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DIFFERENT APPROACHES TO DEVELOP RADIO- AND CHEMOTHERAPY FOR TREATMENT OF HUMAN CANCER

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Institutet

Stockholm 2014

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Published by Karolinska Institutet.
Printed by Åtta.45 Tryckeri

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ISBN 978-91-7549-649-8

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ABSTRACT

Despite huge advancements in our knowledge and understanding of the molecular mechanisms of carcinogenesis, the prognoses of many malignant diseases have not improved dramatically. Improvements in the therapeutic efficacy of several anti-cancer therapies including radiotherapy and chemotherapy would be required to achieve effective treatment. In the present thesis we attempt to improve radiotherapy by proposing new molecular targets for overcoming radio-resistance and identify novel potent drugs effective against human malignancies.

Ion beams can be used to achieve therapeutic effects on tumors which are resistant to conventional radiation/photons. However the treatment planning system currently used in ion therapy centers are still based on data from conventional radiation to describe parameters for ion therapy. In paper I, we used two mathematical models to compare the cellular response to photons and ions. We found that the parameters determined for photons, using the RCR model could be used to predict the response to ion beams. The data also indicated that cells having efficient DNA repair capability are more sensitive to ion beams. In paper II, we compared photons and ion beams by analysis of the global phosphoproteome of a photon resistant cell line and identify signaling pathways responsible for photon resistance. We identified GSK3β to be important for cell proliferation and to have a protective effect on photon-induced tumor cell death. We also confirm the role of p38MAPK in photon resistance.

Cells propagated on plastic surfaces in monolayer culture do not represent accurate models of in vivo tumor tissue. The 3-D microenvironment of tumor tissue, including the presence of hypoxic regions, is better mimicked using the multicellular tumor spheroid model. Spheroids can be used for drug screening projects aimed to identify compounds effective on solid tumors. In paper III, we describe a novel small molecule capable of inducing apoptosis in 3D tumor spheroids and xenograft tumors. The compound triggered rapid increases of intracellular calcium levels. The drug was effective in inducing cell death of all cells of colon cancer spheroids, including cells in the hypoxic nutrient deficient cores. Interestingly, and in contrast to cells in peripheral cell layers, apoptosis did not appear to be induced in the hypoxic core regions. The results showed that novel drugs can be identified which have significantly stronger cytotoxic effects on multicellular spheroids as compared to conventional cancer therapeutics.

In paper IV, we report a novel inhibitor of the ubiquitin-proteasome system (UPS) that is cytotoxic to a number of cancer cell lines and patient tumor cells. This compound HRF-3, induces accumulation of polyubiquitinated proteins in the absence of a proteasomal blocking. Our results indicate that HRF-3 inhibits the UPS at a pre-proteasomal step and generates ROS similar to proteasomal inhibitors. Our data supports the notion that the UPS can be inhibited at several steps resulting in tumor cytotoxicity.

In paper V, we identified the 19S DUB inhibitor b-AP15 analogue VLX1570 which has similar biochemical activity as the hit compound. VLX1570 has strong anti-tumor activity in multiple myeloma cells and is capable of overcoming bortezomib resistance. The findings suggest that VLX1570 is a promising candidate for the clinical drug development against multiple myeloma.

LIST OF SCIENTIFIC PAPERS

I. **Mohanty C***, Zielinska-Chomej K*, Edgren M, Hirayama R, Murakami T, Lind B, Toma-Dasu I.
Predicting the sensitivity to ion therapy based on the response to photon irradiation--experimental evidence and mathematical modelling.
Anticancer Res. 2014; 34(6): 2801-6.
** Equal contribution*

II. Ståhl S, Mamede-Branca R, **Mohanty C***, Zielinska-Chomej K*, Efazat G, Juntti T, Tu J, Hååg P, Stenerlöw B, Lewensohn R, Lehtiö J, Viktorsson K.
Phosphoproteomic profiling of high and low LET irradiated non small cell lung cancer cells reveals differences in growth factor signalling cascades and indicate a role of p38MAPK and GSK3β in low LET radiotherapy cellular response
Manuscript
** Equal contribution*

III. **Mohanty C**, Fayad W, Olofsson H M, Larsson R, De Milito A, Fryknäs M, Linder S.
Massive induction of apoptosis of multicellular tumor spheroids by a novel compound with a calmodulin inhibitor-like mechanism
J Cancer Ther Res. 2013; 2:19. http://dx.doi.org/10.7243/2049-7962-2-19

IV. Haglund C*, **Mohanty C***, Fryknäs M, D'Arcy P, Larsson R, Linder S, Rickardson L.
Identification of an inhibitor of the ubiquitin–proteasome system that induces accumulation of polyubiquitinated proteins in the absence of blocking of proteasome function
Med. Chem. Comm., 2014. 5(3): 376-85.
** Equal contribution*

V. Wang X, **Mohanty C**, Fryknäs M, D'Arcy P, Olofsson H M, Bossler F, Larsson R, Gullbo J, Linder S.
Development of the proteasome deubiquitinase inhibitor VLX1570 for treatment of multiple myeloma.
Manuscript

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LIST OF ABBREVIATIONS

2/3D	2/3 dimentional
Å	Ångström
AAA+ATPases	ATPases associated with diverse cellular activities
ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
AML	Acute myelocytic leukemia
Apaf-1	Apoptosis protease-activating factor 1
APC	Anaphase-promoting complex
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM- and Rad3-related
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
Bcl-xL	B-cell lymphoma extra large
Bid	BH3 interacting-domain death agonist
Bik	Bcl-2 interacting killer
Bim	B-cell lymphoma 2 interacting mediator of cell death
Ca^{2+}	Calcium
Caspase	Cysteinyl aspartate proteinase
CD	Cluster of differentiation
Cdc48	Cell division cycle 48
CDK	Cyclin dependent kinases
CHK	Checkpoint kinase
CKIs	Cyclin-dependent kinase inhibitors
CLL	Chronic lymphatic leukemia
CP	Core particle
CSCs	Cancer stem cells
DISC	Death inducing signaling complex

DNA-PK	DNA-dependent protein kinase
DSB	Double strand break
DUBs	Deubiquitinating enzymes
EGFR	Epidermal growth factor receptor
eIF	Eukaryotic initiation factor
ERAD	Endoplasmic reticulum-associated degradation
ERK	External signal receptor kinase
ES-1	Eeyarestatin-1
FADD	FAS-associated death domain
FDA	Food and drug administration
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase 3 β
Gy	Gray
HECT	Homologous to E6-associated protein C-terminus
HIF	Hypoxia inducible factor
HMOX-1	Heme oxygenase-1
HNSCC	Head and neck squamous cell carcinoma
HR	Homologous recombination
IAPs	Inhibitors of apoptosis proteins
IGF 1R	Insulin like growth factor 1 receptor
IMRT	Intensity modulated radiation therapy
IU1	USP14 inhibitor 1
IκB	Inhibitor of kappa-light-chain-enhancer of activated B cells
JNK	C-Jun N-terminal kinase
keV/µm	Kiloelectron volt per micrometer
LC	Liquid chromatography
LEM	Local effect model
LET	Linear energy transfer
LQ	Linear-quadratic
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog

MK	Microdosimetric kinetic
MRN	Mre11-Rad50-Nbs1
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
NAE	NEDD8-activating enzyme
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
NSCLC	Non-small cell lung cancer
ODC	Ornithne decarboxylase
PARP	Poly (ADP-ribose) polymerase
PI3K	Phosphatidylinositide 3-kinases
PMP	Pseudomyxoma peritonei
POH1	Pad one homolog-1
Pru	Pleckstrin-like receptor for the ubiquitin
PUMA	p53 upregulated modulator of apoptosis
RBE	Relative biological effectiveness
RCR	Repairable-conditionally repairable
RING	Really interesting new gene
RIP	Receptor interacting protein
ROS	Reactive oxygen species
RP	Regulatory particle
Rpn	Regulatory particle non-ATPase subunit
Rpt	Regulatory particle triple-A protein
SCF	Skp1-Cullin-F-box
SCLC	Small cell lung cancer
SCX	Strong cation-exchange chromatography
Smac	Second mitochondria derived activator of caspase
SSB	Single strand breaks
TNF	Tumor necrosis factor

TPS	Treatment planning systems
TRAIL	TNF-related apoptosis-inducing ligand
UBA	Ubiquitin associated
UBA6	Ubiquitin-like modifier activating enzyme 6
UBE1	Ubiquitin-activating enzyme E1
UBL	Ubiquitin like
UBX	Ubiquitin regulatory X
UCH	Ubiquitin C-terminal hydrolases
UIM	Ubiquitin-interacting motif
UPS	Ubiquitin proteasome system
USP	Ubiquitin-specific proteases
VCP	Valosin containing protein
YFP	Yellow-fluorescent protein

1 INTRODUCTION

1.1 Cancer- a complex disease

Cancer is the term for a collection of several diseases caused by uncontrolled growth of abnormal malignant cells, which can originate from any part of the body. Without proper treatment, cancer is usually lethal. Generally it is considered as age related pathology, greatly prevalent in the older age group, however it could occur at any stage of life [1]. The latest assessments of global cancer statistics shows that there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012, making it the leading cause of deaths in developed countries and second leading cause of death in developing countries [2]. Cancer metastasis is a form of cancer progression, when the local invasion and circulation of the tumor cells from its primary site spreads through blood stream and/or the lymphatic system to other parts of the body. Metastasis is the most common cause of death in patients with solid malignancies [3].

Carcinogenesis or initiation of cancer is caused by chromosomal changes such as deletions, mutations, genetic and epigenetic alterations. Due to the changes in genetic material, normal cells in a multi-step process acquire the hallmarks of cancer and transform into tumor cells [4, 5]. Several studies have been done to identify alerted genes and their association with cancer development and progression [6, 7]. The tumor suppressor gene p53 is the most common mutation found in several tumor types [6, 8]. Apart from that genes involved in mitogen-activated protein kinase/MAPK pathways (external signal receptor kinase/ERK, Ras-Raf pathway [9]), phosphatidylinositol-3 kinase [10] and receptor tyrosine kinase (Epidermal growth factor receptor/EGFR and insulin like growth factor 1 receptor/IGF 1R) [11] are also major contributors to tumor initiation [6].

Cancer treatment usually involves a suitable selection of either one or more treatment types, such as surgery, radiotherapy and chemotherapy. The overall aim is to have complete remedy of the disease or substantially increase life expectancy while improving the patient's quality of life. Surgery is considered as the best treatment choice as it achieves complete removal of the tumor from the body. However the tumor is not always accessible or may sometimes be inseparable from vital organs or tissues or may have advanced metastasis, making surgery impractical. Radiotherapy and chemotherapy can then be considered as the treatment of choice [1, 12, 13]. Advanced metastatic cancer is treated by chemotherapy, hormonal therapy and targeted therapies as surgery and radiation therapies are not feasible options [14, 15].

Cancer is a multifaceted disease often controlled by a number of mutated genes. And targeted therapies against a single gene often leads to adaptation of the tumor cells, leading to resistance [16, 17]. Improvement in cancer therapy could be achieved by finding the right balance between destroying the malignant tumor and avoiding undesirable damage to healthy

normal tissues. In terms of radiation therapy, it could mean applying more proficient radiation qualities than the conventional radiation class and also finding new biological interventions which could improve the efficacy of conventional radiation therapy [18]. For chemotherapy discovery of novel drugs which have unique molecular targets to discriminate tumor cells from non-cancerous cells, thereby increasing treatment efficacy will be the foremost requirement. Detailed knowledge about the cellular processes regulating cancer sets a strong foundation for better diagnostics and improved treatment options for patients.

1.2 Radiation therapy

Ionizing radiation is a form of energy that is able to travel, penetrate and release energy onto cells, depending on the inherent physical and biological property of the radiation type [19]. The effect of radiation on living cells can be based on the type of radiation source and the sensitivity of the particular cells towards radiation. The earliest evidence for tumor treatment using radiation comes from Stockholm in 1899 with the treatment of basal cell carcinoma using X-rays [20]. Today radiation therapy is one of the leading treatment options for cancer patients along with surgery and chemotherapy, many a time used in combination and also used for palliative care. The main cellular target of radiation is DNA [21-23]. Radiation causes DNA damage and death of dividing cells by either direct or indirect interaction with the DNA [19, 23]. When DNA has become damaged different DNA repair pathways are activated. DNA lesions may be repaired leading to cells continuing dividing. Incorrectly repaired lesions may lead to mutations which can affect cellular functions and could also lead to carcinogenesis. Extensive DNA damage may lead to irreparable damage and cell death.[24]. Tissues can be early or late responding based on how fast a reaction is seen in response to radiation. Tumor tissues, because of their highly proliferative nature show an early response to radiation whereas normal cells that are efficient in DNA damage repair, are generally late responding tissues [19]. DNA damage is more harmful to cells that are synthesizing DNA and rapidly dividing tumor cells are more sensitive to radiation compared to normal cells, making DNA damage response mechanism a suitable anti-cancer target [25-27].

There are two important physical aspects that regulate the biological response to ionizing radiation. The first being the dose deposited or absorbed within the tissues and second is the linear energy transfer (LET). The dose is generally measured in units of Gray (Gy) and is usually a measure of the energy deposited by the ionizing radiation on the cells [19]. The dose deposited is not randomly distributed but is localized in defined tracks. Depending on the type of radiation the energy deposited on these tracks can be dense or sparse [19]. The LET is the energy deposited per unit length of the track and is measured in kiloelectron volt per micrometer (keV/μm) [19]. These two physical criteria form the basis for defining two radiation qualities described below.

1.2.1 Low LET radiation

Radiation types that deposit very low density of energy in the ionization tracks are generally referred to as low LET radiation [19]. Low LET radiation consists of photons which are generated by natural decay of radioactive isotopes such as 60Co or 137Cs and man-made X-rays [19]. X-rays and gamma rays are routinely used photons in radiation therapy to treat various cancers and are stated as conventional radiation. The LET of photons is usually below 1keV/µm. Low LET radiation creates ionizing events that generate free electrons in their path and these electrons in turn create secondary ionizations until their energy is completely diminished [28, 29]. The energy is thereby deposited in unequal amount through the whole track, which is maximal in the beginning of the track and lowest at the end of the track [30]. Due to their low mass and energy, photons create a large area of interaction where the energy is deposited. Low LET radiation is therefore not beneficial for deep seated tumors or tumors near vital organs. The energy can be deposited straight on the DNA molecule causing direct interactions or the interaction can be indirect where free radicals are produced by radiation induced ionization of H2O molecules, which would then damage the DNA [19, 31]. It is known that about 70% of the DNA damage caused by low LET is due to indirect interactions [19].

