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ANALYSIS AND CHARACTERIZATION OF CHEMO- AND RADIATION THERAPY SENSITIZING STRATEGIES IN TUMOURS WITH FOCUS ON EFFECTS OF PHENOTHIAZINES ON DNA DAMAGE RESPONSE SIGNALLING

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Analysis and characterization of chemo- and radiation therapy sensitizing strategies in tumours with focus on effects of phenothiazines on DNA damage response signalling

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To My Beloved Ones

ABSTRACT

Cancer is, despite rapid progress in development of new targeted therapies, still mainly treated with surgery, chemotherapy and/or radiation therapy. The last two mentioned conventional therapies often meet general obstacles such as normal cell toxicity and tumour cell resistance. Further efforts are, hence, required to increase efficacy of both radiotherapy and chemotherapy in order to increase patients survival. In this thesis, we analysed and characterised strategies to improve chemotherapy and radiation therapy sensitivity in tumours, with focus on lung cancer.

Particle radiation therapy offers an opportunity to overcome tumours' resistance to conventional photon irradiation. Unfortunately, treatment planning systems describing parameters for ion irradiations, used in particle therapy centres worldwide, are still based on the survival data from conventional radiation. In **Paper I**, we used two mathematical models, the linear-quadratic (LQ) and the repairable-conditionally repairable damage (RCR), to compare the effects of high LET accelerated ions with those elicited by conventional low LET photons, in different types of tumour cells. We show that the data on response to low LET irradiation can be used to create models that can predict cellular response to high LET with radiobiological parameters assessed with the RCR model. Moreover, results achieved with the RCR - but not with LQ model, suggest that tumour cells with high DNA repair capacity can benefit from radiation therapy with accelerated particles.

Another cancer treatment strategy presented in this thesis is focused on the use of phenothiazines, alone or as sensitizers to chemotherapy. Phenothiazines are drugs clinically used for psychiatric disorders, but which also have been shown to possess cytotoxic activity in various regimens and tumours. In **Paper II**, we uncovered that monotherapy with phenothiazines caused decreased cell viability and cell death of small cell lung cancer (SCLC) cells. Furthermore, we showed that lysosomal dysfunction induced by phenothiazines was responsible for the observed higher responsiveness of SCLC cells. Our studies presented a new context of use and activity of phenothiazines in tumour cells and allow for a potential treatment opportunity for SCLC. In **Paper III**, we showed that phenothiazines interfere with DNA damage response (DDR) machinery in tumour cells by inhibition of one of the DNA double strand breaks (DBSs) repair pathways - the non-homologous end joining (NHEJ). We demonstrated that this phenothiazine-mediated inhibition of DNA repair was associated with increased chromatin-centred DNA-PK/ATM signalling, resulting in augmented substrate phosphorylation and protracted checkpoint arrest. This novel tumour cell selective feature of phenothiazines preferentially caused chemosensitization to genotoxic agents that induce DNA DSBs, but also opens up for possible combinations with DNA repair inhibitors. In **Paper IV**, *in silico* gene expression analysis suggested similarities in mode of action between phenothiazines and epigenetic signalling modulators. Accordingly, we showed that phenothiazines can be used to treat SCLC or the epigenetically deregulated tumour cells, neuroblastoma (NB) and acute myeloid leukemia (AML), either alone or in combination with chromatin-modifying drugs. The cytotoxicity, cell death signalling and hyperactivation of DNA repair signals observed with phenothiazines in tumour cells, were comparable in magnitude with the effect of the chromatin-modifying drugs, pan-HDACi panobinostat or the BRD4 antagonist JQ1. Moreover, the model phenothiazine compound trifluoperazine, TFP, was also found to prolong phosphorylation of DNA-PKcs in chromatin fractions of SCLC cells. The tandem treatment with TFP and either panobinostat or JQ1 was also demonstrated to increase cytotoxicity and triggered both apoptotic and autophagic cell death signalling in SCLC and NB cells. Thus, our findings in **Paper IV** suggest a novel therapeutic utility of phenothiazines and chromatin-modifying drugs in cancer therapy.

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 Research 2014; 34: 2801-2806*
* equal contribution

II. Zong D., **Zielinska-Chomej K.**, Juntti T., Mörk B., Lewensohn R., Hååg P., Viktorsson K. Harnessing the Lysosome-Dependent Antitumor Activity by Phenothiazines in Human Small Cell Lung Cancer. *Cell Death and Disease 2014; 5, e1111; doi:10.1038/cddis.2014.56*

III. Zong D., **Zielinska-Chomej K.**, Hååg P., Yakymovych I., Lewensohn R., Viktorsson K. Phenothiazines Trigger Hyperactivation of Chromatin-Associated DNA-PK and ATM Signaling and Offer a Novel Approach to Chemotherapy Sensitization. *Manuscript*

IV. **Zielinska-Chomej K.**, Zong D., Lewensohn R., Viktorsson K., Hååg P. Phenothiazines Modulate Chromatin-Related Epigenetic Processes in Tumour DNA Damage Response and Offer a Novel Strategy for Cancer Therapy. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ALK	anaplastic lymphoma kinase
AML	acute myeloid leukemia
ATM	ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BRD4	bromodomain-containing protein 4
CaM	calmodulin
CFSE	carboxyfluorescein diacetate N-succinimidyl ester (assay)
Cmap	Connectivity Map
CNS	central nervous system
CPZ	chlorpromazine hydrochloride
D2R	dopamine receptor D2
DDR	DNA damage response
DNA DSB	DNA double strand break
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-PK catalytic subunit
EGFR	epidermal growth factor receptor
HDAC	histone deacetylase(s)
HDACi	histone deacetylase inhibitor(s)
HR	homologous recombination (repair)
ICLs	(DNA) interstrand crosslinks
keV/μm	kiloelectron volt per micrometer
LC	lung cancer
LC3-II	light chain 3
LEM	local effect model
LET	linear energy transfer
LQ	linear-quadratic model
MKM	microdosimetric kinetic model
MOMP	mitochondrial outer membrane permeabilization
MRN complex	Mre11/Rad51/Nbs1 complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (assay)
NB	neuroblastoma
NHEJ	non-homologous end joining (repair)
NSCLC	non-small cell lung cancer
PARP-1	Poly(ADP-ribose) polymerase-1

