Lam R, Lahat G, Hoffman A, Cormier JN, Feig BW, Lazar AJ, Lev D, Pollock RE (2013) Long-Term Outcomes in Patients with Radiation-Associated Angiosarcomas of the Breast Following Surgery and Radiotherapy for Breast Cancer. *Ann Surg Oncol* **20:** 1267-1274

Toruno C, Carbonneau S, Stewart RA, Jette C (2014) Interdependence of Bad and Puma during Ionizing-Radiation-Induced Apoptosis. *Plos One* **9:** e88151

Tripathi DN, Chowdhury R, Trudel LJ, Tee AR, Slack RS, Walker CL, Wogan GN (2013) Reactive nitrogen species regulate autophagy through ATM-AMPK-TSC2-mediated suppression of mTORC1. *Proc Natl Acad Sci U S A* **110:** E2950-2957

Tsai WB, Chung YM, Takahashi Y, Xu ZH, Hu MCT (2009) Functional interaction between FOXO3a and ATM regulates DNA damage response (vol 10, pg 460, 2008). *Nat Cell Biol* **11:** 1387-1387

Tsimberidou AM, Iskander NG, Hong DS, Wheler JJ, Falchook GS, Fu S, Piha-Paul S, Naing A, Janku F, Luthra R, Ye Y, Wen S, Berry D, Kurzrock R (2012) Personalized medicine in a phase I clinical trials program: the MD Anderson Cancer Center initiative. *Clin Cancer Res* **18:** 6373-6383

Tsukamoto H, Shibata K, Kajiyama H, Terauchi M, Nawa A, Kikkawa F (2008) Aminopeptidase N (APN)/CD13 inhibitor, Ubenimex, enhances radiation sensitivity in human cervical cancer. *Bmc Cancer* **8:** 74

Turcotte S, Chan DA, Sutphin PD, Hay MP, Denny WA, Giaccia AJ (2008a) A molecule targeting VHL-deficient renal cell carcinoma that induces autophagy. *Cancer Cell* **14:** 90-102

Turcotte S, Sutphin PD, Giaccia AJ (2008b) Targeted therapy for the loss of von Hippel-Lindau in renal cell carcinoma: a novel molecule that induces autophagic cell death. *Autophagy* **4:** 944-946

Ueno T, Sato W, Horie Y, Komatsu M, Tanida I, Yoshida M, Ohshima S, Mak TW, Watanabe S, Kominami E (2008) Loss of Pten, a tumor suppressor, causes the strong inhibition of autophagy without affecting LC3 lipidation. *Autophagy* **4:** 692-700

van de Sluis B, Mao X, Zhai Y, Groot AJ, Vermeulen JF, van der Wall E, van Diest PJ, Hofker MH, Wijmenga C, Klomp LW, Cho KR, Fearon ER, Vooijs M, Burstein E (2010) COMMD1 disrupts HIF-1alpha/beta dimerization and inhibits human tumor cell invasion. *J Clin Invest* **120:** 2119-2130

van Meerten E, Muller K, Tilanus HW, Siersema PD, Eijkenboom WMH, van Dekken H, Tran TCK, van der Gaast A (2006) Neoadjuvant concurrent chemoradiation with weekly paclitaxel and carboplatin for patients with oesophageal cancer: a phase II study. *Brit J Cancer* **94:** 1389-1394

Vaupel P, Mayer A (2007) Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metast Rev* **26:** 225-239

Verbrugge I, de Vries E, Tait SWG, Wissink EHJ, Walczak H, Verheij M, Borst J (2008) Ionizing radiation modulates the TRAIL death-inducing signaling complex, allowing bypass of the mitochondrial apoptosis pathway. *Oncogene* **27:** 574-584

Vitale I, Galluzzi L, Castedo M, Kroemer G (2011) Mitotic catastrophe: a mechanism for avoiding genomic instability. *Nat Rev Mol Cell Biol* **12:** 385-392

Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ, Press M (2002) Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol* **20:** 719-726