1.2.2 High LET radiation

Charged particulate radiation such as α particles, β particles, protons and ions (boron, carbon, neon etc.) are referred to as high LET radiation. These radiation types are more advantageous, as they differ from low LET irradiation in their energy deposition through the ionization tracks. High energy particles begin to slow down from collisions and release small amounts of energy along the track. Towards the end of their range they begin to decelerate rapidly, depositing the maximum amount of energy in a very short distance or in a peak, which is stated as the Bragg peak, first described in 1907 by William Bragg [32]. Beyond the Bragg peak the radiation energy weakens rapidly and there is almost no or very little dose deposition [28, 33, 34]. Due to this physical property high LET radiation has a better dose localization to the tumor site and is therapeutically superior by sparing normal tissues surrounding the tumor site [34]. High LET radiation also differs in the way it interacts with the different targets within the cells. In contrast to photons, heavy ions deposit energy in more localized tracks, due to their substantial mass. This results in more clustered damage within the cells [31]. Most of the energy from high LET radiation is directly deposited on the DNA molecule with less damage due to indirect radical ionizations.[35].

1.2.3 Cell survival models

As high LET radiation is the newer trend for treatment, comparisons are often made between conventional radiation and high LET radiation. The primary comparison is done by taking the proliferative ability of the cells as the biological endpoint. Tumor cells have the ability to undergo unlimited cell division and after irradiation, loss of this capacity is seen

after either one or several cell divisions. The preferred method to determine proliferative capacity after treatment with ionizing radiation is the clonogenic assay, and this assay is also commonly used to determine the efficacy of other cytotoxic modalities [36, 37]. In this assay, the ability of a single cell to divide and form a colony of at least 50 cells is determined [37]. Even though clonogenic assay detects the long term effects of cytotoxic agents, it does not differentiate between cellular senescence and cell death. The data is plotted on to a survival curve where the dose is plotted on a linear scale (abscissa) and the survival on a logarithmic scale (ordinate).

Mathematical modelling has been useful in describing the dose response relationship for various types of ionizing radiation. These models are used worldwide for treatment planning systems (TPS), measuring tumor control probability and treating with combination therapies. A number of mathematical models with varying degree of complexities are used to describe survival data and convert the physical absorbed dose into a more clinically relevant value to be used for actual treatment strategy. A cell survival model describes the relationship between the absorbed dose and the total fraction of cells retaining their clonogenic ability, with the formation of a cell survival curve. For high LET radiation, cell survival models are used to evaluate the relative biological effectiveness (RBE) for use in treatment planning systems. The concept of RBE is defined later in the text. Here the cell survival models used in the paper I included in this thesis are described.

1.2.3.1 The linear-quadratic cell survival model

The linear-quadratic (LQ) model is the most common cell mathematical survival model used to describe the response of normal and tumor cells to ionizing radiation. Initially this model was used to describe chromosomal damage [38] and later on was developed to relate cell survival and the radiation dose [39, 40] The basis of the linear quadratic model is focused on the assumption that DNA is the critical target to be damaged in order for the cell to lose proliferative capacity or to die. This is assumed to require at least one DNA double strand break, either by a single event or as a result of two single strand breaks [19].

The model describes cell survival (*S*) after a single dose of radiation (*D*) for by the following equation:

$$S(D) = e^{-\alpha D - \beta D^2} \qquad (1)$$

where α and β are the linear and quadratic components of cell killing by radiation respectively [39, 41]. The linear component is proportional to the dose whereas the quadratic component is proportional to the square of the dose. The ratio of these two parameters α/β gives the dose at which both the linear and quadratic component of cell killing are equal and its value describe the innate sensitivity of a cell type [19]. The α/β ratio also gives an estimate of the response to radiation in different tissue types and is helpful in determining the fractionation schedules for treatment. For late responding tissue the α/β ratio is low (~3Gy),

which is in the case of normal tissues and for early responding tissues such as tumor cells the α/β ratio is high (~10Gy) [42]. However there are exceptions , where tumor cells show a low α/β ratio as in the case of prostate cancer [43, 44]. The inverse of this ratio β/α is perceived to be correlated with the repair capacity of irradiated cells [45].

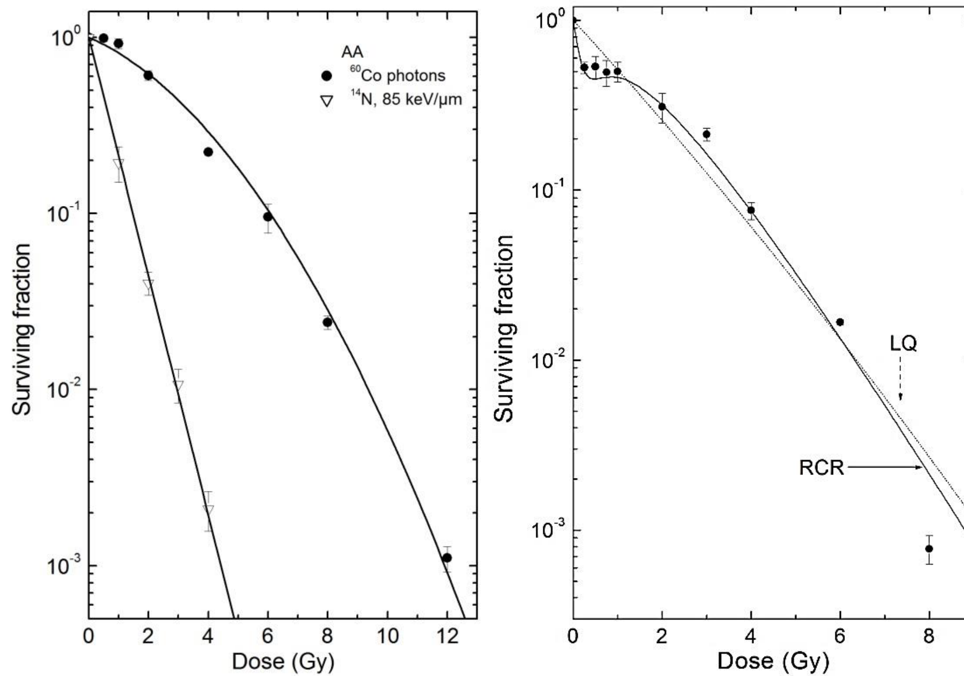

Figure 1: Cell survival curves showing difference between low and high LET radiation (right panel) and the LQ and RCR model (left panel). *In the right panel cell survival curve of a melanoma cell line irradiated with photons and high LET ion beam radiation is shown. The survival curve for photons has a constant curvature while the high LET curve is a straight line (refer to text). The figure on the left panel shows RCR model fitting the low dose hypersensitivity data better than the LQ model.*

Even though the LQ model is efficient in accurately predict cell survival to experimental photon therapy, it does not predict low dose hypersensitivity, which is observed in cells irradiated with high LET [46-48]. This phenomenon is also observed in paper I using both low and high LET irradiation. The survival curves in the LQ model shows a constant curvature which does not agree with the actual experimental data, where at higher doses the curve becomes linear as shown in Figure 1. Also the LQ formalism does not agree with the hypofractionated regimen of radiation therapy [49, 50]. So to include the specific effects of high LET radiation and recent radiation therapy procedures, models based on the LQ model were developed, such as the microdosimetric kinetic (MK) model [51, 52] and the local effect model(LEM) [53-55], which are currently used in clinical settings in Japan and Germany respectively.

1.2.3.2 The repairable-conditionally repairable damage model

The repairable-conditionally repairable (RCR) model takes into account the low dose hypersensitivity and high dose effect which were the drawbacks of the LQ model [56]. This model is based on the assumption that a cell could survive radiation either by not receiving any damage or by efficiently repairing the damage. The damage caused could either be potentially repairable or conditionally repairable, both depending on the absorbed dose [56]. The potentially repairable damage can trigger the repair mechanisms in the cells and only then the conditionally repairable damage can be repaired [56]. The cell survival (*S*) at a given dose (*D*) based on the RCR model is expressed as:

$$\mathrm{S\,(D)} = \mathrm{e}^{-\mathrm{aD}} + \mathrm{bD}^{-\mathrm{cD}} \qquad (2)$$

where a, b and c are the three parameters of the model. The first term $\mathrm{e}^{-\mathrm{aD}}$ gives the fraction of cells that are not damaged at all and the second part bD^{-cD} gives the fraction of cells that have successfully repaired the inflicted damage. The parameter b is associated to the repair capacity of the cells and the ratio of *b/a* would in fact give a better evaluation of the portion of damaged cells that have been repaired. It has been shown that RCR model is better suited for modelling of hypofractionated radiation therapy in comparison with LQ model [49]. In paper I we show that the RCR model gives a better fitting to the low dose hypersensitivity data as compared to the LQ model (Figure 1).

1.2.4 Relative biological effectiveness

As described above, since the same dose of low and high LET radiation will yield different responses, comparison cannot be done based on the absorbed dose. The efficiency of low and high LET radiation is compared on the basis of their capacity to kill cells or decrease cell survival. The term RBE (relative biological effectiveness) is generally used when performing such comparisons. RBE is defined as the ratio of the dose required to create the same biological response between a reference radiation type (commonly low LET radiation) and the corresponding high LET radiation; given that other conditions are equal [19].

$$\mathrm{RBE} = \frac{\mathrm{D_{Ref}}}{\mathrm{D}} \qquad (3)$$

RBE varies according to the tissues irradiated and the end point taken for measurement of biological response [33, 57]. RBE also varies as a function of LET, with higher LET the RBE values increase and reaches a peak at about 100keV/µm [58-60]. If the cells or tissues are treated with different radiation sources and several biological end points are measured, the RBE calculation becomes complicated.

Generally RBE calculations are carried out by calculating the ratio of doses at a specific survival fraction. It is often useful to use the D_{10} dose i.e. the dose required to inhibit the proliferative capacity of 90% of the tumor cells (Figure 2). However while comparing the

doses required to acquire a specific amount of tumor control appears to be simplified, the RBE value would differ based on which survival level is chosen [19]. So a calculation method which takes into account the whole survival curve by comparing a single parameter depicting all the doses used in the survival curve, would be more appropriate. The mean inactivation dose (*Dbar* or $\overline{D}$) was proposed to have a single factor characterizing the intrinsic radiosensitivity of different cell lines [61] and it was seen that $\overline{D}$ indeed gave good correlation when different experiments were compared and also was more consistent with the clinical radiosensitivity of different tumor types [62, 63].

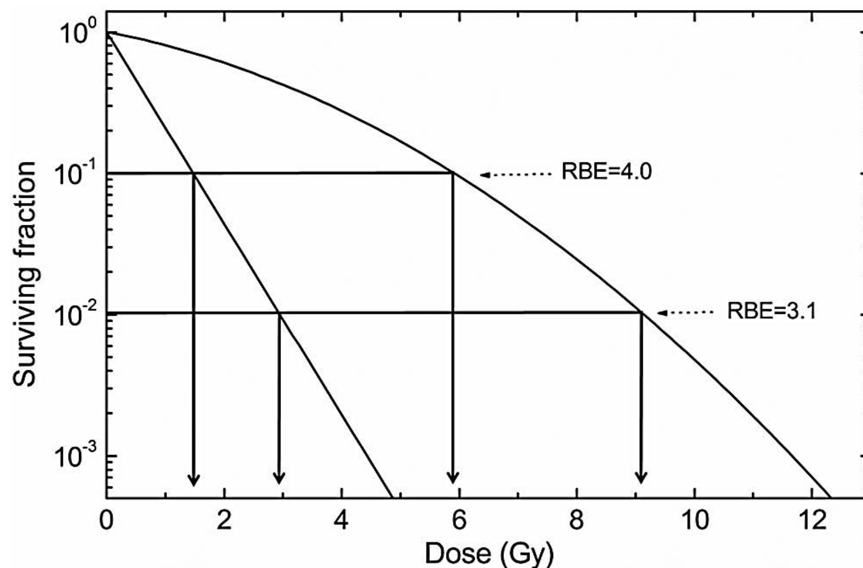

Figure 2: RBE calculations at two different survival levels. *The values of RBE would differ based on the survival level selected and the type of radiation.*

1.3 Cellular response to radiation

The biological effect of radiation is essentially due to the interaction of the absorbed radiation with DNA. As mentioned before the interaction could either be direct or indirect depending on the mechanism by which radiation damages the DNA. Tumor cells are rapidly dividing and successful cell division requires that the integrity of the DNA has to be maintained. Initiation of the DNA repair mechanisms and cell signaling pathways are the initial events that occur to counteract the radiation damage. Inability to correctly repair the DNA results in cell cycle arrest and may lead to subsequent induction of cell death (Figure 3). The radiation response is described below:

1.3.1 Radiation induced DNA damage

It is well known that radiation induces a wide spectrum of DNA lesions. These could be resulting from damage to nucleotides (base damage), DNA single strand breaks (SSB), DNA double strand breaks (DSB), DNA-DNA cross links and DNA-protein cross links depending on the dose of radiation and also the type of radiation [19]. Studies on DNA lesions on individual sites show minor importance of DNA SSBs in inducing a damage

response and cell death in mammalian cells [64]. DNA DSBs are the most destructive to the cells and could lead to chromosomal abnormalities, genetic mutations and cell death. It has been reported that there is a direct correlation between the number of DNA DSBs and the loss of clonogenicity of irradiated cells [65, 66]. It has been shown by mathematical modelling that in response to high LET about 70% of strand breaks are DSBs and that these are of complex nature involving two or more DSBs in close proximity, whereas only 20-30% of strand breaks induced by low LET are complex DSBs [67]

1.4 DNA damage response

The formation of DNA DSBs in response to radiation is the major cause of the damage response in cells and that the other types of DNA lesions are repaired efficiently without any significant damage to the genetic material. The DNA damage induced by irradiation leads to activation of several checkpoints which lead to cell cycle arrest. By arresting the cell cycle progression and DNA synthesis, the cell optimizes the possibilities of successful repair. DSBs are repaired by two distinct processes, i.e. the homologous recombination (HR) and non-homologous end joining (NHEJ) [68].