PARPi	PARP inhibitor
PE	plating efficiency
PIKKs	phosphatidylinositol 3'-kinase-related protein kinases
RBE	relative biological effectiveness
RCR	repairable–conditionally repairable model
RT	radiation therapy
SAHA	suberoylanilide hydroxamic acid, vorinostat
SCLC	small cell lung cancer
SOBP	spread-out Bragg peak
TFP	trifluoperazine dihydrochloride
TMRE	tetramethylrhodamine ethyl ester perchlorate (assay)
TPS	treatment planning system
TSA	trichostatin A
Å	Ångström, a unit of length equal to 10−10 m (one ten-billionth of a metre) or 0.1 nm

1 INTRODUCTION

1.1 CANCER AND NEED FOR NOVEL THERAPEUTIC STRATEGIES

Cancer is a complex disease for which there is an urgent need for improving therapy approaches allowing the treatment outcomes to be better. The main reason of the increasing incidence of cancer is the aging of the worldwide population. Moreover, there are risk factors i.e. smoking, overweight, lack of physical activity, infections associated with modern civilisation's lifestyle and economic development [1]. According to global cancer statistics, it is roughly estimated that 'about 14.1 million new cancer cases and 8.2 million deaths occurred in 2012 worldwide' [1, 2]. According to a recently published report from the Swedish Cancer Society (Cancerfondsrapporten 2014), around 57.000 new cases were registered in 2012 and around 22.000 cancer deaths recorded in the same year in Sweden [3].

Lung cancer (LC) is the main tumour type in focus of this thesis and is also the main cause of cancer associated death among males, and has been even superior to breast cancer incidence in females [1]. For LC the primary and main risk factor is the use of tobacco.

LC has traditionally been classified according to histology and immunohistochemical characteristics into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC can be further divided into the histopathologic subtypes: lung adenocarcinoma, lung squamous cell carcinoma and large cell carcinoma [4, 5], respectively. NSCLC is often found to be intrinsically resistant to both chemo- and radiation therapy (RT). The treatment procedure of NSCLC are guided by the stage of the tumour disease and for early stage disease (which constitute about 20% of all LC patients) surgery is the main procedure [6]. For more advanced stages of disease or inoperable tumours, RT with or without chemotherapy (i.e. mainly used cisplatin, carboplatin, paclitaxel, pemetrexed) still is the main treatment option, although for EGFR mutated cases and cases with EML4-ALK translocation targeted agents are approved for treatment [7]. In non-resectable, locally advanced LC as well as in cases with metastatic disease (around 50% of all diagnosed LC cases) chemotherapy remains the only available treatment option [5, 7]. SCLC accounts for approximately 14% of all diagnosed LC and displays neuroendocrine features. SCLC is, in contrast to NSCLC, very responsive to first-line treatment with 20 – 40% of all cases showing complete remissions on chemotherapy alone or in combination with RT and with about 50% of the SCLC patients with limited disease demonstrating complete remission after the same treatment [8, 9].

Genomic analysis of LC has demonstrated that the majority of the cases harbor high levels of somatic mutations, alterations in copy number and chromosomal rearrangements [10]. Some of these genetic analyses have, in combination with histomorphological and immunohistochemical parameters evaluation, been started to be implemented in clinical routines to better define relevant clinical (sub)groups of NSCLC patients and to enable a personalized cancer medicine approach [5]. For instance, testing for the status of the epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) rearrangement in adenocarcinomas, has been incorporated into regular treatment procedures,

allowing for targeted therapies towards these aberrations to be applied. Nevertheless, these two alterations are only found in about 10-15% of all adenocarcinoma NSCLC cases, illustrating that ways of improving chemotherapy/RT-responsiveness still are of great importance to make therapy response of NSCLC patients more effective. Albeit high mutation rate and other genetic aberrations also have been revealed in SCLC e.g. PTEN loss mutations, FGFR1 and SOX2 amplification [11-14], it has not yet resulted in molecular targeted approaches for this disease in the clinical settings [15]. Hence, ways to improve chemotherapy response of SCLC are still urgently needed, especially for the therapy of refractory cases where the second line treatment options are limited.

Although, we possess knowledge about molecular alterations within human cells that lead to the malignant transformation, so called "hallmarks of cancer" [16, 17], and information and concepts about cancer's origin are available, relatively little progress has been made in exploiting etiology and mechanisms of disease, to prevent the cancer disease and, equally important, to treat cancer. Most of the anti-cancer therapies which still constitute the main treatment options i.e. chemotherapy, were in fact developed decades ago, when the development of therapeutics was not yet supported and driven by detailed knowledge of the genetic, molecular and biochemical, cellular mechanisms of cancer pathogenesis [18]. More recent cancer therapy approaches, like small-molecular-weight drugs or monoclonal antibodies targeting aberrant growth factor receptor signalling and gene therapy strategies including viral vectors, have been developed with the aim to target cancer pathogenesis, but still only few percent of these new solutions have so far come from the lab bench to the bedside [19, 20].

Although, the big effort on developing new targeted therapies, still the conventional ways of treating cancer play a key role in the clinic. Thus, surgery is still main treatment of choice, whenever possible, as it has the highest chance for complete cure. If surgery is not an option, chemotherapy and/or RT are considered. Ionising radiation and most conventional chemotherapeutic agents cause DNA damage and have more severe effect on rapidly proliferating cells, yet neither of these treatment modalities can distinguish between tumour and normal cells, resulting in significant normal tissue toxicity. Therefore, the development of new strategies, which can be more accurate in treatment delivery or dose delivery in case of RT, and which selectively can sensitise tumour cells to enhance the efficacy of chemo- or radiotherapy, is still needed.

1.2 RADIATION THERAPY

Radiation therapy of tumours goes back to the 19th century, when the ionising phenomenon was reported, and at a point when technical solutions for delivery of such treatment, i.e. linear accelerators, synchrotrons and cyclotrons, were revealed. At this point radiobiology as a discipline describing effects of ionising radiation on living organisms was also initiated [21, 22].