Von Hoff DD, Stephenson JJ, Rosen P, Loesch DM, Borad MJ, Anthony S, Jameson G, Brown S, Cantafio N, Richards DA, Fitch TR, Wasserman E, Fernandez C, Green S, Sutherland W, Bittner M, Alarcon A, Mallery D, Penny R, Grp BS (2010) Pilot Study Using Molecular Profiling of Patients' Tumors to Find Potential Targets and Select Treatments for Their Refractory Cancers. *J Clin Oncol* **28:** 4877-4882

von Muhlinen N, Akutsu M, Ravenhill BJ, Foeglein A, Bloor S, Rutherford TJ, Freund SMV, Komander D, Randow F (2012) LC3C, Bound Selectively by a Noncanonical LIR Motif in NDP52, Is Required for Antibacterial Autophagy. *Mol Cell* **48:** 329-342

von Pawel J, von Roemeling R, Gatzemeier U, Boyer M, Elisson LO, Clark P, Talbot D, Rey A, Butler TW, Hirsh V, Olver I, Bergman B, Ayoub J, Richrdson G, Dunlop D, Arcenas A, Vescio R, Viallet J, Treat J (2000) Tirapazamine plus cisplatin versus cisplatin in advanced non-small-cell lung cancer: A report of the international CATAPULT I study group. *J Clin Oncol* **18:** 1351-1359

Vrouwe MG, Pines A, Overmeer RM, Hanada K, Mullenders LHF (2011) UV-induced photolesions elicit ATR-kinase-dependent signaling in non-cycling cells through nucleotide excision repair-dependent and -independent pathways. *J Cell Sci* **124:** 435-446

Wakita A, Ohtake S, Takada S, Yagasaki F, Komatsu H, Miyazaki Y, Kubo K, Kimura Y, Takeshita A, Adachi Y, Kiyoi H, Yamaguchi T, Yoshida M, Ohnishi K, Miyawaki S, Naoe T, Ueda R, Ohno R (2012) Randomized comparison of fixed-schedule versus response-oriented individualized induction therapy and use of ubenimex during and after

consolidation therapy for elderly patients with acute myeloid leukemia: the JALSG GML200 Study. *Int J Hematol* **96:** 84-93

Wallace SS (2014) Base excision repair: A critical player in many games. *DNA Repair* **19:** 14-26

Wang CR, Lu QB (2010) Molecular Mechanism of the DNA Sequence Selectivity of 5-Halo-2 '-Deoxyuridines as Potential Radiosensitizers. *J Am Chem Soc* **132:** 14710-14713

Wang Y, Li J, Booher RN, Kraker A, Lawrence T, Leopold WR, Sun Y (2001) Radiosensitization of p53 mutant cells by PD0166285, a novel G(2) checkpoint abrogator. *Cancer Res* **61:** 8211-8217

Wang Y, Schulte BA, LaRue AC, Ogawa M, Zhou D (2006) Total body irradiation selectively induces murine hematopoietic stem cell senescence. *Blood* **107:** 358-366

Ward JF (1994) DNA-Damage as the Cause of Ionizing Radiation-Induced Gene Activation. *Radiat Res* **138:** S85-S88

Warr MR, Binnewies M, Flach J, Reynaud D, Garg T, Malhotra R, Debnath J, Passegue E (2013) FOXO3A directs a protective autophagy program in haematopoietic stem cells. *Nature* **494:** 323-327

Webley K, Bond JA, Jones CJ, Blaydes JP, Craig A, Hupp T, Wynford-Thomas D (2000) Posttranslational modifications of p53 in replicative senescence overlapping but distinct from those induced by DNA damage. *Mol Cell Biol* **20:** 2803-2808

Wenzl T, Wilkens JJ (2011) Modelling of the oxygen enhancement ratio for ion beam radiation therapy. *Phys Med Biol* **56:** 3251-3268

Weston VJ, Oldreive CE, Skowronska A, Oscier DG, Pratt G, Dyer MJ, Smith G, Powell JE, Rudzki Z, Kearns P, Moss PA, Taylor AM, Stankovic T (2010) The PARP inhibitor olaparib induces significant killing of ATM-deficient lymphoid tumor cells in vitro and in vivo. *Blood* **116:** 4578-4587

WHO. (2012) GLOBOSCAN 2012: Estimated cancer incidence, mortality and prevalence worldwide in 2012. Available from: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.