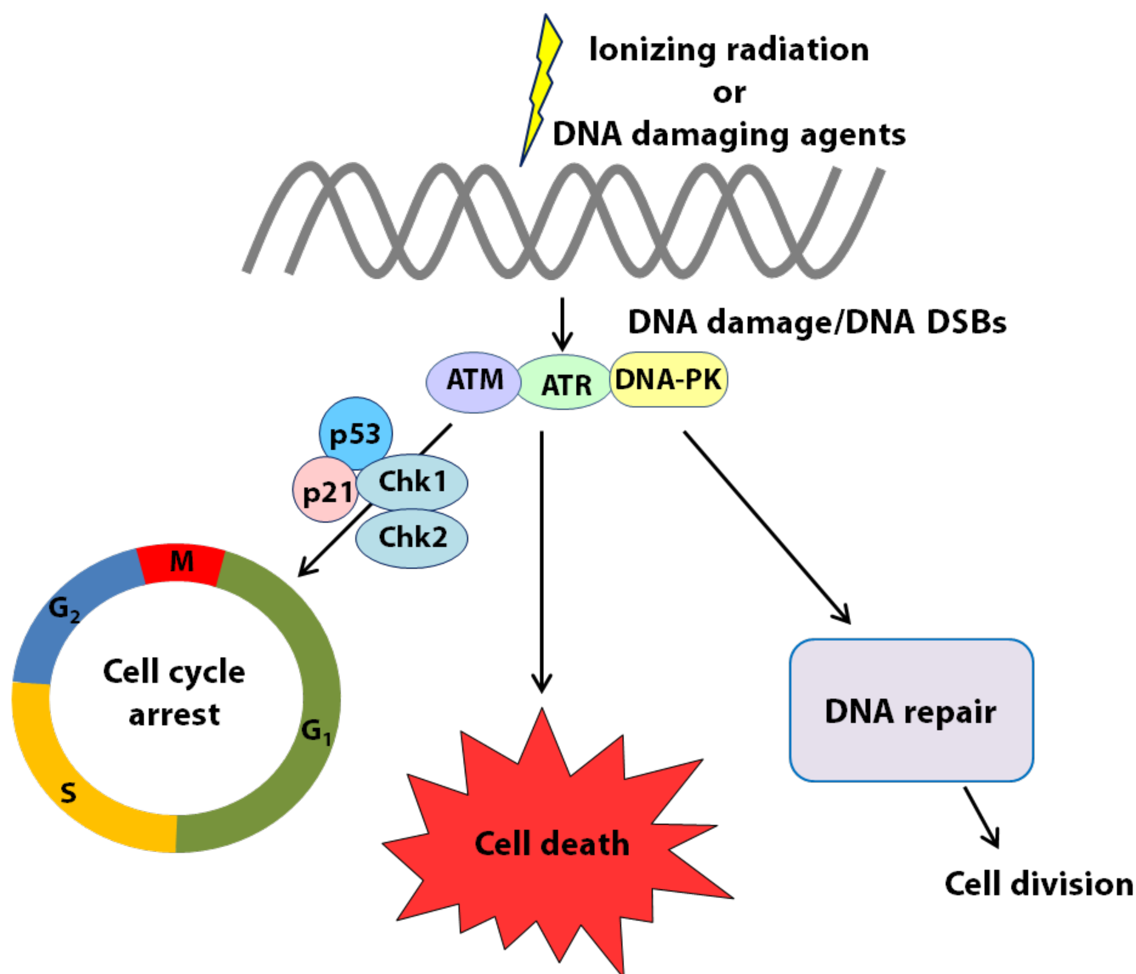

Figure 3: The DNA damage response. *More detailed description in the text.*

The DNA damage response is predominantly initiated by the activation of the kinases, ataxia telangiectasia mutated (ATM), ATM- and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) by the DNA DSBs [69-71] (Figure 3). The Mre11-Rad50-Nbs1 (MRN) mediator complex recruits ATM to the site of DNA DSB, acting as a sensory component [72, 73]. Activation and phosphorylation of ATM leads to phosphorylation of downstream target proteins, most noticeably the phosphorylation of DNA DSB marker H2AX at Ser139 into γH2AX [70, 74] as well as P53 and Chk2 [75]. Phosphorylation of H2AX could also be mediated by ATR and DNA-PK.[74]. The phosphorylation of γH2AX activates a number of different factors for checkpoint regulation and repair pathways [76].

1.4.1 Cell cycle arrest

As mentioned earlier, DNA damage in response to irradiation leads to initiation of cell cycle arrest in order to allow accurate DNA repair process to be accomplished [77]. Exposure to ionizing radiation causes delays of the cell cycle in either the G1 or G2 phases. Activation of the tumor suppressor protein p53 plays a vital role in the cell cycle block induced by ionizing radiation. The p53 protein is a transcription factor which regulates many different pathways involved in cell cycle regulation. The main purpose of p53-dependent G1 arrest could be for the removal of cells with DNA damage whereas the radiation induced G2 arrest is mostly a protective mechanism from cell death [78].

Cyclin dependent kinases (CDK) are a family of protein kinases known to regulate cell cycle progression and are inhibited by $p21^{Cip1}$. Activation of $p21^{Cip1}$ is dependent on the tumor suppressor p53 [79] and is a critical requirement for cell cycle arrest [80, 81] (Figure 3). Inhibition of cyclin E-CDK2, cyclin D-CDK4/6 by $p21^{Cip1}$ is preceded by the p53 dependent increased transcription of $p21^{Cip1}$ [82, 83]. The inhibition of cyclin E-CDK2 leads to G1 arrest. In a parallel pathway phosphorylation of Chk2 by ATM leads to phosphorylation of cdc25A which in turn leads to G1 arrest [84]. The G1 check point is however abrogated in many tumors due to mutations in the p53 gene [85], leading to a dependency on the G2/M checkpoint in order to avoid cell cycle progression with unrepaired damaged DNA.

The G2 checkpoint is activated by the detection of DNA damage at the G2 phase by activation of ATM and ATR kinases which phosphorylate and activate Chk1 and Chk2 [86, 87]. The Chk kinases phosphorylate and inactivate cdc25A/B/C, which prevents the activation and formation of the cyclinB-cdc2(CDK1) complex [88]. The p53-dependent CDK inhibitor $p21^{Cip1}$ also has a prominent role in regulation of the G2 checkpoint arrest by inhibiting CDK1 [89, 90]. In a similar cascade p38MAPK regulated MK2 has also a role in the G2 checkpoint arrest [91], which is active mostly in case of p53 mutations [88, 92]. In absence of p53- $p21^{Cip1}$ activation, the p38MAPK dependent activation of MK2 leads to degradation of cdc25A/B and subsequent cell cycle arrest in response to DNA damaging agents [93].

1.4.2 Cell death

The cell cycle checkpoint arrest allow a timely repair process of the damaged DNA, failing to which the cells could either undergo irreversible growth arrest or cell death via a number of diverse mechanisms depending on the severity of the DNA damage and sensitivity of the tumor type [94]. Cellular senescence is the permanent arrest of metabolically active cells in G1 phase, in case of severe DNA damage [95].The modes of cell death are apoptosis, necrosis, autophagy and mitotic catastrophe, with apoptosis and necrosis being the culminating outcome of DNA damage response [94]. Nevertheless it is still not clear, how the cells decide between permanent growth arrest or cell death.

1.4.2.1 Apoptosis

Apoptosis is a highly regulated, programmed cell death mechanism characterized morphologically by chromatin condensation, nuclear fragmentation, shrinkage of cellular volume, plasma membrane blebbing and ultimately disintegrating into apoptotic bodies [96]. The principal regulators of apoptosis are caspases, which are a family of cysteine proteases. Caspases are divided based on their function in the apoptotic process, as upstream initiators or downstream effectors. The initiator caspases (caspase-2,-8, -9 and-10) activate the effector caspase (caspase-3, -6 and -7), which perform proteolytic cleavage of a number of cellular structures and stimulate DNA fragmentation resulting in cell death [97].

Apoptosis could be operated in two main pathways, the intrinsic and extrinsic pathways. The extrinsic or death receptor pathway is initiated by the binding of death ligands (e.g., FasL/CD95, TRAIL) to cell surface death receptor (e.g., Fas. TRAILR1, TRAILR2) leading to the recruitment of FAS-associated death domain (FADD) and caspase-8 for the formation of a death inducing signaling complex (DISC) [98]. DISC activates the initiator caspase-8, which in turn activates the effector caspase-3 and -7 leading to apoptotic cell death [99]. The intrinsic pathway of apoptosis is mainly activated by the release of several proteins such as cytochrome c, apoptosis inducing factor (AIF), Smac/DIABLO, EndoG and OMI/HTRA2 by the permeabilization of the mitochondrial outer membrane [97]. The most important pro-apoptotic protein is cytochrome c, which along with the adaptor molecule apoptosis protease-activating factor 1 (Apaf-1) and dATP forms a complex, the apoptosome, which activates the initiator procaspase-9 into caspase -9. Activation of the initiator caspase-9 leads to activation of the effector caspase-3 and -7, which continue the apoptotic cascade [100]. Smac/DIABLO interact with the inhibitors of apoptosis proteins (IAPs) and remove the caspase inhibitory effect of IAPs [101] (Figure 4).

The intrinsic pathway is regulated by a balance between a group of pro-apoptotic and anti-apoptotic proteins, both belonging to the Bcl-2 family. The anti-apoptotic proteins (e.g., Bcl-xL, Bcl-2 and Mcl2) maintain the pro-apoptotic Bcl-2 proteins (e.g., Bak, Bax, Bid, Bim, Bad, PUMA and NOXA) in an inactive state [102]. The pro-apoptotic proteins Bak and Bax undergo conformational changes in response to DNA damage and initiate release of

cytochrome c into the cytosol [103]. The extrinsic and the intrinsic pathways of apoptosis are entwined with each other, by the caspase-8 mediated cleavage of Bid, which triggers the mitochondrial outer membrane permeabilization and release of cytochrome c [104]. Resistance to apoptotic stimuli is seen in many human cancers due to deregulation of the Bcl2 class of proteins.

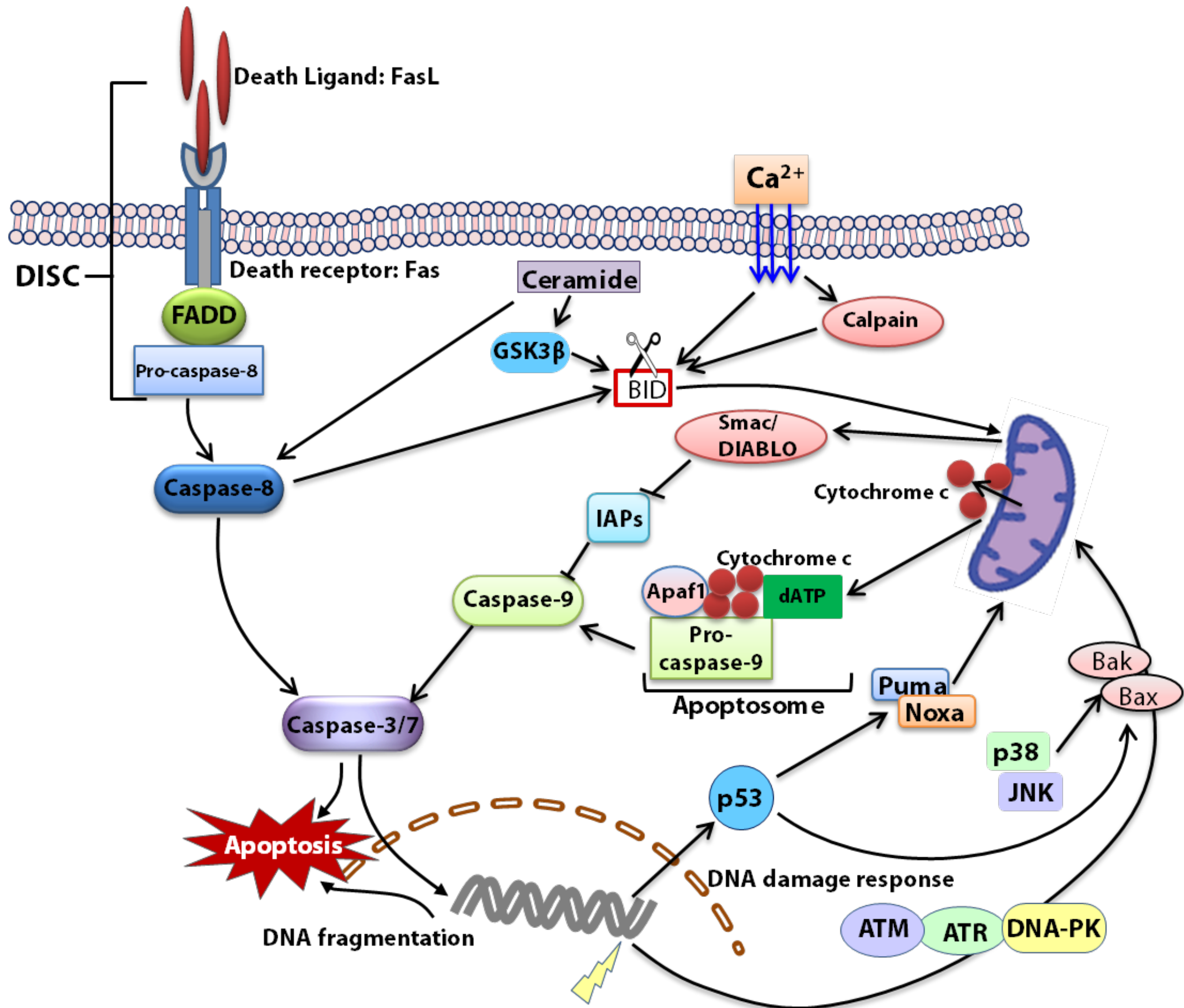

Figure 4: The intrinsic and extrinsic pathways of apoptosis. *Detailed description in the text*

Apoptosis can be initiated by various stress influences such as DNA damage, ER stress, ROS formation etc. and modulated by different factors including the tumor suppressor gene p53, cell surface ceramide and influx of Ca2+ ions. Activation of p53 leads to an increased expression of pro-apoptotic proteins (FasL, TRAIL) and pro-apoptotic Bcl-2 family proteins Puma, Noxa and Bax triggering the mitochondrial release of cytochrome c and Smac/DIABLO [105]. Translocation of Bax to mitochondria is promoted by JNK through phosphorylation of 14-3-3 [106]. Ceramide formation is induced by various tumor-treating agents such as chemotherapeutic drugs and ionizing radiation [107]. Activation of p38MAPK by ceramide can induce translocation of Bax to mitochondria [108, 109], which is the key mediator of ceramide mediated apoptosis [110]. Ceramide can also induce activation of caspase-8 mediated cleavage of Bid by activation of GSK3β [111]. Influx of Ca2+ ions into

the cytosol can induce cell death pathways, leading to apoptosis or necrosis. The increase in intracellular Ca2+ levels activates a number of Ca2+ dependent proteins such as proteases, enzymes, endonucleases and most importantly calpain to trigger the advancement of apoptosis [112, 113]. Activated calpain induce apoptosis and necrosis by increasing plasma membrane permeability and caspase cleavage [113, 114]. Increased levels of cytosolic Ca2+ ions can deregulate the protein folding and modifying function of the ER [114] and lead to ER stress related apoptosis. Along with ER stress, mitochondria is effected both by increased Ca2+ levels and also by activation of calpain [115]. Calpain activation leads to cleavage of Bid and cytochrome c release culminating at cell death by apoptosis [116].