When energy from radiation is absorbed by biological material, excitation results in higher energy level of electron. If radiation with its energy ejects orbital electrons from the molecule, it results in ionization and ionising radiation can be electromagnetic i.e. x-rays or γ-rays, or particulate i.e. protons, α-particles, charged ions [21, 22]. In this thesis effects of both electromagnetic and the charged particle radiation qualities have been studied and some fundamental differences among them are therefore discussed below.

One fundamental difference between x-rays or γ-rays and charged particles, is the energy decrease within tissue, resulting in different dose delivery. Thus, when a tissue is irradiated with x-rays or γ-rays, the energy and thereby delivered dose decrease with the depth of the tissue, whereas charged particles deposit only little amount of energy on the surface of the tissue and, instead, increase energy deposition with depth, reaching the maximum of energy release in region described as the Bragg peak (**Figure 1**). The narrow energy disposition range for particles is taken as an advantage in tumour treatment where the Bragg peak is placed over the tumour in order to adjust the energy to be delivered to the tumour with the best possible dose localization and to save the surrounding healthy tissue. Moreover, beams of different energy can be broaden for better energy distribution and dose deposition into the tumour region by an approach called Spread-Out Bragg Peak (SOBP) (**Figure 1**) [23-27]. Thus, analyses of Bragg curve characteristics of particles have shown that particle therapy can provide excellent dose distributions in tumours of patients [26, 28, 29].

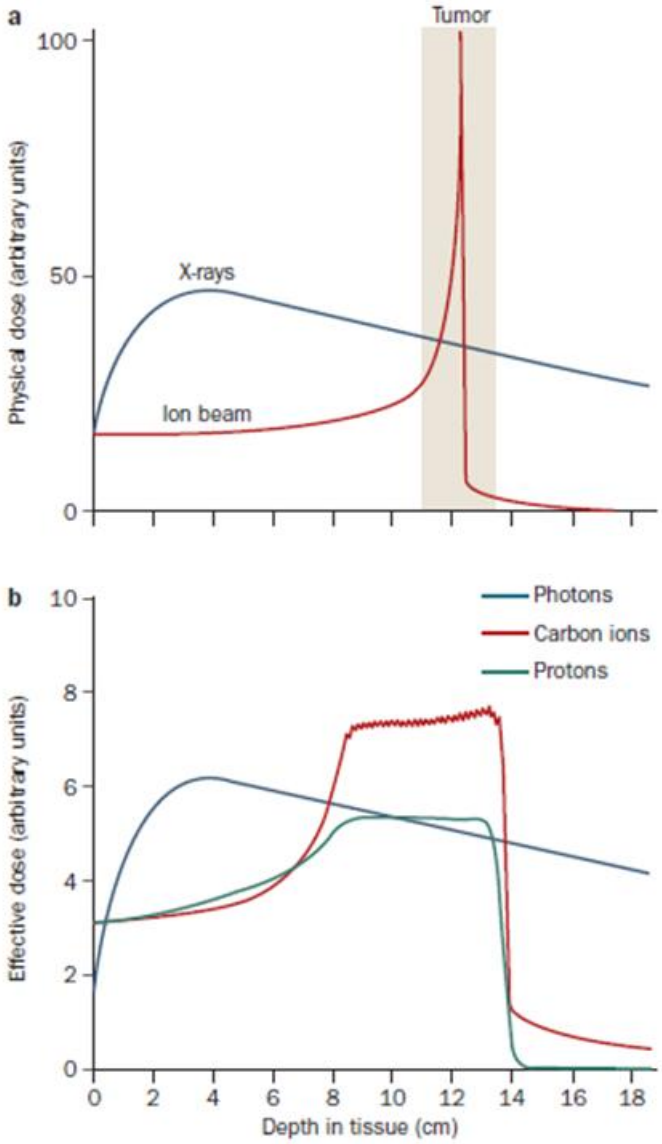

Figure 1. Comparison of the depth-dose relationships for the x-rays and high-energy charged particles *(adapted from Durante M. et al. 2010 [26] with permission from the publisher)*.

1.2.1 Characteristics of direct and indirect action of radiation and concept of the Linear Energy Transfer (LET)

The action mechanism of ionising radiation can briefly been categorized to be a consequence of indirect or direct ionization (**Figure 2**). Indirectly ionising x-rays of γ-rays electromagnetic radiation do not interact directly with the matter and do not cause damage by themselves, but giving up their energy to fast moving charged particles which cause damage while going through the target e.g. DNA [21, 22]. This type of ionization is typical for co called sparsely ionising radiation, where ionising events are separated in space. Moreover, indirect action of radiation, which for instance cause free radicals production, which while interacting the molecules cause damage (**Figure 2**), is the reason for about 70% of DNA damage as an effect of low LET radiation [21].

Direct ionization is a typical mechanism of action of charged particles, whose kinetic energy is high enough to cause chemical modifications and, hence, biological damage directly, when

passing through molecules such as DNA, and is described as densely ionising radiation consisting of ionization tracks [21, 22]. When interacting with the target e.g. DNA, atoms can be ionised/excited, which starts cascade of biological changes in response to radiation (**Figure 2**). Such direct action of radiation is typical for high LET radiation, resulting in clustered damage (with multiple damage sites) [30].

For description of particulate radiation the linear energy transfer (LET) is used to define 'the average energy deposited/transferred per unit length of a track with the special unit - kiloelectron volt per micrometer (keV/µm) of unit density material' [21]. Low LET, sparsely ionising radiation, consists of photons generated by natural decay of radioactive isotopes such as ^{60}Co and ^{137}Cs as well as x-rays with very low dose per single track, and its commonly used in conventional radiation therapy. High LET, densely ionising radiation is characterised by high dose per single track and is typical for e.g. ions, also used therapeutically in charged particle therapy centres [24, 27, 31-37].