Wilkinson S, O'Prey J, Fricker M, Ryan KM (2009) Hypoxia-selective macroautophagy and cell survival signaled by autocrine PDGFR activity. *Gene Dev* **23:** 1283-1288

Williams A, Sarkar S, Cuddon P, Ttofi EK, Saiki S, Siddiqi FH, Jahreiss L, Fleming A, Pask D, Goldsmith P, O'Kane CJ, Floto RA, Rubinsztein DC (2008a) Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. *Nat Chem Biol* **4:** 295-305

Williams JR, Zhang Y, Zhou H, Gridley DS, Koch CJ, Slater JM, Little JB (2008b) Overview of radiosensitivity of human tumor cells to low-dose-rate irradiation. *Int J Radiat Oncol Biol Phys* **72:** 909-917

Wilson PF, Nagasawa H, Fitzek MM, Little JB, Bedford JS (2010) G2-phase chromosomal radiosensitivity of primary fibroblasts from hereditary retinoblastoma family members and some apparently normal controls. *Radiat Res* **173:** 62-70

Wilson WR, Hay MP (2011) Targeting hypoxia in cancer therapy. *Nat Rev Cancer* **11:** 393-410

Wojtkowiak JW, Rothberg JM, Kumar V, Schramm KJ, Haller E, Proemsey JB, Lloyd MC, Sloane BF, Gillies RJ (2012) Chronic Autophagy Is a Cellular Adaptation to Tumor Acidic pH Microenvironments. *Cancer Res* **72:** 3938-3947

Yao KC, Komata T, Kondo Y, Kanzawa T, Kondo S, Germano IM (2003) Molecular response of human glioblastoma multiforme cells to ionizing radiation: cell cycle arrest, modulation of the expression of cyclin-dependent kinase inhibitors, and autophagy. *J Neurosurg* **98:** 378-384

Yazbeck VY, Buglio D, Georgakis GV, Li Y, Iwado E, Romaguera JE, Kondo S, Younes A (2008) Temsirolimus downregulates p21 without altering cyclin D1 expression and induces autophagy and synergizes with vorinostat in mantle cell lymphoma. *Exp Hematol* **36:** 443-450

Yeager CL, Ashmun RA, Williams RK, Cardellichio CB, Shapiro LH, Look AT, Holmes KV (1992) Human Aminopeptidase-N Is a Receptor for Human Coronavirus-229e. *Nature* **357:** 420-422

You S, Li R, Park D, Xie M, Sica GL, Cao Y, Xiao ZQ, Deng X (2014) Disruption of STAT3 by niclosamide reverses radioresistance of human lung cancer. *Mol Cancer Ther* **13:** 606-616

Yu L, Gu C, Zhong D, Shi L, Kong Y, Zhou Z, Liu S (2014) Induction of autophagy counteracts the anticancer effect of cisplatin in human esophageal cancer cells with acquired drug resistance. *Cancer Lett* **355:** 34-45

Zhang H, Berel D, Wang Y, Li P, Bhowmick NA, Figlin RA, Kim HL (2013) A comparison of Ku0063794, a dual mTORC1 and mTORC2 inhibitor, and temsirolimus in preclinical renal cell carcinoma models. *Plos One* **8:** e54918

Zhang H, Wong CCL, Wei H, Gilkes DM, Korangath P, Chaturvedi P, Schito L, Chen J, Krishnamachary B, Winnard PT, Raman V, Zhen L, Mitzner WA, Sukumar S, Semenza GL (2012) HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs. *Oncogene* **31:** 1757-1770

Zhang Y, Gao XS, Saucedo LJ, Ru BG, Edgar BA, Pan DJ (2003) Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat Cell Biol* **5:** 578-581

Zhao H, Traganos F, Darzynkiewicz Z (2008) Phosphorylation of p53 on Ser15 during cell cycle caused by Topo I and Topo II inhibitors in relation to ATM and Chk2 activation. *Cell Cycle* **7:** 3048-3055

Zhao J, Brault JJ, Schild A, Cao P, Sandri M, Schiaffino S, Lecker SH, Goldberg AL (2007) FoxO3 coordinately activates protein degradation by the Autophagic/Lysosomal and proteasomal pathways in atrophying muscle cells. *Cell Metab* **6:** 472-483

Zhao S, Weng YC, Yuan SSF, Lin YT, Hsu HC, Lin SCJ, Gerbino E, Song MH, Zdzienicka MZ, Gatti RA, Shay JW, Ziv Y, Shiloh Y, Lee EYHP (2000) Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. *Nature* **405:** 473-477

Zhuang W, Li B, Long L, Chen L, Huang Q, Liang Z (2011) Induction of autophagy promotes differentiation of glioma-initiating cells and their radiosensitivity. *Int J Cancer* **129:** 2720-2731