1.4.2.2 Necrosis

Necrosis is often negatively termed as the cell death process in the absence of apoptosis and autophagy [117]. It is characterized morphologically by swelling of cytoplasm and cytoplasmic organelles, slight chromatin condensation and rupture of plasma membrane following loss of intracellular material [96]. Necrosis was traditionally thought to be a disorganized mode of cell death occurring by accident; however it is suggested that necrosis could be an alternate form of programmed cell death regulated by specific proteins so pertinently called as regulated necrosis or necroptosis [118].

Necrotic cell death occurs in response to extreme alterations in physiological conditions including DNA damage, hypoxia (restricted oxygen supply), restricted blood supply, nutrient or energy (ATP) deprivation and other pathological trauma. Cellular ATP levels are important in determining the mode of cell death. Apoptotic cell death process requires several ATP dependent steps, which could be disadvantaged in low energy conditions. It is known that ATP generation either by glycolysis or by mitochondria oxidative phosphorylation is an unavoidable step for the final execution of apoptosis [119]. In tumor tissues where blood supply is low leading to both oxygen and nutrient deprivation, necrotic regions are commonly found. It is conceivable that necrosis is due to poor metabolic status of the cells in these regions and that apoptosis is not possible due to ATP depletion.

Necrosis can be mediated by a number of different substrates most important of which is poly (ADP-ribose) polymerase (PARP), which is activated in response to DNA damage or oxidative stress. Activation of PARP leads to inhibition of glucose dependent ATP production causing ATP depletion and hence necrosis [120]. Necrosis is also mediated by tumor necrosis factor (TNF) along with the kinases receptor-interacting protein 1 (RIP1) and RIP3, mainly in the presence of caspase inhibitors which inhibit the normal apoptosis process. This pathway involves the formation of a death complex "necrosome" that results in increased ROS production, calcium mobilization and release of lysosomal enzymes leading to cell death [121] (Figure 5).

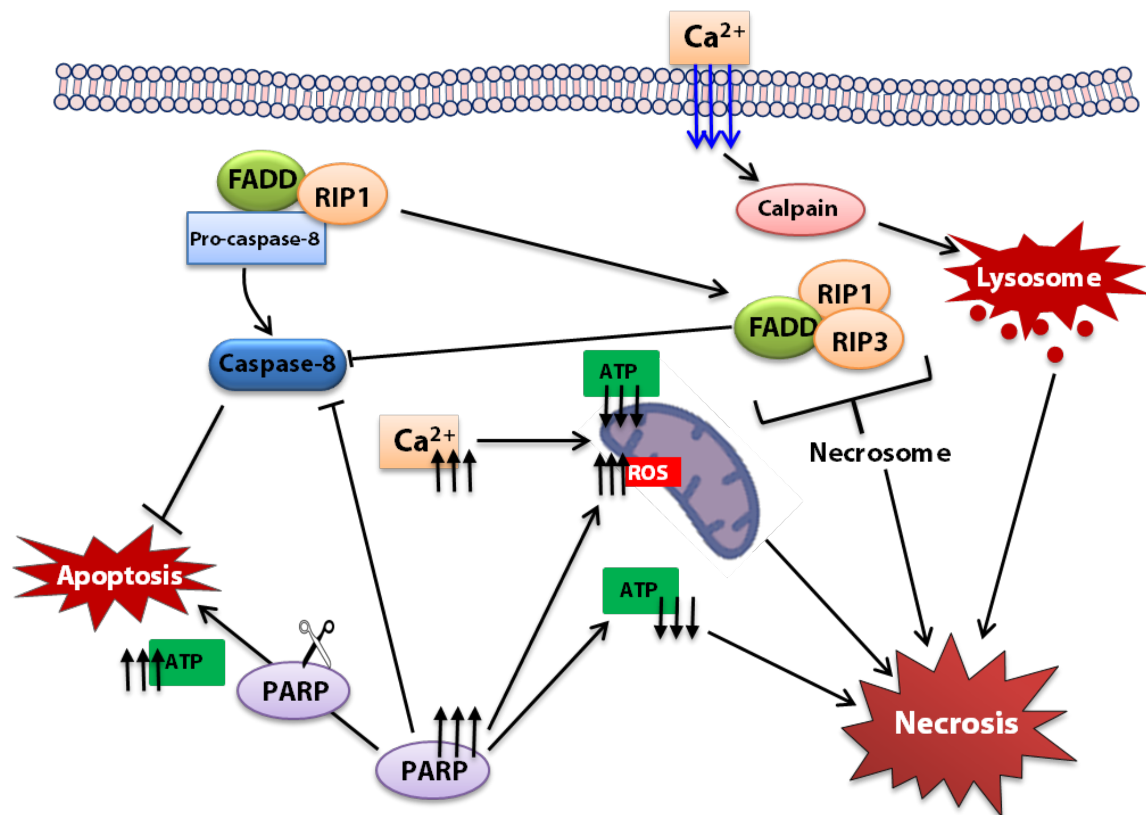

Figure 5: The pathways leading to necrosis*. ATP levels regulate the decision between apoptosis and necrosis*

1.5 Resistance to radiation therapy

Clinically radioresistance in cancer tissues is acknowledged when either a lack of response in tumor control is seen or the tumor relapses with/without distant metastasis after successful treatment. The resistance could be intrinsic i.e., resistance to therapy during initial treatment; or acquired due to selection of radioresistant cells during treatment. There are several biological factors regulating tumor radioresistance including hypoxia, intrinsic radiosensitivity and differential expression of DNA repair and cell death regulating genes.

The tumor suppressor protein p53 is a major player in DNA repair and apoptotic response towards DNA damage by ionizing radiation. p53 is mutated in many human tumors and p53 mutations are associated with a weak apoptotic response to low LET radiation [122]. High LET radiation is, however able to overcome this type of resistance by induction of p53 independent apoptosis [123, 124]. Cellular radiation sensitivity is largely dependent on the capacity to efficiently induce a DNA damage response and to repair the damage. So several proteins involved in the DNA damage response, DNA repair, cell cycle checkpoints and apoptosis induction have been targeted to abort radioresistance. Inhibition of ATM, ATR, Chk1/2, PARP by chemotherapeutic drugs have been studied to evaluate their radio sensitizing capabilities with promising responses[125]. Increased expression of EGFR is involved in survival and proliferation of tumor cells. It has been reported to be associated with inherent and induced radioresistance [126, 127]. Another growth factor receptor, the

IGF-1R is also a key mediator of radiation resistance in breast [128] and non-small cell lung cancer [129, 130]. Inhibition of these growth factors receptors has been shown to sensitize cells to radiotherapy [131]. Discovery of novel radio-sensitizing pharmaceutical agents is a promising and interesting field of research, with a large potential to improve cancer therapy.

1.6 Hypoxia and resistance

As previously mentioned hypoxia is seen in many human tumors and is present in specific regions in solid tumors. Low oxygen levels confers resistance to both chemo and radiotherapy in tumor tissues [132, 133]. In the case of head and neck cancers, it has been demonstrated that reoxygenation of tumor tissue gives a better treatment outcome to radiation therapy[134]. There are several reasons for hypoxia induced resistance. Hypoxic cells are present in the deep tumor parenchyma lacking adequate blood supply. These cells are also inaccessible to many anti-cancer drugs. Many of these hypoxic cells are quiescent. Ionizing radiation and many important cancer therapeutical drugs are most effective on actively proliferating cells [135]. The presence of oxygen increases the efficiency of DNA strand break formation and DNA repair process [136]. Hypoxia induces changes in the DNA damage repair pathway and cell death pathways leading to a more malignant and resistant characteristic [137]. Tumor cells have a higher transcriptional activity of hypoxia inducible factors (HIFs), especially at low oxygen conditions and HIF-1 plays a major role in the regulation of hypoxia related tumor radioresistance [138] and also is linked to multi drug resistance in chemotherapy.

Regions of hypoxic cells in tumors could be of two different types: chronic and acute hypoxia. Chronic hypoxia is the result of limited diffusion distance of oxygen, due to metabolic activity of proliferating cells in the tumor. These cells are present at the furthest distance from blood vessels and are less likely to recover and generally contribute to the necrotic region of the tumor [19]. Acute hypoxia is formed due to frequent malformation in the tumor vasculature resulting in temporary variations in blood flow and these cells can revert back to normoxic conditions with the availability of oxygen a phenomenon referred to as cycling hypoxia[139]. The tumor is gradually divided from proliferating tumor cells to quiescent hypoxic cells rather than having specific borderlines. Cells which have a decrease in oxygen concentration that render them resistant to anti-cancer agents but can still proliferate and contribute to regrowth of cancer, are present between the hypoxic and normoxic regions of the tumor [19]. It has also been suggested that hypoxic regions with increased HIF stabilization could drive the formation of cancer stem cell like properties [140]. Cancer stem cells are a small subpopulation of the tumor which are pluripotent and have the potential to self-renew and are resistant to most of the conventional tumor therapies [141]. These cells are the major contributor for repopulation after therapy and are crucially dependent on HIFs for their survival, self-renewal and tumor regrowth [141]. So targeting tumor hypoxia is a major necessity for advancement of cancer therapy and as hypoxic regions

are only present in solid tumors, it can be a distinguishing factor between normal and cancerous tissue to increase the therapeutic index[135].

Several strategies to overcome tumor hypoxia in radiation therapy have been attempted including different ways to deliver oxygen directly at the hypoxic regions, reoxygenation of acute hypoxia by fractionated radiotherapy, radiosensitizing the hypoxic areas by use of nitroimidazole derivatives and increasing specificity of radiation dose delivery by intensity modulated radiation therapy (IMRT). The hypoxic cells can be specifically targeted by the use of hypoxic cytotoxins, HIF 1 inhibitors and gene therapy [142]. Fractionated radiotherapy and IMRT have shown successful results in the clinic and several chemotherapeutic drugs targeting hypoxia such as tirapazamine have shown potential benefits in patients with lung and head and neck cancer used in combination with radiation [142]. However there is still an extensive need to develop drugs that are active on both hypoxic and normoxic tumor cells.

1.7 Cancer stem cells

Malignant tumor consists of a mixture of phenotypically and functionally different cell types often referred to as tumor heterogeneity [143]. Cancer stem cells (CSC) are a subpopulation of tumor cells that retain the ability to self-renew , maintain tumor cells and differentiate into several types of cells that form the tumor [144]. CSCs are present in most types of hematopoietic and solid tumors. They play a major role in tumor initiation, maintenance. These are also involved in resistance to anti-cancer therapies and relapse of tumors [145, 146]. CSCs are identified in solid tumor tissues by their expression of specific cell surface proteins or markers such as CD133, CD44, CD24 and several others [147]. CSCs have an increased capability to repair DNA damage, protect from oxidative DNA damage by ROS scavenging and regulate cell cycle checkpoints which is the basis of resistance to several therapeutic agents [147]. In case of CD44+/CD24−/low human breast cancer and CD133+glioma, CSCs have increased phosphorylation of ATM. CHK1/2 conferring radioresistance [147]. In NSCLC chemotherapeutic resistance is seen in CSCs due to an increased activation of CHK1 [148]. Activation of several cell survival pathways including PI3K/Akt, WNT/β-catenin, notch and hedgehog signaling also contribute to the resistant phenotype of CSCs [149-151]. Targeting pathways specific to CSCs together with conventional anti-cancer agents would greatly improve tumor therapy.

1.8 Spheroids as a model for solid tumors

Experimental models that realistically mimic solid tumors would give an advantage to challenge hypoxia-related radio- and chemo resistance. Most studies of the anticancer agents are performed on tumor cell lines grown as 2D monolayer, a poor representation of the actual tumor microenvironment. As mentioned previously, the problems in tumor tissues arising from limited diffusion of oxygen and nutrition are not correctly represented by monolayer cultures. The presence of necrotic region, region with chronic hypoxic and quiescent cells and

normoxic proliferating cells are not distinguished in a monolayer cell culture as all the cells represent the highly proliferating region.

3D multicellular tumor spheroids were developed as a more appropriate model to represent solid tumors in vitro for the purpose of biological and therapeutic studies [152]. Spheroids are produced by growing tumor cells in a 3D configuration and are of intermediate complexity between monolayer tumor cell lines and solid tumors in vivo [153, 154]. Similar to solid tumors, spheroids show defined regions of a necrotic core surrounded by hypoxic quiescent cells and an outer layer of highly proliferative tumor cells [155]. Cancer cell lines when grown in multicellular spheroids show dissimilar sensitivity to anti-cancer agents i.e. ionizing radiation and chemotherapeutic drugs [156]. Also differences in sensitivity to radiation and drugs, between the areas of tumor spheroids have been found with altered cell cycle distribution [157]. As previously, stated hypoxic quiescent cells within solid tumors, when exposed to adequate amount of oxygen and nutrients would proliferative and repopulate, which is a typical characteristic of multicellular tumor spheroids[158]. So it is of huge importance that anti-cancer drugs should be developed which are effective not only on the proliferative tumor cells but also on the resistant quiescent hypoxic cells, especially in case of solid tumor treatment.

1.9 Chemotherapy

Cancer chemotherapy is the use of pharmaceutical drugs to damage tumor cells. The main aim of chemotherapy is to cure cancer completely in addition to reduce the risk of cancer relapse or metastasis. Chemotherapy is frequently used in combination with surgery or radiotherapy to increase the effectiveness of the treatment and sometimes also as a palliative care. In contrast to radiation, where DNA is the main aim, chemotherapeutic drugs are directed against various targets within the tumor cells. As compared to normal cells, most tumor cells have a higher proliferative status and altered gene expression which is one of the main targets of chemotherapeutic drugs.