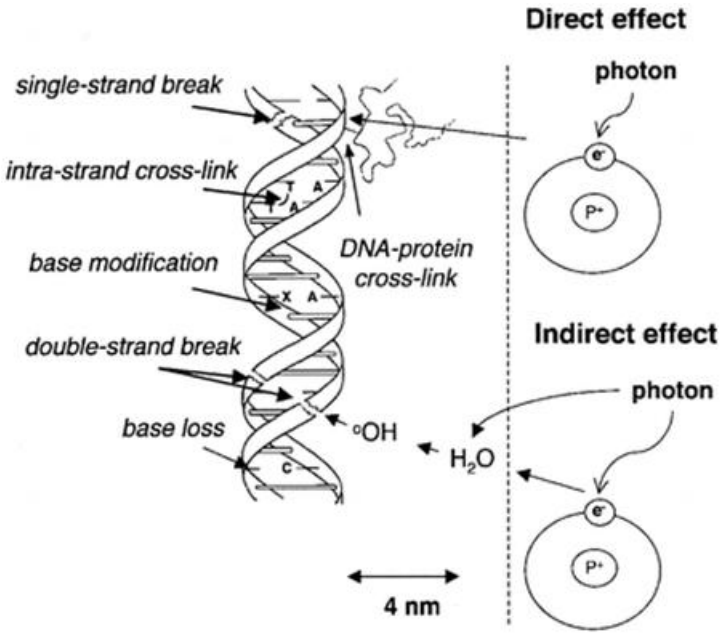

Figure 2. Direct and indirect actions of radiation (*adapted from Pouget JP. et al. 2001 [30], with permission from the publisher*).

1.2.2 Relative Biological Effectiveness (RBE)

Comparison between different radiation modalities cannot be based on the absorbed dose with gray or rad as unit (a physical quantity describing the amount, quantity of radiation as the energy absorbed per unit mass of tissue), as different types of radiation, even with the equal doses, will cause various biological effect because of differences in the pattern of energy deposition. To define these differences another parameter was introduced, already in 1954, to describe the efficiency of low and high LET radiation in context of their capacity for cell killing or cell survival decrease - relative biological effectiveness (RBE). RBE is defined as 'the ratio between the dose of reference radiation (usually 250 kV x-rays as standard) and the dose of test radiation required to produce the same biological effect' [21, 22, 38]:

$$RBE = \frac{Dose\ of\ reference\ radiation}{Dose\ of\ test\ radiation}$$

RBE is a complex parameter which depends on different factors i.e. types of particles ('for carbon ions the RBE increases with increasing depth of the tissue' [26, 38]), radiation quality (LET), cell or tissue type, dose, number of dose fractions, dose rate and oxygen status [24, 39], but also chosen biological system or end point influence the RBE values. Thus, tissues which accumulate and repair the most of sublethal damage will have high RBE values, for others, which do not possess such ability, the RBE values are low [38].

The relation between two main parameters i.e. LET and RBE, describing different radiation qualities is presented by cell survival curves obtained in clonogenic survival assay (see section 1.2.3, **Figure 3**). As the curve becomes steeper with an increase of the LET and the shoulder of the curve becomes smaller, accordingly with the RBE, which also changes as a function of LET [40, 41]. With an increasing LET, the RBE first increases slowly, then more rapidly to reach a maximum at LET about 100 keV/µm [40, 41]. Above this LET value, the RBE values decrease again, which has important implications for the choice of LET for use in particle therapy. Thus, the RBE reaches its peak for radiation with LET of about 100 keV/µm, and this relation between LET and RBE is almost the same for a broad range of mammalian cells, but also for mutation or for cell killing as the end points in the RBE assessment [42]. This relation is taken into consideration for dose planning in the medical practice, as at such ionization tracks density, the separation between ionising events is almost equal the diameter of the DNA double helix (20 Å or 2 nm). In consequence, such radiation has the highest probability and efficiency of causing DNA double strand break by a single charged particle track of radiation.

RBE is usually calculated as the ratio of doses at a specific survival fraction e.g. D_{10} dose, as in **Paper I**, which is required to inhibit tumour growth with 90%, thus, RBE differs between different survival levels. Another calculation method taking into account the whole survival curve is the mean inactivation dose (D_{bar} or $\overline{D}$), also considered in **Paper I** [43]. This factor better characterises intrinsic radiosensitivity and has accordingly been shown to have more accurate correlation when comparing experimental results and being more consistent with radiosensitivity of various tumour types in the clinical settings [43].

1.2.3 Clonogenic cell survival and mathematical models of radiation effects

In 1956 the first publication by Puck and Markus on the survival response of human cancer cells cultured *in vitro* and exposed to radiation, provided a valuable tool for radiobiological studies, the clonogenic survival assay [44]. Since then clonogenic assays have been the golden standard method to describe radiosenstivity of a given cell type. In this assay the capability of a single cell to grow into colony of at least 50 cells, is taken as a proof of retained reproductive integrity and ability to continue to proliferate, so called clonogenicity [45]. Thus, a cell survival curve describes the relationship between the radiation dose (a linear

scale), and the proportion of cells that survive - surviving fraction (a logarithmic scale) (**Figure 3**).

Several theories have been developed to explain the shape of the survival curve resulting in different biophysical models, of which the **linear-quadratic (LQ) model** is still the most universal repair model. This model and others, such as the repairable-conditionally repairable damage (RCR) model [46] (applied in **Paper I**), but also the microdosimetric kinetic model (MKM) [47], and the local effect model (LEM) [48] (both based on LQ), are experimentally evaluated or used in treatment planning systems (TPS) of RT for tumour control probability and clinical response modelling, involving both low and high LET RT facilities worldwide [49, 50]. In this thesis, clonogenic survial assay alongside with two repair models, RCR and LQ, were used in **Paper I**, to describe the response of tumour cells to conventional RT and different accelerated particles/ions and are further described in depth below.

1.2.3.1 The linear-quadratic (LQ) model

The linear-quadratic (LQ) model which is the most commonly used model, is based on the assumption that there are 'two components contributing to radiation-induced cell killing, one which is proportional to dose and one which is proportional to the square of the dose' [21]. The DNA is a critical target for RT to be damaged and LQ model assumes that at least one DNA double strand break is required in order to lose clonogenic capacity of a cell. The expression for the cell survival (S) curve described by the LQ model is as follows:

$$S = e^{-\alpha D - \beta D^2}$$

where '(S) is the fraction of cells surviving a dose (D), α is a linear component of cell killing proportional to the dose, and β is a quadratic component of cell killing proportional to the square of the dose' (**Figure 3**). The initial slope of the survival curve is determined by α and bend(ing) of the curve at higher doses depends on the quadratic component of cell killing β. Parameters from the LQ model are used to describe the innate radiosensitivity of a given cell types, i.e. the dose of radiation which is able to cause cell inactivation. For that purpose the α/β ratio is used as it is equal a dose at which the linear and quadratic components of cell killing are equal (**Figure 3**) [21, 22, 51-53]. During the last decades, the linear-quadratic (LQ) model has been used extensively, both for cell survival modelling and for estimating the effects of changes in the fractionation schedule with doses near the standard clinical dose of 2 Gy per fraction.

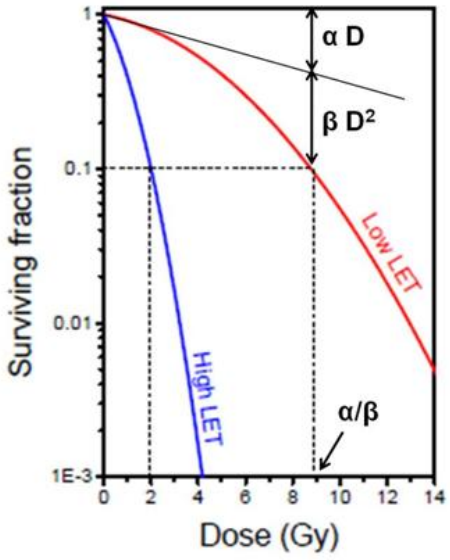

Figure 3. Clonogenic survival curve for cells irradiated with low LET radiation fitted with the linear-quadratic (LQ) model (red) (*Hall EJ. et al. 2006 [21], modified*).

1.2.3.2 The repairable-conditionally repairable damage (RCR) model

Albeit often used, the LQ model has its limitations as pointed out by Wouters BG. et al., i.e. it does not appropriately describe cell survival at high doses, due to the used quadratic dose term [54]. Moreover, the LQ model does not account for low-dose hypersensitivity [54]. Other cell survival models have therefore been developed, of which the repairable–conditionally repairable (RCR) model [46] is one which also was applied in this thesis. As described by Lind B. et al. [46], in the RCR model distinguish is made: 'between two different types of damage: the potentially repairable, possibly also may be lethal if unrepaired or not repaired correctly, and the conditionally repairable, which may be repaired or may lead to cell death if not repaired correctly' [46]. According to Lind B. et al. [46], the RCR model is based on the following assumptions: 'the total amount of initially induced damage is proportional to the absorbed dose (D) (at low to medium doses), the total amount of damage can be divided into potentially repairable (PR) and conditionally repairable (CR) damage and only the potentially repairable damage can trigger activation of the cellular DNA repair system'. The cell survival (S) at a given dose (D) described by the RCR model is as follows:

$$S = e^{-aD} + bD^{-cD}$$

where a, b and c are the parameters of the model. As outlined by Lind B. et al.: 'the first exponential term of the model, describes the fraction of cells that have not been damaged by radiation and, hence, the parameter 'a' gives the initial mean number of damage events per unit dose. The second term of the model, describes the fraction of cells that have been damaged and subsequently, correctly repaired and, thus, represents survival after complete repair of sublethal damage. The parameter 'b' describes the maximum amount of damage that can be repaired per unit dose and the 'c' parameter is related to the probability that a potentially repairable event is actually repaired' [46].

It has been proposed that the resulting RCR expression of cell survival has 'the unique ability to fit experimental data over the entire dose range i.e. low doses within the initial hypersensitive range, intermediate doses found on the shoulder of the survival curve, and high doses situated on the quasi-exponential region of the survival curve' [46]. It has been also shown that the RCR model seems to be more appropriate for hypofractionated stereotactic irradiation than the LQ model, as more biological parameters are taken into consideration in the RCR model than in the standard LQ model [55].

1.3 DNA DAMAGE RESPONSE (DDR)

Every day each cell of the human body is exposed to tens of thousands of DNA lesions. Such amount of DNA damage, their recognition and repair processes influence cellular processes by inhibition of the progression of the cell cycle, replication or transcription. When DNA damage are not correctly repaired or left unrepaired, they lead to establishment of mutations in the DNA sequence of the cell or may even cause more serious genomic aberrations, such as deletions, translocations, aneuploidy, resulting in genomic instability, which is dangerous for the cell and the whole organism and may also increase risk of cancer [56-58]. Some DNA damage appear as a consequence of physiological processes e.g. DNA replication, hydrolytic or non-enzymatic reactions or reactive oxygen species (ROS) formation by oxidative respiration, products of lipid peroxidation or by macrophages or neutrophils during infections and inflammation [59]. Moreover, DNA damage may also be a result of environmental agents such as physical factors i.e. ultraviolet (UV) light, ionising radiation generated during radioactive compound decay or in therapeutic settings of tumours, but also after exposure to chemical factors i.e. cancer-causing DNA-damaging chemicals such as those found in cigarette smoke or aflatoxins in contaminated food [57]. Both endogenous processes and exogenous factors which attack DNA, lead to formation of diverse DNA damage, such as base modifications or loss, DNA interstrand crosslinks or DNA single or double strand breaks (DNA SSBs and DSBs). These diverse DNA damage can all lead to alteration in the DNA sequence and, hence, DNA rearrangements and/or loss of genetic information and may therefore cause genomic instability [56, 60]. The cellular response to DNA damage includes inhibition of the cell cycle, which allow for repair of the damage, or lead to induction of cell death if the damage cannot be correctly repaired. Importantly, inappropriate DNA repair may cause cellular transformation which in the whole organism can result in tumour formation, premature ageing or inherited defects [59].