Tumor cells, due to their high proliferative rate, require systematic degradation of misfolded proteins, which would otherwise cause cytotoxicity. Two different pathways are involved in the degradation of misfolded proteins in eukaryotic cells: the lysosomal system and the ubiquitin proteasome system (UPS). However it was observed that UPS is the major pathway for intracellular protein degradation in eukaryotic cells [159]. In this thesis two compounds have been studied which inhibit different parts of the ubiquitin proteasome system (UPS) as an anti-cancer strategy.

1.10 The ubiquitin proteasome system

The Nobel Prize in chemistry in 2004 was awarded to Avram Hershko, Aaron Ciechanover and Irwin Rose for the discovery of a small protein ‘ubiquitin’ which mediated proteasome degradation. The UPS accounts for about 80-90% of intracellular protein

degradation [160, 161]. Removal of unused or deformed proteins from cells is indispensable for basic biological process. The degradation of proteins by UPS regulates several cellular pathways such as cell-cycle and apoptosis regulatory proteins, DNA replication, damage response and repair proteins, inflammatory response, gene transcription, antigen presentation, protein quality control and maintaining source of amino acids [162-164]. The UPS system is critical for tumor sustenance and is involved in degradation of tumor suppressors, proto-oncogenes and components of signal transduction system [163]. Defects in the UPS could also lead to cancer initiation. Besides cancer UPS is involved in several human diseases including neurodegenerative diseases [165], cardiovascular diseases [166], inflammation and microbial infections [167].

The UPS mediated proteolysis can be roughly divided into two distinct steps: conjugation of a polyubiquitin chain to the substrate protein destined for degradation (ubiquitination/ubiquitylation) and the actual degradation of the protein by the 26S proteasome (proteasomal degradation) (Figure 6). The 26S proteasome is composed of two sub-complexes: the proteolytic active 20S core particle (CP) capped by the 19S regulatory particle (RP).

1.10.1 Ubiquitination

Ubiquitin is a highly conserved 76-residue protein which specifically attaches by isopeptide formation at its C-terminus to the ϵ-amino groups of lysine side chains of the protein targeted for degradation [168]. The conjugation of ubiquitin to a substrate protein is governed by the concerted action of three different enzymes – ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2 and finally a ubiquitin ligase E3 [163, 169]. There are two isoforms of the E1 enzyme UBE1 and UBA6. The E2 enzyme has a number of isoforms which have different specificities for the E1 enzyme [170]. Three major classes of E3 have been identified, termed the HECT (homologous to E6-associated protein C-terminus), RING (really interesting new gene) finger and U-box (a modified RING motif) [171].

Ubiquitination is a post translational modification which is executed in a three step manner. First a cysteine residue in the catalytic site of the E1 enzyme, in an ATP dependent manner, forms a thiol-ester bond with the carboxyl group of G76 at the C-terminus of ubiquitin leading to its activation. Secondly the ubiquitin conjugating enzyme E2 rapidly takes over the activated ubiquitin by the formation of another thiol-ester linkage. And finally the substrate specific ubiquitin ligase E3 transfers the activated ubiquitin from E2 to a lysine residue in the substrate protein. Ubiquitin is a protein that itself can function as a substrate for the attachment of another ubiquitin. The attachment of one ubiquitin molecule to the target protein leads to repetitive conjugations via the E1, E2 and E3 enzymes and localizes the substrate to the proteasome [172, 173]. Sometimes another ubiquitination enzyme E4 is required along with the existing three enzymes, for extending the polyubiquitin chain [174] (Figure 6).

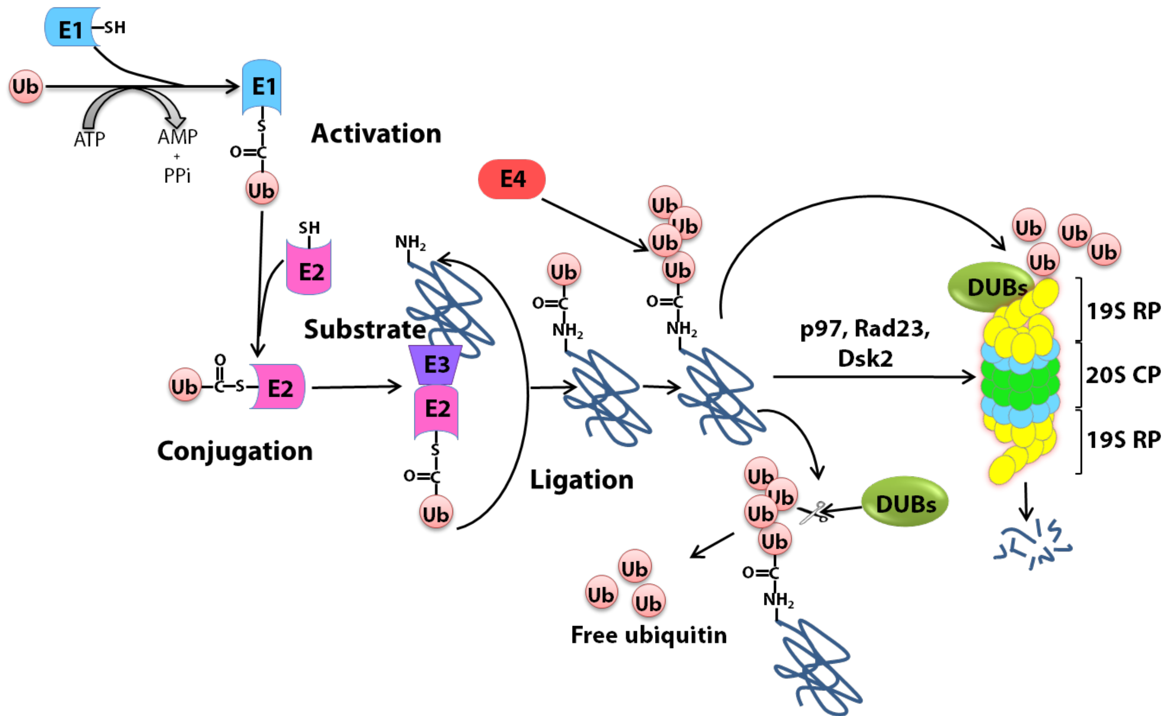

Figure 6: The ubiquitin proteasome pathway. *The three steps of ubiquitination as described in the text*

Polyubiquitin chains can be formed by linking one of the seven ubiquitin lysine (K) residues (which are K6, K11, K27, K29, K33, K48, and K63) or the ubiquitin amino terminal Met1 residue (which generates linear chains) and can lead to different chain conformations [175]. Out of these the most abundant K48 linked polyubiquitin chains are a principal target signal for proteasomal degradation [169]. The other prominent chains of K63-linked polyubiquitin play a role in DNA damage response [176], DNA repair [177], regulation of stress response via JNK pathway [178] and activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) [179]. However in some studies it has been found that K63 linked chains could be targeted for proteasomal degradation [180]. Recently it has been suggested that all other polyubiquitin chains, except for K63 linkage, are targeted for proteasomal degradation and specifically K11 linked chains are involved in protein removal by the endoplasmic reticulum-associated degradation (ERAD) pathway [181, 182].

Ubiquitination can also modify proteins by attachment of a single ubiquitin molecule (mono ubiquitination) which has a role in membrane trafficking, transcription regulation, DNA replication and repair [183]. Like several other protein modification ubiquitination is also reversible by the function of specific deubiquitinating enzymes (DUBs). The chain lengthening by E3 ligase and shortening by DUBs is balanced for appropriate proteasomal function. Most substrates require polyubiquitination to bind to ubiquitin receptors on the proteasome. An exception to this is the enzyme ornithine decarboxylase (ODC), which is degraded by the proteasome via binding of polyamine-induced protein (antizyme) [184, 185].

1.10.2 Delivering ubiquitinated substrates to the 26S proteasome

Protein substrates attach to the proteasome at its 19S RP by the help of ubiquitin receptors and are then translocated into the 20S core particle (CP), where they are chopped off. The polyubiquitinated proteins can be delivered to the proteasome in different ways. There are five ubiquitin receptors identified so far: two intrinsic receptors which are subunits of the 19S RP: Rpn10/S5a/hRpn10 and Rpn13/ARM1/hRpn13, and three proteasome associated extrinsic receptors Rad23, Dsk2, and Ddi1 [186]. The Rpn10/S5a is the most important and the first ubiquitin receptor to be described [187], localized at the 19S particle and recognizes only substrates with specific lengths of polyubiquitin [188, 189]. The Rpn10 has a small C-terminal domain called the ubiquitin-interacting motif (UIM) which recognizes and binds to the polyubiquitin chains [190, 191]. In mammals, the Rpn10/S5a receptor has two UIM domains [191]. The other ubiquitin receptor, Rpn13/ ARM1, binds ubiquitin via pleckstrin-like receptor for the ubiquitin (Pru) domain [192], . Rpn13/ ARM1 have higher affinity for K48-linked ubiquitin. Rpn10 and Rpn13 can simultaneously bind to a single K48 linked polyubiquitinated substrate [193]. The C-terminal domain of this ubiquitin receptor serves as the receptor site for Uch37, a 19S RP DUB, linking chain recognition and disassembly [194-196].

The three intrinsic receptors Rad23, Dsk2, and Ddi1 have two distinct domains: the ubiquitin like (UBL) domain and the ubiquitin associated (UBA) domain and are therefore collectively called the UBL/UBA proteins [197]. This UBL/UBA family of proteins acts as shuttling factors to deliver selective substrate proteins directly at the 26S proteasome (Figure 7). Each of these receptors connects with one of the 19S RP subunits Rpn1, Rpn10 and Rpn13 via the UBL domain and conjugates with ubiquitin via the UBA domain [198]. Dsk2 has been seen to associate with free Rpn10 (the Rpn10 receptors not bound to the 19S RP) to deliver substrates to the proteasome. The UIM motif of Rpn 10 binds to the UBL domain of Dsk2 and regulates the binding of Dsk2 to the proteasome [199, 200]. However it is not clear whether Rpn10 also acts as a shuttling factor similar to the extrinsic receptors.

1.10.2.1 The p97/VCP/CDC48 chaperone

The p97 or valosin containing protein (VCP) in mammals or Cdc48 (cell division cycle) in yeast is an ATP dependent molecular chaperone and has a prominent role in protein homeostasis, membrane trafficking, regulation of mitosis and DNA repair [201]. It is a very abundant protein representing about 1% of all cytoplasmic proteins [202]. P97 belongs to the hexameric AAA+ ATPase family (ATPase associated with diverse cellular activities) of proteins that has two ATPase domains D1 and D2 and a globular N terminal domain. The chaperone is loosely related to the ATPase ring on the 19S RP of the 26S proteasome. The hydrolysis of ATP in the D2 domain is critical for D1 domain rearrangement in p97 and subsequent attachment of polyubiquitin proteins to the N terminal domain [203-205]. A number of ubiquitin receptors or cofactors are present for p97, which are similar to the UBL/UBA family of proteins [206, 207]. These receptors have an ubiquitin regulatory X

(UBX) domain which binds to the Cdc48/p97 chaperone and a UBA domain for substrate binding [208]. The most common of these UBX/UBA proteins are the Npl4/Ufd1, Ufd2, Ufd3, Ubx2 and p47/UbxD1 [201]. Cdc48/p97 can bind to structurally modify the substrate protein either directly by its N terminal domain or via the UBX/UBA co factors. These substrates are targeted to be either recycled or degradation by proteasome. Cdc48/p97 recruits multiple enzymes including E3 ligase, E4 and DUBs for polyubiquitin chain editing to make the chain length appropriate for proteasome recognition and degradation [209, 210]. It has been suggested that p97 can deliver substrates to the proteasome via the shuttle factors Rad23 and Dsk2 [211] (Figure 7). The 19S RP sometimes requires preprocessing or unfolding of ubiquitinated substrates by p97 for recognition [212, 213].

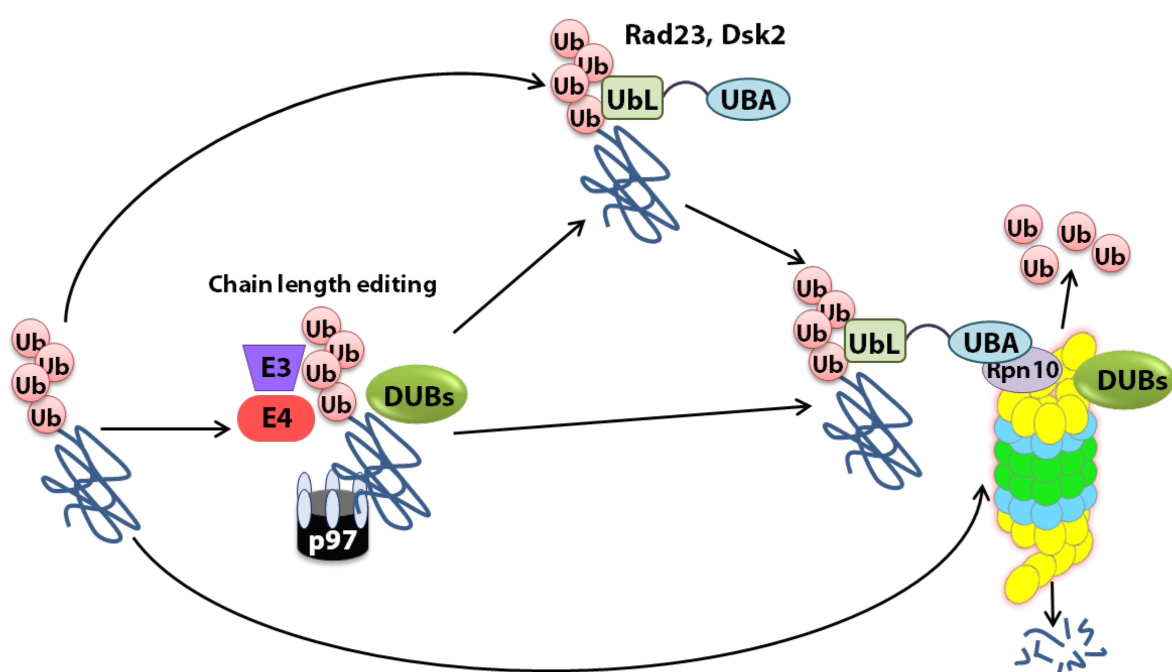

Figure 7: The role of p97 chaperone and shuttle factors (Rad23, Dsk2) in substrate delivery to the proteasome. *The substrate could be delivered either directly to the proteasome or it could go through the p97 chaperone with or without the shuttle factors.*

1.10.3 Degradation of proteins by the 26S proteasome

The degradation of misfolded and unwanted proteins within the cells is done by a multicatalytic enzyme complex, the 26S proteasome. As mentioned previously the 26S proteasome is composed of two major subunits: the central 20S CP capped on either end by the 19S RP.

The 19S Regulatory particle is responsible for recognition of polyubiquitinated proteins, releasing the ubiquitin chain and translocation of the substrate into the 20S core particle. The 19S RP consists of 19 subunits with molecular masses ranging from 10 to 110 kDa and can be divided into the lid and base sub complexes [213].

The lid complex includes 9 non-ATPase subunits, (Regulatory particle non-ATPase) Rpn3, Rpn5–Rpn9, Rpn11, Rpn12, and Sem1. Rpn 11/POH1 is an essential DUB for deubiquitination along with two other DUBS, (ubiquitin C-terminal hydrolases) UCH37/UCHL5 and (ubiquitin-specific proteases) Ubp6/USP14 [214, 215]. RPN11 cleaves the link between the substrate and the ubiquitin chain, whereas UCHL5 and USP14 remove each ubiquitin molecule sequentially from the distal tip of the polyubiquitin chain [214].

The base of the 19S RP is composed of ten subunits out of which six are AAA+ ATPase (Rpt1-Rpt6) that form a barrel shaped hetero-hexameric ring with the specific order of Rpt1-Rpt2-Rpt6-Rpt3-Rpt4-Rpt5. The rest four subunits are non-ATPase: Rpn1, Rpn2, and ubiquitin receptors Rpn10 and Rpn13 [216, 217]. The ATPases provide the energy required for substrate unfolding required to enter the 20S CP [218, 219]. Rpt2 is involved in the channel opening into the 20S CP [220] and Rpt5 plays a role in polyubiquitin chain recognition [221]. The non-ATPase base subunits Rpn1, Rpn10 and Rpn13 bind to the shuttle factors Rad23, Dsk2 and Ddi1 [198].

The 20S core particle is a barrel-shaped structure that is composed of four stacked rings. The two outer rings are of seven unique α subunits and the two inner rings are composed each of seven unique β subunits. These rings form a hollow cylinder, with the proteolytically active sites of the β1, β2, and β5 subunits facing towards the center of the 20S core particle [222, 223]. These catalytic subunits are responsible for caspase-like, trypsin-like, and chymotrypsin-like activities and are able to cleave peptide bonds at the C-terminal side of acidic, basic, and hydrophobic amino-acid residues [224, 225]. The channel leading into the catalytic chamber is narrow (~ 13 Å in diameter) and is composed of α2, α3 and α4 subunits. Protein substrates pass through this narrow channel and are cleaved by the active sites on α rings, into oligopeptides and then hydrolyzed into amino acids by different peptidases [224, 226-228].

1.11 Inhibition of the UPS in tumor therapy

It is understood that UPS via its multi-step system, controls several biological process within the cell by balancing the abundance and specific degradation of several regulatory proteins. Tumor cells have an increased rate of protein synthesis, translation and protein quality controls that is required for their survival and progression and are thus dependent on the efficiency of the UPS. The UPS plays an important role in cell cycle control, cell death/apoptosis regulation, DNA repair, ER and oxidative stress response in tumor cells. Inhibition of the UPS can lead to a toxic accumulation of misfolded proteins and tumor suppressors leading to tumor cell death. The major cellular response of malignant cells to proteasome inhibitors is seen among others as NFκB inhibition, increase in pro-apoptotic proteins, loss of DNA damage response, cell cycle arrest and inhibition of angiogenesis [229]. Targeting the UPS is therefore a lucrative option in the field of cancer therapy.

UPS inhibitors induce cell cycle arrest by blocking the degradation of cyclins (D, E, A, B) and cell cycle regulatory proteins and cause an accumulation of p21 and p27 in malignant cells [230-232]. Inhibition of NEDD8-activating enzyme (NAE), an E1 enzyme by MLN4924 results in loss of tumor cell growth both in vitro and in vivo [233]. MLN4924 stalls DNA replication and is currently in clinical trials for advanced solid tumors, lymphoma, myeloma, melanoma and acute myeloid leukemia [234]. PYZD-4409 and PYR-41 are two inhibitors of the E1 enzyme that stabilize p53 and suppress NFκB activation leading to apoptosis in tumor cells [235, 236] Selective inhibition of the Cdc34, an ubiquitin conjugating E2 enzyme by the compound CC0651 inhibits human cancer cell line proliferation [237]. CC0651 prevents p27 ubiquitination and results in cell cycle arrest in G1 phase [237, 238]. E3 ubiquitin ligases are responsible for substrate recognition and ubiquitination. Several E3 enzymes including SCF (Skp1-Cullin-F-box) and APC (anaphase-promoting complex) are involved in regulation of cell cycle, which are responsible for the ubiquitination of the CKIs (cyclin-dependent kinase inhibitors) or cyclins [230]. Inhibitors of SCF E3 ligase is involved in the ubiquitination of p21 [239]. Inhibition of F-Box proteins which is a component of SCF by the compound SMER3, blocks the mTOR pathway and increases sensitivity to rapamycin [240, 241]. Another compound TAME, that inhibits the E3 ligase APC results in mitotic arrest by failure of cyclin B1 regulation [242].

UPS inhibitors can upregulate several proapoptotic factors including p53, pro-apoptotic members of the Bcl-2 family (Bax, Bak, Bad, Bim, Bik, and Bid) and NOXA, and downregulate anti-apoptotic proteins such as Bcl-2 and IAP proteins [243].

MDM2, a E3 ligase is a negative regulator of p53 and targets it for proteasomal degradation [244, 245]. MDM2 also regulates the degradation of MDMX, that binds to and negatively regulates p53, however MDMX does not act as an E3 ligase [246]. Several compounds have been described that inhibit MDM2/MDMX such as Serdemetan [247, 248], Nutlins [249, 250], NSC-207895 [249] and subsequently activate p53. This results in activation of p53 downstream targets to inhibit cell proliferation and induce apoptosis.

The proteasome ubiquitin receptor RPN13, present on the 19S RP is essential in recognition of Ub K48 linked chains. The compound RA190 binds to RPN13 and inhibits proteasomal degradation. This compound causes rapid accumulation of polyubiquitinated proteins in tumor cell lines and is able to sensitize bortezomib resistant multiple myeloma cell lines, inducing apoptosis via ERAD pathway[251].

P97/Cdc48 is often upregulated in several human cancers and is correlated with poor prognosis, metastasis and decreased ability to undergo apoptosis [201]. P97 regulates many key cancer-related proteins / pathways such as IκBα, an inhibitor of pro-survival function of NFκB [252, 253], Akt mediated survival pathway and DNA damage [201]. Small molecule inhibitors of p97/Cdc48/VCP have gathered much interest in recent years for tumor control. It has been shown that a small molecule inhibitor of p97 associated DUBs, Eeyarestatin I

blocks the ERAD pathway in mammalian cells [254] and inhibits NSCLC proliferation both in vitro and in vivo [255]. Another compound NMS 873, which is a specific inhibitor of p97/VCP ATPase induces cell death in tumor cells [256]. Recent studies describe several other p97/Cdc48/VCP inhibitors such as: 2-anilino-4-aryl-1,3-thiazoles that inhibit both the ATPase and protein degradation activity [257], DBeQ that inhibits ERAD and activates caspases [258], Syk inhibitor III that irreversibly inhibits p97/CDC-48 ATPase activity of the D2 domain [259] and Sorafenib that prevents p97/CDC-48 tyrosine phosphorylation and control tumor progression in hepatocellular carcinoma [260]. Inhibition of p97/Cdc48/VCP leads to disruption of the ERAD pathway and lethal accumulation of proteins and eventually cell death.

Inhibitors of the 20S proteasome are generally targeted against the three peptidase activities (caspase-like, trypsin-like, and chymotrypsin-like) of the proteasome. Bortezomib (PS-341, Velcade ®; Millennium Pharmaceuticals) is a boronic acid analogue that is the first in class of 20S CP inhibitors and has shown tangible success in the molecular targeting of the proteasome. Bortezomib is cytotoxic to a number of different tumor types and has been approved by the US Food and Drug Administration (FDA) for the treatment of multiple myeloma and mantle cell lymphoma [261-263]. Bortezomib is a reversible inhibitor of the chymotryptic-like and caspase-like and to some extent trypsin-like activities of the 20S CP [264-266]. Bortezomib's main cytotoxic effect on tumor cells is through the direct induction of apoptosis after accumulation of excessive protein [267]. Tumor cells respond to Bortezomib through a number of different mechanisms such as: inhibition of the NF-κB activation [268, 269], induction of p53, p21 and p27 [270, 271], activation of JNK pathway [272] and induction of pro-apoptotic protein NOXA [273, 274]. However disparate reports of inhibition of NF-κB pathway in response to bortezomib and other proteasome inhibitors have been found [275]. It was shown that bortezomib stimulated two NFκB activating kinases and promoted non-proteasomal degradation of IκB in multiple myeloma cells [276]. These discrepancies in the results lead to the conclusion that inhibition of NFκB activation may not be the main reason for induction of apoptosis, in case of bortezomib and could also be a possible explanation of acquired resistance to the drug.

However there were several toxic side effects of bortezomib including peripheral neuropathy, low platelet and erythrocyte counts and joint pain. Along with that non-responsiveness and resistance was seen in many patients [277-279]. There a huge need for development of new proteasome inhibitors with focus on overcoming resistance to bortezomib and improve patient outcome.

Carfilzomib is a new second generation proteasome inhibitor approved for treatment of relapsed or refractory multiple myeloma by the FDA [280, 281]. Unlike bortezomib, carfilzomib is an irreversible inhibitor of the chymotrypsin-like activity of the 20S CP [281, 282]. Clinically carfilzomib has shown complete response in bortezomib treated and untreated multiple myeloma patients, without the bortezomib related toxicity [281]. Several

other proteasome inhibitors such as Marizomib/NPI-0052, MLN9708, CEP-18770 and ONX0912 have been described that are able to sensitize bortezomib resistant cells [229].

Many of the DUBS are over expressed or altered in human cancers, making these as a potential target for development of anti-cancer agents [283, 284]. Various pan-DUB inhibitors targeting both proteasomal and non-proteasomal DUBs have been described. WP1130 is a partially selective inhibitor of several DUBS including USP9X, USP5, USP14 and UCHL5. WP1130 induced rapid accumulation of polyubiquitinated proteins, down-regulation of anti-apoptotic and up-regulation of pro-apoptotic proteins, leading to aggresome formation tumor cell apoptosis [285]. WP1130 is synergistic in action with Bortezomib [286]. Another compound P5091 which is a -specific inhibitor of USP7 induces apoptosis in multiple myeloma cells and is able to overcome acquired resistance to bortezomib [287]. Another inhibitor of the DUB USP7, P22077 induces tumor cell death [288]. The compound NSC 632839/F6 which inhibits USP2 and USP7 induces apoptosis in tumor cell lines [289]. AC17 is a curcumin analogue which is an irreversible inhibitor of the DUB activity of the 19S RP. The compound inhibits the activation of NF-κB and activates p53 [290]. A novel small molecule inhibitor of proteasome associated DUB activity, *RA-9*,inhibits tumor growth in ovarian cancer cell lines and primary patient cell cultures. This compound induces apoptosis and ER stress in cancer cells [291]. The compound *b-AP15* is a novel, small molecule, reversible inhibitor of the proteasomal DUBs USP14 and UCHL5. The compound has shown anti-cancer activities in a number of tumor types including solid tumors and hematological malignancies. [292]. b-AP15 is cytotoxic on multiple myeloma cells that had acquired resistance to bortezomib [293].

There are few compounds that inhibit DUBS, but do not have any cytotoxic effect on tumor cells. *IU1* is a reversible inhibitor of USP14 and is shown to increase the efficiency of proteasomal degradation at very high concentrations. This compound has been suggested for the treatment of neurodegenerative diseases that are associated with accumulation of misfolded and aggregated proteins [294]. A selective inhibitor of UCHL1, *LDN-57444*, results in increased cell proliferation of lung tumor cell lines [295].

2 AIMS

The work presented in this thesis was focused on developing various treatment modes for human cancer including comparative study of different radiation types used for therapy and identification of drugs targeting specific properties of tumor cells.
The specific aims of each project were:

- To compare cell survival in response to low and high LET radiation and use mathematical modelling to predict whether cellular response to low LET could predict the response to high LET radiation **(Paper I)**.

- To compare the phosphoproteome of a NSCLC cell line in response to low and high LET radiation and predict putative targets responsible for low LET resistance in the cell line **(Paper II)**.

- To identify and characterize a novel compound effective on multicellular tumor spheroids **(Paper III)**.

- To identify and characterize novel inhibitor of the ubiquitin proteasome system **(Paper IV)**.

- To identify the optimised lead of the sucessful DUB inhibitor, b-AP15 and to illustrate the activity of the lead compound in multiple myeloma **(Paper V)**.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Predicting the Sensitivity to Ion Therapy Based on the Response to Photon Irradiation – Experimental Evidence and Mathematical Modelling

High LET ionizing radiation is emerging as a superior radiation type as compared to conventional low LET radiation, due to its physical properties. Leading ion therapy centers around the world use cellular response to photons and ions *in vitro* for their TPSs [296]. In this study we compare the response of tumor cell lines towards low and high LET radiation and use mathematical modelling to determine how cellular response to low LET could predict the response to high LET irradiation.

A panel of five different cell lines: one small cell lung cancer (SCLC) cell line (U-1690), one head and neck squamous cell carcinoma (HNSCC) cell line (FaDu), one melanoma cell line (AA) and two prostate cell lines (PC-3 and DU-145) were irradiated with low and high LET radiation for clonogenic cell survival experiments. Low LET photons (^{60}Co or ^{137}Cs) were used at doses 0-12Gy and high LET ions (^{12}C or ^{14}N or ^{36}Ar) were used at doses of 0-4Gy. The clonogenic data was fitted to the LQ and RCR model. We observed expected differences between the two radiation modalities where high LET radiation was highly effective in cell killing even using lower doses. Low dose hypersensitivity was seen in few of the cell lines and to avoid this phenomenon RBE values were calculated for doses higher than 1Gy which is more clinically relevant. We calculated and compared the RBE values using either D_{10} or $\overline{D}$ values from LQ and RCR model for each cell line. No significant difference was found with either of the methods and models for RBE calculation. This indicates that the choice of models (LQ or RCR) is not vital while comparing RBE between different cell lines. So to compare the response of different cell lines; we plotted the ratio of two parameters for photon irradiation from each of the two models i.e., β/α for the LQ model and b/a for the RCR model, against the RBE calculated as $\overline{D}$ ratios. It was seen that the plot using RCR model displayed correlation between the b/a and RBE values, which was not the case with LQ model.