In order to counteract these DNA damage and maintain genomic integrity cells developed several defence mechanisms called the **DNA damage response (DDR)** (**Figure 4**), and which consist of multiple signalling networks [61]. These networks comprise sensors for detection of DNA lesions, signal tranducers which transmit information of the presence of DNA damage, and downstream effector molecules which mediate cell cycle arrest, localised chromatin remodelling and promotion of the DNA repair (**Figure 4**) [62-67]. Thus, if the DNA damage is correctly repaired, DDR signalling is inactivated, cell cycle restarts and cell survives. When DNA lesions are not correctly repaired or cannot be eradicated, persistent

DDR signalling cause cell inactivation by either death (apoptosis) or by senescence – a form of permanent cell cycle blockade, which both have antitumour potential [57, 59, 68, 69].

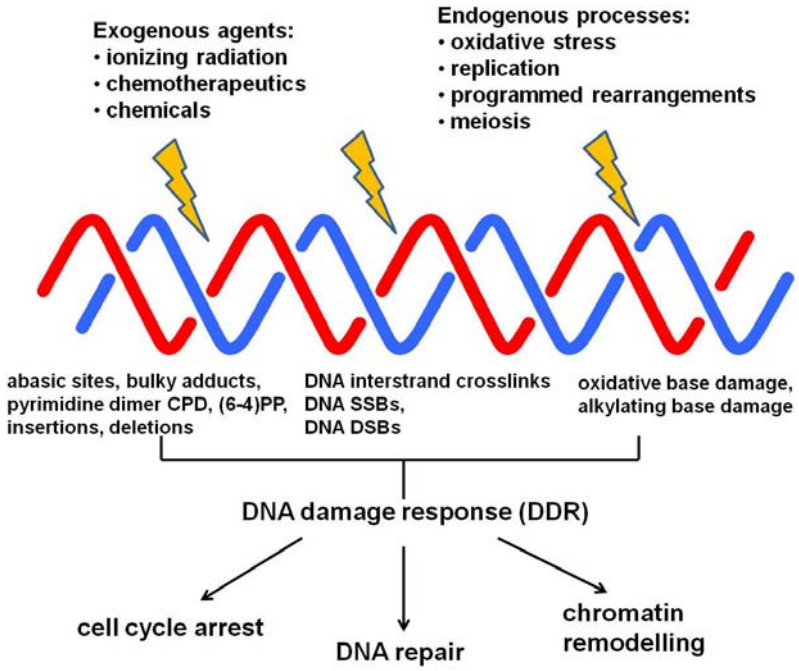

Figure 4. Overview of different DNA damage and the cellular DNA damage response (DDR) network.

1.3.1 Detection of DNA damage, cell cycle arrest and chromatin remodelling

The principle of DDR is a triage of molecules that sense (sensors), transduce (signal transducers) and effect (effectors) the cellular response to DNA damage (**Figure 4**) [56, 63]. Eukaryotic cells developed a detection system of DNA lesions consisting of multiprotein complexes (sensors) which, dependent on the type of damage induced, are recruited to the DNA damage site. For DNA DSB the sensors includes Ku (Ku70/Ku80) complex, MRN (Mre11/Rad51/Nbs1) complex, Rad52 and PARP-1 (poly(ADP-ribose) polymerase 1) [70-74]. In turn these sensors activate the transducers, which in relation to DNA DSBs are ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related), but also DNA-PK (DNA-dependent protein kinase), all which belong to the PIKKs (phosphatidylinositol 3'-kinase-like protein kinases) family [75-79]. In mammalian cells the key DDR signalling components, ATM/ATR/DNA-PK, are activated by replication protein A (RPA)-coated single strand DNA and DSBs [80-82]. This causes the stalled replication forks and DNA single strand breaks are formed, which activate ATR. ATM is a main mediator of the repair of DNA DSBs [75, 82-85]. The checkpoint serine/threonine kinases Chk1 and Chk2 are targets of ATM and ATR. When they are activated, these kinases reduce cyclin-dependent kinase (Cdk) activity, phosphorylate a number of substrates, which in turn initiate a cascade of reactions resulting in block of cell cycle progression allowing the DNA repair to take place (**Figure 5**) [81, 86-89]. Thus, inhibited Cdks cause cell cycle arrest at the G1-S, intra S, or in G2-M phases, allowing DNA repair of inflicted damage to be completed before replication or prior to mitotic division (**Figure 5**) [90-92].

When activated, Chk1 also phosphorylates serine residues on the protein phosphatase Cdc25a, leading to its destabilization and recognition by ubiquitin ligases. In turn the ubiquitination marks Cdc25a for proteolytic degradation via the proteasome, thus, limiting its capacity for cell cycle progression through the S phase, causing a p53-independent S phase arrest (**Figure 5**) [93]. In the G2 phase, inhibition of cell cycle results from Cdc25c phosphorylation by Chk1, which also prevents dephosphorylation and activation of Cdk1.

The G2-M checkpoint suppresses the promitotic activity of cyclin B/cdc2 and block the mitosis entry preventing possible chromosomal aberrations when DNA lesions remain unrepaired (**Figure 5**) [86, 94, 95].

Activation of Chk2 is primarily triggered in response to single strand breaks (SSBs), formed either endogenously during replication stress, or in response to exogenous agents such chemotherapeutics. The effects of activated Chk2 on the effector proteins, Cdc25a, Cdc25c, and p53, are similar to the ones described for Chk1 [93, 96, 97]. Even if the downstream pathways seem to be similar for Chk1 and Chk2, they have distinct roles in response to DNA damage. Thus, the phenotypic effects of the pharmacological modulation of Chk1 or Chk2 gives different outcomes and both have been shown to be useful therapeutic targets to enhance DNA damage-induced cytotoxicity in tumour cells [98-100].