As the *b/a* value from RCR model corresponds to the repair capacity of cells [56]. We observed that the cell lines with higher repair capacity, which are resistant to low LET radiation, are more sensitive to high LET ionizing radiation. Resistance to low LET radiation is observed in many tumor types and is limiting to effective radiotherapyMolecular targets to sensitize resistant cells towards low LET radiation are studied in paper II.

3.2 PAPER II

Phosphoproteomic profiling of high and low LET irradiated Non small cell lung cancer cells reveals differences in growth factor signaling cascades and indicate a role of p38MAPK and GSK3β in low LET radiotherapy cellular response

As seen in paper I, high LET radiation has a stronger biological effect on tumor cell types which are resistant to low LET photons. In this paper, we study global phosphoproteomic changes in a radioresistant NSCLC cell line in response to high and low LET radiation, along with direct signaling pathway analysis. The aim of this study was to reveal critical signalling events which may explain how high LET accelerated particles can overcome low LET photon resistance and thereby enabling identification of biomarkers of response and potential targets with low LET IR sensitizing capacity of NSCLC.

The NSCLC cell line U1810 was irradiated with different doses of high and low LET radiation. In accordance with published data low LET radiation did not cause induction of cell death in this NSCLC cell lines even when 8 Gy was used whereas high LET radiation resulted in induction of a cytotoxic response already with a dose as low as 1 Gy. Importantly, in contrast to low LET photons high LET accelerated particles resulted in activation of the Bcl-2 proteins Bak and Bax implicating a clear activation of apoptotic signaling in response high LET IR in these cells.

We then profiled changes in phospho proteome of these cells which occurred in response to either low or high LET radiation. A SCX fractionation and TiO_2 beads enrichment based method along with nano-LC and MS based analysis was utilized for this purpose. The analyzed phospho proteins were subsequently mapped on to signaling networks to identify specific signaling pathways and putative kinases that were differentially activated or deactivated in response to high and low LET radiation respectively. The phosphoproteomic analysis revealed higher signaling activity in low LET irradiated U-1810 cells as compared to high LET IR samples. This could be due to diminished signaling activity in response to high LET IR, which is still present in low LET samples as this treatment does not cause a cytotoxic response in these cells. Pathway analysis was done in a way to recognize networks that were still active in after low LET IR but inactivated in response to high LETIR. The top differentially regulated pathway identified by this approach was the regulation of transcription translation initiation by eIF.

Within this pathway multiple kinases were found to have decreased phosphorylation in response to high LET IR including GSK3β which was further analyzed. In order to identify specific kinases that regulate the altered signaling pathways the bioinformatic tool NetworKIN was used. GSK3β and p38MAPK were identified as one the kinases involved in the phosphorylation of the specific substrates of the eIF along with CDK2 and CKIIα. GSK3β has a complex role in tumor therapy as it can either inhibit or promote tumor progression [297]. We checked the phosphorylation of this kinase after low and high LET

irradiation by western blotting and could validate that high LET decreased its phosphorylation at Ser9. We further observed in a panel of NSCLC cells a higher degree of phosphorylation of GSK3β at Ser9 was in part correlated to low LET IR resistance. This is in contrast to previously reported pro-death activity of GSK3β in a number of tumor types and kinase inhibitory activity of Ser 9 phosphorylation in particular [297].These results indicate that inhibition of this kinase could sensitize cells to low LET irradiation. By using siRNA knockdown of GSK3β a decreased cell survival of NSCLC cells were evident with a slight increase in cytotoxic response when applied in combination with low LET IR. GSK3β is shown to arrest cells at G0/G1 phase [298], which could explain why we had only a slight increase in effect after combination treatment as compared to GSK3β knockdown alone. Irradiation in these p53-null NSCLC cells mainly causes its effect in the G2/M phase of cell cycle. Further analyses of cell cycle distribution in response to GSK3β knockdown would therefore be interesting.

p38MAPK has previously been shown to protect against low LET photon irradiation in NSCLC in part by participating in IGF-1R-mediated signaling [129, 299]. Interestingly, a clear decrease in p38MAPK phosphorylation was evident in response to high LET irradiation of these NSCLC cells suggesting that the kinase plays a critical role in the cytotoxic effect of high LET radiation. Accordingly, when p38MAPK was inhibited by SB203850 in combination with low LET radiation, a decrease in cellular proliferation capacity was evident but with only minor effect on PARP-1 cleavage suggesting a non-apoptotic pathway to contribute to the observed sensitizing effect.

In conclusion, our data suggest that signaling pathways involving GSK3β and p38MAPK are associated with radio resistance in NSCLC cells. Furthermore, global phosphoproteomics analysis is a powerful tool to characterize signaling pathways regulating the response to radiation and to discover new molecular targets that sensitize resistant cells to low LET radiation.

3.3 PAPER III

Massive induction of apoptosis of multicellular tumor spheroids by a novel compound with a calmodulin inhibitor-like mechanism

Monolayer cell cultures are not adequate models for studies of the sensitivity of in vivo tumor tissue to chemotherapeutic drugs. Multicellular spheroids are similar to solid tumors by mimicking a hypoxic treatment resistant core and 3D tumor microenvironment [300, 301]. This study was designed to identify and characterize novel drugs effective on multicellular spheroids

Multicellular spheroids from HCT116 cells, consisting of outer layers of dividing cells and a non-dividing hypoxic core were treated with a small library of drugs pre-selected for biological activity (the Mechanistic set of 827 compounds from the Developmental Therapeutics Program of the US National Cancer Institute). The spheroids were examined for viability by acid phosphatase assay and apoptosis induction using the M30 Apoptosense^R ELISA. The screening revealed the compound NSC647889 to be highly effective on multicellular spheroids, eliciting substantial induction of apoptosis within 6 and 8 hours of treatment. NSC647889 induced massive apoptosis of the outer cellular layers of the spheroids. In contrast, we did not observe apoptosis (activation of caspase-3) in cell populations in the center. We conducted clonogenic assays after treatment in order to measure survival and proliferation capability of all the cells in the spheroids, including the hypoxic core. These experiments showed that NSC647889 was able to reduce clonogenic survival by > 90%, suggesting that the compound was effective on cells in the core of the spheroids as well. These data suggest that the compound is cytotoxic to the hypoxic cells in the core regions, but that these cells do not die by apoptosis. An interesting possibility is that the poor metabolic state of these cells results in non-apoptotic cell death, since ATP is required for apoptosis [119].

Anti-neoplastic activity of the compound has previously been seen on acute lymphoblastic leukaemia, promyelocytic leukemia and chronic myelogenous leukemia cell lines in the NCI60 cell lines panel. Due to the high hydrophobicity of the compound (XLogP 7.3) validation of the effect of the compound was difficult to perform *in vivo* (hydrophobic compounds are difficult to formulate for animal experiments). We treated FaDu xenografts in SCID mice with NSC647889 dissolved in DMSO and checked the examined the levels of human cytokeratins in plasma. Cytokeratins are released from dying epithelial cells and can be used to evaluate cytotoxic effects using mouse plasma [302]. Determination of both cytokeratin 18 and caspase-cleaved cytokeratin 18 can be used to distinguish between apoptosis and necrosis [303]. We found that levels of total and cleaved cytokeratin-18 increased with response to NSC647889, indicating apoptosis to be operational during the response.

In order to characterize the molecular mechanism of action of the compound, we used a bioinformatics based approach, Cmap [304], which matches a compilation of gene expression signatures from drug-treated tumor cell lines to our compound of interest. We found that NSC647889 induces a gene expression profile which is similar to that induced by the calmodulin inhibitor calmidazolium and several other compounds that are described to be calmodulin inhibitors. Based on this hypothesis, we tried to examine the level of calcineurin activity in NSC647889-treated cells. Calcineurin is a calcium-dependent serine-threonine phosphatase, which regulates the activation and binding of calmodulin to calcium (Ca^{2+}) [305] . Interestingly the calcineurin levels increased in response to NSC647889 and contrary to our positive control calmodulin inhibitor W7. This indicated that the mechanism of cell death in response to NSC647889, was not due to inhibition of calmodulin, but the very high levels of calcineurin/calmodulin could be a possible result of higher calcium levels in the cells. Continual increase in intracellular calcium levels has been related to induction of apoptosis in cells during later stages [306]. We then checked the intracellular calcium levels in NSC647889 treated cells and found that the cells treated with NSC647889 had increased levels of intracellular calcium, just after 1 hour of treatment. The calcium levels were increasing with dose and had a direct correlation with the dose dependent apoptosis of NSC647889 treated cells. The apparent discrepancy between the Cmap analysis and increase in calcineurin/calmodulin activity could be explained by the fact that calmodulin inhibitors induce apoptosis by stimulating intracellular calcium influx, which is similar to the mode of action of NSC647889.

In conclusion, we report a novel compound NSC647889, capable of inducing massive apoptosis in 3D tumor spheroids and is also able to induce apoptosis in human tumor xenografts. The compound is comparably more effective than currently used drugs in inducing apoptosis/cell death of spheroids. This compound seems to act by increasing levels of intracellular calcium; however the actual mechanism of action is unknown. The finding that hypoxic regions of spheroids appear resistant to apoptosis could be of importance for studies where the efficacies of various treatment modalities are examined on solid tumors. If apoptosis is used as the single read-out, cell death of hypoxic cells may escape detection

3.4 PAPER IV

Identification of an inhibitor of the ubiquitin–proteasome system that induces accumulation of polyubiquitinated proteins in the absence of blocking of proteasome function

Human tumor cells have an increased level of protein synthesis and thereby require a high level of proteasomal activity to eliminate misfolded and non-essential proteins. This makes the UPS system a viable target for drug treatment in tumor cells. Bortezomib (Velcade®) is a 20S proteasome inhibitor which is approved for treatment of multiple myeloma and mantle cell lymphoma [307]. Here we identify a novel UPS inhibitor with anti-neoplastic activity.

An image based screening of 382 compounds was done for both cytotoxicity and proteasomal inhibition. Cytotoxicity was measured using FMCA method in HCT116 cells treated for 3 days with the compounds. A reporter cell line HEK 293 was used which expresses an ornithine decarboxylase (ODC)-fusion green-fluorescent protein that is rapidly degraded by the active proteasome. We identified a single compound (HRF-3) to induce the accumulation of the ODC-fusion protein at a level of >3 S.D. above that of untreated control. To verify the proteasomal blocking in another reporter cell line, we used a human melanoma cell line, MelJuSo Ub^{G76V}-YFP expressing ubiquitin fused to yellow-fluorescent protein (YFP). We performed live cell imaging of the cells treated with HRF-3 and bortezomib as a positive control. Even though we did find fluorescent signals indicating a possible proteasomal blocking in both the cell lines, the signals were considerably weaker as compared to the 20S inhibitor bortezomib. As described in Paper III, we analyzed the gene expression signature of HRF-3 treated cells using Cmap. The gene expression profile showed significant similarity to the profile of several other known UPS inhibitors, such as MG-262, thiostrepton, 15δ-PGJ2 and withaferin A.

Inhibition of the proteasome generally leads to the accumulation of polyubiquitinated proteins, which can be detected by immunoblotting. We observed an increase in the levels of K48-linked polyubiquitinated protein in response to HRF-3 which was both time and dose dependent. The accumulation of polyubiquitinated proteins was similar to that observed using the 20S inhibitor bortezomib. Co-treatment of HRF-3 and bortezomib did not show any significant increase in the accumulation of K-48 linked polyubiquitinated proteins. Surprisingly, we observed no increase in the Ub^{G76V}-YFP proteasome substrate in the MelJuSo Ub^{G76V}-YFP reporter cell line at the concentrations where polyubiquitin starts to accumulate (i.e. 5μM). Apparently, K-48 linked polyubiquitinated proteins accumulation in the cells despite no or minimal blocking of the proteasome. To ascertain whether or not HRF-3 directly inhibited the 20S subunit we measured the enzymatic activity of the 20S proteasome using Suc-LLVY-AMC. Cytotoxic concentrations of HRF-3 did not inhibit the 20S subunit, whereas very high concentrations of the compound i.e., 50μM did show a weak inhibitory effect. At this concentration we observed proteasomal blocking also *in vivo*.

Bortezomib used as a positive control showed complete inhibition of the 20S proteasome. According to the Cmap analysis HRF-3 did induce expression of several chaperone genes, which is consistent with other proteasomal inhibitors [308]. Nevertheless the 20S inhibitor bortezomib and 19S DUB inhibitor b-AP15 express Hsp70B as the final line of action against proteasomal stress [309, 310] , which was completely absent in the response to HRF-3 at 5-10µM. The absence of Hsp70B induction despite strong accumulation of polyubiquitinated (and presumably misfolded) proteins is quite surprising. These findings suggest that HRF-3 is not a direct inhibitor of the proteasome; rather it inhibits other targets within the UPS.

We then compared HRF-3 with a panel of inhibitors of the UPS. Eeyarestatin-1 (ES-1) is recently described to inhibit p97/VCP-associated DUB activity [311] and NMS859 inhibits the ATPase activity of the same enzyme [312]. The compound piperlongumine inhibits the UPS at levels other than the 20S and 19S subunits of the proteasome [313]. We found that ES-1, piperlongumine and NMS859, similarly to HRF-3, does induce accumulation of polyubiquitinated proteins but do not appear to block proteasome function (at least in terms of stabilization of the Ub^{G76V}-YFP reporter). These data suggest that HRF-3 could have a mechanistic effect at pre- proteasomal steps, similar to ES-1 and NMS859. Induction of oxidative stress has been related to proteasomal inhibition and has been regarded as one of the viable reasons for cytotoxicity of these compounds [309, 310, 314]. In response to HRF-3 we found accumulation of HMOX-1, a gene associated with oxidative stress in a dose dependent manner, which was also the case with all the other UPS inhibitors used for comparison.

Proteasomal inhibitors such as bortezomib and b-AP15 have been seen to be highly effective on myeloma cell lines and leukemia cell lines as compared to other tumor types [292, 315]. This was consistent with the findings of HRF-3 response to tumor cell lines. We also treated primary tumor cells from colon carcinoma, pseudomyxoma peritonei (PMP), acute myelocytic leukemia (AML) and chronic lymphatic leukemia (CLL) patients with HRF-3 and bortezomib and calculated IC_{50} values. Interestingly we found that primary tumor cells have lower IC_{50} values as compared with the established cell lines of the same tumor types, for both HRF-3 and bortezomib.

In summary we present a novel UPS inhibitor that is cytotoxic to tumor cell lines and primary tumor cells from patients, with myeloma and leukemia cell lines being most sensitive. The compound is capable of accumulating polyubiquitinated proteins in the absence of a direct inhibition of the proteasome. It can be projected from the findings that HRF-3 and other similar compounds such as piperlongumine inhibit some pre proteasomal step during the transport or attachment of polyubiquitinated proteins to the proteasome; however the exact mechanism of action of these compounds is not known yet. These results suggest that the UPS is a highly druggable system and further studies on novel compounds targeting several steps of the UPS could be discovered for enhanced treatment of human cancer.

3.5 PAPER V

Development of the proteasome deubiquitinase inhibitor VLX1570 for treatment of multiple myeloma

The compound b-AP15 was recently described as an inhibitor of the proteasome-associated DUBs USP14 and UCHL5 [292]. In this paper we identify and characterize the compound VLX1570 which is an optimized lead based on b-AP15.

Cueing to the success of b-AP15 on multiple myeloma cells both *in vitro* and *in vivo* [293], a number of b-AP15 analogues were synthesized and their cytotoxicity to HCT116 colon carcinoma cells were evaluated. Based on criteria of potency, solubility and the ability to be formulated for future clinical use we selected the compound VLX1570. VLX1570 inhibited the DUB enzymes USP14 and UCHL5 and lead to accumulation of polyubiquitinated proteins similar to b-AP15. The Cmap analysis of VLX1570 showed that it induces a similar gene expression profile as b-AP15. VLX1570 induced similar responses as other UPS inhibitors which included expression of chaperone genes, induction of ER stress and oxidative stress [292, 309, 310]. It has been previously shown that siRNA knockdown of USP14 and UCHL5 in combination leads to increase in accumulation of polyubiquitinated proteins [316]. We here used a small molecule inhibitor of USP14, IU1, which is shown to enhance proteasomal activity and decrease oxidative in vitro [294], in comparison with VLX1570 and the 20S inhibitor bortezomib. We find that IU1 accumulates K-48 linked polyubiquitinated proteins and HMOX-1 in HCT116 cells, however the level of accumulation is not as much as is seen with VLX1570 and bortezomib. We also performed siRNA knockdown experiments of UCHL5 in combination with IU1 treatment. We found no increase in polyubiquitinated proteins in the combination of siRNA UCHL5 and IU1 as compared to siRNA UCHL5 and USP14. These results are currently unexplained and indicate that differences in how USP14 inhibition affect proteasome function by various drugs and by siRNA knock-down.

In order to find off target activities of the compound VLX1570 a kinase panel comprising of 211 individual enzymes were tested for inhibition. It was found that VLX1570 did not show any significant inhibition of kinases except for Cdk4 with only 4% median level of inhibition, suggesting that off-target activities are not severe despite the presence of Michael acceptors. The alpha,beta-unsaturated ketones on b-AP15/VLX1570 are expected to be relatively “soft” electrophiles which react primarily with cysteine thiolates, not expected to occur in most kinases. The hit compound b-AP15 was found to be highly effective on myeloma cells [293] and we found that VLX1570 was more effective in induction of cytotoxicity and apoptosis in myeloma cells compared to b-AP15. Apoptotic response with activation of caspase-3 and PARP cleavage was seen in response to both b-AP15 and VLX1570. We also found increase in phosphorylation of stress activated kinases JNK and p38-MAPK and a decrease in phosphorylation of ERK. It has been previously shown that

pharmacological inhibition of JNK in b-AP15 treated cells leads to a decrease in cell death and phosphorylation of JNK is a downstream effect of oxidative stress induced by b-AP15 [309]. Our observations strongly suggest that apoptosis induction by VLX1570 occurs by the same mechanisms as by b-AP15.

It has been reported that b-AP15 can overcome bortezomib induced drug- resistance in multiple myeloma cells [293]. We examined the response of the myeloma cell line OPM2 and a bortezomib resistant variant of OPM2 to VLX1570 and b-AP15. We found that VLX1570 could indeed reduce cell survival in bortezomib resistant cells, similar to b-AP15 and the response of the resistant cells towards VLX1570 and b-AP15 was similar as its parental cell line. However there was less induction of apoptosis and lower levels of caspase-3 and PARP cleavage in the resistant cell line. The decrease in apoptosis induction was simultaneous with increase in phosphorylation of p38-MAPK and ERK. The resistant cell lines also showed a lower induction of k48 linked polyubiquitinated proteins and Hsp70B.

Next we examined the effect of VLX1570 on two different human multiple myeloma xenograft mouse models. VLX1570 increased survival rates in both these models and the anti-neoplastic activity of the compound was associated with a decrease in phosphorylation of ERK detected by immunohistochemistry. It was previously shown that b-AP15 increased levels of K-48-linked polyubiquitinated proteins in b-AP15-treated mice [293]. We could not reproduce the same effect in VLX1570 treated mice, probably due to different properties of antibody used in the assay. Further investigation is required to identify appropriate pharmacodynamics biomarkers for validation of these kinds of studies.

In summary we present VLX1570, an optimized lead of the previously described compound b-AP15. The compound is highly effective on multiple myeloma *in vitro* and *in vivo* and is under preparation for future clinical trials.

4 CONCLUSIONS AND FUTURE PRESPECTIVES

In paper I, we evaluate the response of tumor cells towards low and high LET radiation which is seen by the clear difference in the overall clonogenicity and survival. Using the RCR model it was found that cell lines with high *b/a* ratio, which corresponds to cells with high DNA repair capability, are more sensitive to high LET damage. It was found that the parameters from the low LET radiation response could predict the high LET radiation response in tumor cells with the RCR model. However the LQ model did not show any correlation between low and high LET response. These results indicate that utmost consideration should be given while using parameters from the LQ model for radiotherapy treatment planning.

In paper II, we show that by comparing the phosphoproteome of a radio-resistant cell line in response to low and high LET and network analysis of the data, various pathways that are responsible for low LET resistance could be predicted. We describe GSK3β to have a role in cell survival and low LET radiation response and confirm the role of p38MAPK in radio-resistance of NSCLC. Specific kinase inhibitors have been used for cancer treatment with several benefits along with potential side effects. However further research is required to identify newer targets to increase the therapeutic efficiency and also confirm their role in cancer therapy.

Hypoxic cells are usually resistant to anti-cancer agents and this phenomenon is one of the most significant limiting factors for treatment of solid tumors. We identify a novel drug that is capable of inducing apoptosis in multicellular tumor spheroids and a xenograft tumor model in paper III. Even though the compound did not induce apoptosis in the core hypoxic regions of the multicellular tumor spheroids the clonogenicity of the cells was reduced to ~1%. This shows that this compound was able to kill the hypoxic cells as well. These data indicate that drug screening assays targeted for solid tumors should be performed on cellular models mimicking the actual tumor such as multicellular tumor spheroids. In absence of energy, the cell death mode might shift towards necrosis instead of apoptosis, so appropriate assays leading to correct detection of cell death type should be used in case of experiments with hypoxic cells. Advancement of solid tumor treatment requires identification of novel drugs that are cytotoxic to hypoxic nutrient deficient cells as well as the proliferative tumor cell population.

The UPS is the principal pathway for protein degradation and inhibitors of this pathway have been effective in the treatment of multiple myeloma. We describe a novel small molecule inhibitor of the UPS, HRF-3, in paper IV. HRF-3 induces the accumulation of polyubiquitinated proteins and a gene expression profile similar to other known UPS inhibitors. The compound is cytotoxic to a number of tumor cell lines and primary patient tumor cells with the strongest activity in myeloma and leukemia cell lines. However at cytotoxic concentrations, the compound has weak accumulation of proteasome substrates and

inhibits the 20S proteasome only at very high concentrations. Our data suggests that HRF-3 is similar to other compounds that inhibit the UPS at pre-proteasomal steps with strong accumulation of ROS. There are a number of novel compounds identified as UPS inhibitors that do not directly inhibit the 20S proteasome. These along with our data suggest that, as the UPS is a multistep process, inhibition of the UPS could be done by targeting several of the components involved. Further investigation is required to identify the exact target of compounds like HRF-3.

In paper V, we characterize the compound VLX1570, an optimized lead of the 19S deubiquitinase inhibitor b-AP15. The compound has similar biochemical and cytotoxic profile as b-AP15 and has shown significant cytotoxicity in multiple myeloma both in vitro and in vivo. An important advantage with VLX1570 is that it is possible to use less toxic excipients when administering these drugs to animals/humans. VLX1570 is marginally more effective than b-AP15 in apoptosis induction and loss of viability in myeloma cell lines. VLX1570 is cytotoxic to bortezomib resistant cell lines, similar to b-AP15. We found that VLX1570 does not show significant inhibitory activity on a protein kinase panel and inhibition of the 19S DUBs USP14 and UCHL5 are specific targets of the compound. However, whether inhibition of these DUBs are the sole reason for cytotoxicity of VLX1570 and b-AP15 or these DUBs regulate other molecular targets leading to the cytotoxicity is presently not known. We conclude that VLX1570 is a strong candidate for clinical management of multiple myeloma and is currently undergoing toxicity testing for future clinical trials.

5 ACKNOWLEDGEMENTS

I would like to extend my deepest gratitude to all those people who have made this dissertation possible. In particular I would like to thank:

My main supervisor **Stig Linder**, for your guidance, encouragement and support. I am immensely thankful for accepting me as your student in very difficult times and bringing me into this wonderful research group. You believed in my ability as a PhD student when no one else did and I would not have imagined finishing this thesis in time without your kind support. Your passion, enthusiasm and dedication for science are truly inspiring. You are so focused yet always full of exciting ideas and have an incredible ability to explain even the most difficult ones in simple terms. Despite your busy schedule, you have always found time to discuss and never hesitated to run into the lab. I am impressed by your optimism, immense patience and consideration for others which motivated me positively and I will always remember working with you. You have provided me with all possible help and support, even when I was not able to express. THANK YOU SO MUCH.

My co-supervisor **Margareta Edgren**, for help and support. You have introduced me to the basics of radiobiology, mathematical modelling and taught me about lab techniques. I am grateful for your willingness to help during the difficult time when there was a lot of uncertainty about the supervisor appointment for my thesis work. I am thankful for the figures and suggestions you have made.

All my co-authors for their contribution to the respective papers, especially, to **Iuliana Toma-Dasu**. My special thanks to you for being a great teacher and invaluable help and discussion for the radiobiological modelling paper.

All present lab members of this excellent research group, for always being helpful and making the lab a great workplace. **Maria**, you have been an inspiration with your organization skills and focus on work. You are always there to look for things, I need. Thank you for showing me around the lab and teaching me about spheroids. **Pádraig D'arcy**, for your suggestions and ideas about the projects. Your immense knowledge and exceptional focus in science is impressive along with your remarkable memory of where things are in the lab. Thank you for everything especially the muffins during lab meeting! **Angelo De Milito**, you are an amazing scientist and I admire your passion for science. Thank you being the defence chairman of my dissertation and for all the chats during bus rides. **Xiaonan**, you bring so much joy and laughter into the lab, thank you for listening when needed and encouraging me. I will miss our philosophical discussions about life☺.

Magda, for your help with siRNA experiments and chats around the lab. You are beautiful☺. **Paola**, for always being helpful and open to talks. **Xin**, for helping out in the lab, during defence application and thesis printing. **Chao**, for your willingness to help. **Ingrid**, thanks for making publication figures and arranging things for the group.

All the past members of our lab. Especially **Angela, Chiara, Maria B, Feli and Francesca**.

Member of the previous groups, I have worked in, thank you everyone for your kind help and support during my studies. Members of MSF: Anders Brahme, Hooshang Nikjoo, Irena Gudowska, Bo Nilsson, Pedro Andreo, Karen Belkić, Dževad Belkić, Thiansin, Reza, Martha Hultqvist, Kristin, Johanna, Björn, Patrick, Eleftheria, Marta, Laura, Minna, Lucilio, Tommy, Nico and Mariann And members of KBC: Rolf Lewensohn, Kristina Viktorsson, Birgitta Mörk, Christina von Gertten, Marianne Langéen, Petra, Lovisa, Dali, Hogir, Katarzyna, Ghazal and Therese. Thank you for everything. Special thanks to **Lil**, your smile and patient attitude has made me feel welcome always.

Administrative staff **Erika**, **Susanne**, **Sören**, **Monica** and **Eva-Lena** for always providing the required help and service.

Manjit Dosanjh, for your diligent efforts in creating a multi-disciplinary scientific network. I am privileged to be a part of the PARTNER network, for thankfully providing me the resources to travel to new places for scientific discussions. I thank all my friends from the PARTNER network, for being so cheerful and making the events successful. Hopefully we will have the chance to meet again. Special thanks to **Katarzyna** and **Marta** for all the travels and experiences together.

Everyone at the National Institute of Radiological Sciences (NIRS), Japan, for making my stay enjoyable in a foreign land. Especially to **Murakami Takeshi**, for providing me the opportunity to work at the international open laboratory (IOL). I would like to extend my gratitude to **Yoshiya Furusawa**, **Ryoichi Hirayama**, **Yoshitaka Matsumoto** and **Sadayuki Ban** for help with the lab and everything else.

My friends outside of work in Sweden and India. To our girls gang, **Kaberi**, **Ashwini**, **Reshu** and **Gargi**, we are always together having fun and laughing at our own jokes☺. **Rini**, you are so younger than me, but I still get inspired by you. Wish you all the best for your future. **Lipsa** and **Ashutosh**, you guys are a great couple and I have so much fun with you. My friends back in India, **Prativa** and **Alok**, thank you for encouraging me and always

being there to listen to my ramblings. All my friends those names I do not mention here, I sincerely thank you.

My dear friends **Lagnajita** and **Rajat** and my darling **Kuhu**, we have had some great times together and I can't image how we are living so far away. I miss you.

My family including my in-laws, for your unconditional love, care and support. **Mama**, for teaching me the importance of freedom and making me confident. **Baba**, for always loving and understanding me and my little brother **Anu**, for all the crazy times. I am lucky and proud to be your sister. **Dulu da** for always caring and inspiring me.

My beautiful daughter **Lisu**, for being so forgiving and making every day brighter than before. I LOVE YOU.

And finally, my husband, **Manoj** for being the hero of my life. With your never-ending love, courage and compassion, you have guided me through the roughest period of my life. Thank you for inspiring me, making me understand the value of education and pushing me but never letting me fall. You have tolerated me when I myself would not have. It is beyond words to express my gratitude for what you have done for me. Rightfully this thesis is dedicated to **you**.

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