ATM/ATR signalling enhances cellular DNA repair in multiple ways: (i) by inducing DNA repair protein expression, (ii) by promoting their recruitment to the DNA damage site, or (iii) by altering their functionality via post-translational modifications (PTMs), i.e. phosphorylation, acetylation, methylation, ubiquitination, SUMOylation and ADP-ribosylation [101, 102]. Activation of ATM, the key DDR factor, results in chromatin relaxation at DSBs sites [103]. Increasing evidence show the significant impact of chromatin structure on DDR and also that chromatin structure is modulated after DNA damage [104-107]. Hence, the organization of chromatin itself, its DNA damage-induced modifications, chromatin movement, alterations in structure surrounding the break sites as well as mobility of DNA DSBs, are all reported to influence repair processes and repair kinetics [108-111]. So far, the best characterised example of a chromatin-based DNA damage signalling is the phosphorylation of histone H2A variant, H2AX, on serine 139 by either of the following kinases in the chromatin - ATM, ATR and DNA-PK, in close vicinity to the DNA DSB sites [112-116]. This also triggers accumulation of DDR factors and also other chromatin-modifying components, apart from γH2AX, like BRG1, which together promote DNA DSBs repair and augment DDR signalling [102, 117-120]. It is also reported that chromatin remodeling takes place in a non-DSB position dependent manner [105, 112], during DNA DSB repair processes by homologous recombination (HR) or non-homologous end joining (NHEJ) (see section 1.3.2). This remodeling is carried out by several ATP-dependent chromatin remodelling complexes, such as the RSC (chromatin structure remodeling), INO80, SWR1, SWI/SNF, and Rad54 or NuA4, but also by histone acetyltransferase TIP60 [121-124]. Moreover, DSB repair is also reported to have slower kinetics and to be less effective in heterochromatin than in euchromatin [108, 125], as was observed when ATM-

dependent, phosphorylated KAP-1 was used as a marker of DSBs in heterochromatin [103, 126, 127]. The reported delay in repair of heterochromatic DNA DSBs suggests that these are more difficult to repair than DSBs found in euchromatic DSBs. This is likely a result of the more tightly compact structure of heterochromatin which requires more manipulation than less condensed chromatin, euchromatin, in order to allow DDR proteins to get access to the DNA break. In addition, the histone acetyltransferase HP1 or TIP60 are considered as important mediators of DSB-induced heterochromatin decondensation, in which they function upstream ATM, causing ATM acetylation resulting in enhanced ATM kinase activity [128]. Apart from KAP-1, other components are also shown to be required for heterochromatic DNA decondensation, relaxation and repair of DNA DSBs in such chromatin regions [108, 125, 129]. These include 53BP1, MDC1, RNF8 (RING-finger protein 8), RNF168, MRE11, and NBS1 [127, 130], but also mediators of heterochromatin formation, i.e. HP1, the HDAC1/2 proteins, the Suv39H1/2 histone methyltransferases, and the DNA methyltransferase DNMT3B [125, 129]. In this thesis, histone deacetylase (HDAC) inhibitors (HDACi) are applied. In this context it is interesting to mention that increasing the accessibility of chromatin to DNA damaging agents has also been proposed as one of the strategy for chemotherapy sensitization in cancer treatment [131].

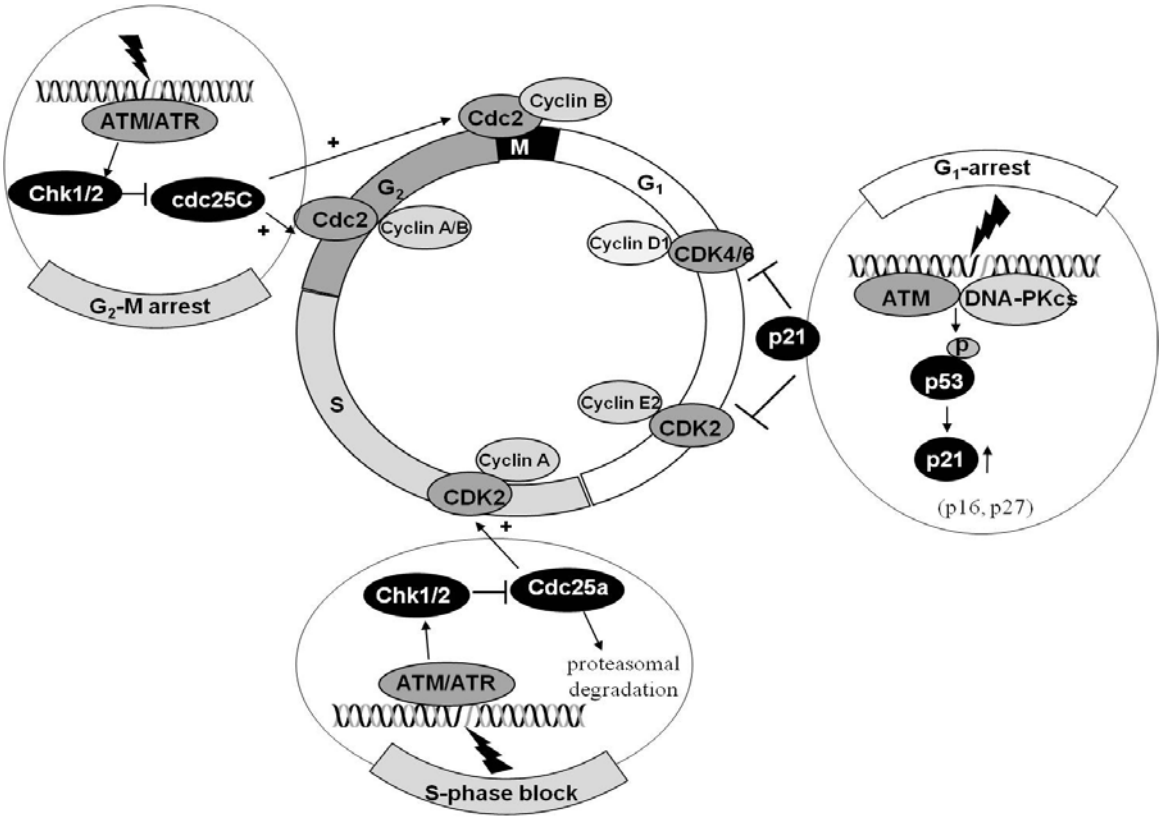

Figure 5. Overview of cell cycle perturbations in response to DNA damage.

1.3.2 DNA Double Strand Breaks (DSBs) repair

It is clear that altered DNA repair capacity influences the risk for development of genomic instability and, hence, tumour development. Thus, observations of patients with rare inherited

diseases i.e. Xeroderma pigmentosum, Ataxia telangiectasia or Bloom syndrome, with common feature of altered DNA repair, have revealed an increased number of cancer incidence [59].

The activation of a particular DNA repair pathway is dependent on the type of DNA damage inflicted, thus, abasic sites, 8-oxoguanine or single-strand breaks (SSBs) caused by x-rays/low LET ionising radiation/photon irradiation, oxygen radicals/reactive oxygen species, alkylating agents are repaired by base excision repair (BER) [132]. The UV light, polycyclic aromatic hydrocarbons which both generate bulky adducts, 6-4 photoproduct (6-4)PP and cyclobutane pyrimidine dimer CPD are handled by nucleotide-excision repair (NER) [133]. Replication errors in DNA resulting in A-G and/or T-C mismatch, insertions and deletions, are attacked and counteracted by mismatch repair (MMR) [134]. Finally, the ionising radiation and anti-tumour agents induce DNA interstrand cross-links, which either primarily or during repair processes generate double-strand breaks (DSB), and are counteracted by either of two principal recombinational repair systems, homologous recombination (HR) and non-homologous end joining (NHEJ), respectively [56, 59, 135, 136] (**Figure 6**).

HR restores genomic integrity by making use of the undamaged homologous chromosome as a model template for the repair, and accordingly, this pathway is instrumental for controlling DNA DSBs repair when DNA replication has almost been completed and such template is available, meaning late S or G2 phase of the cell cycle. NHEJ, on the other hand, does not rely on homology of the terminal DNA sequences and can, hence, be active DNA repair in all parts of the cell cycle (**Figure 6**) [137].

The broken DNA ends are recognised by the MRN complex, then the DNA-binding motifs within MRE11 in this complex, together with RAD51, bind to DNA ends, which sensitise the DNA ends for further processing. NBS1, which is yet another part of the complex, interacts with ATM, which is transformed from the inactive dimer into active monomers in presence of broken DNA ends. (**Figure 6**) [138]. Subsequently, ATM phosphorylates various substrates required for repair via either HR or NHEJ, respectively. Apart from the MRN complex, other factors such as BRCA1 and CtIP, participate in the activation of HR repair pathway in the S and G2 cell cycle phases [135, 139].

Apart from mediating chromatin remodelling around the DNA DSBs, ATM also coordinates DDR by phosphorylating multiple protein factors that drive NHEJ/HR e.g. DNA-PKcs, BRCA1, Rad51, induce chromosome cohesion e.g. H2AX, KAP-1, NBS1 and SMC1, and activates cell cycle checkpoint effectors e.g. Chk1, Chk2 [103, 140-142]. The importance of both ATM and DNA-PK in the maintenance of genomic integrity is illustrated by the hypersensitivity to DNA damaging agents of cells with suppressed expression or activity of these kinases.

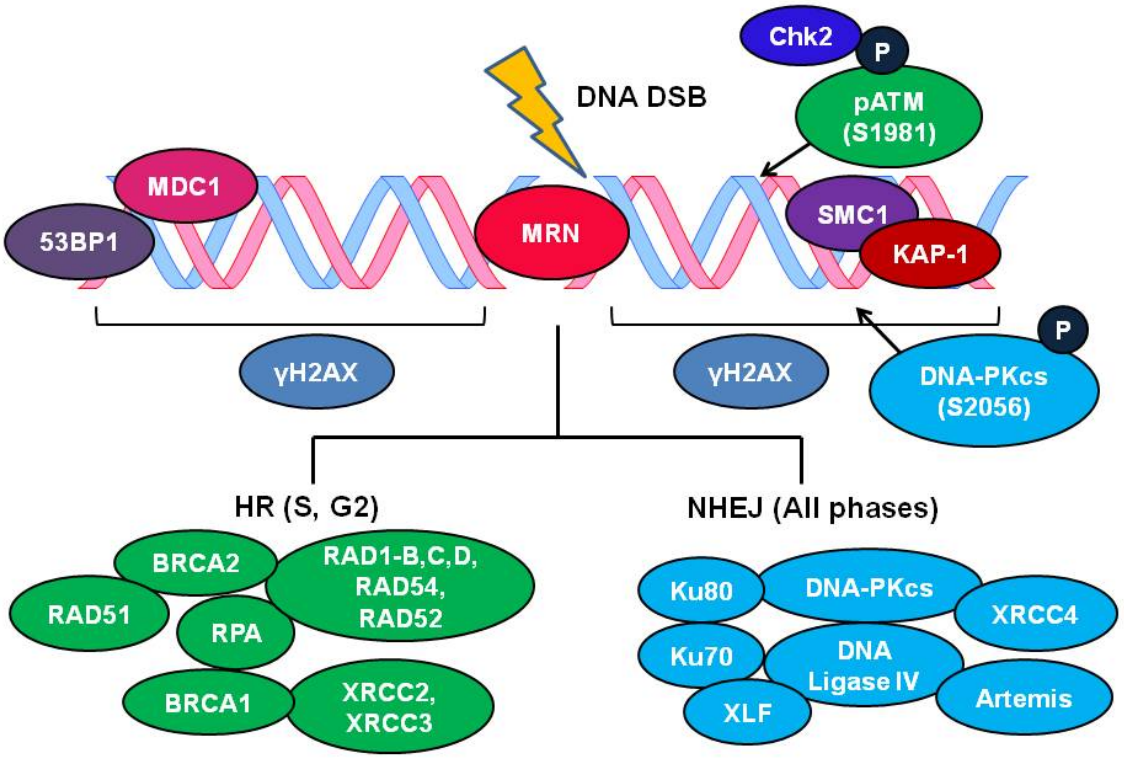

Figure 6. Homologous recombination (HR) and non-homologous end joining (NHEJ).

1.3.2.1 Homologous recombination (HR)

HR is a very precise repair mechanism which takes place in S/G2 phase and relies on a homology of a sister chromatid as a template for the repair. This process involves significant manipulation of both chromosomes which is followed by processes such as resection and invasion of the strand, formation of the D-loop, DNA synthesis, and finally Holliday junction resolution [143]. The 5'-3' exonucleases digest the damaged strands to expose single-stranded regions on both sides of the break. These functions of HR DNA repair machinery is carried out by RAD50, RAD51, RAD52, RAD54, MRE11, RPA, BRCA1, and BRCA2 (**Figure 6**) [144-147]. Thus, the RAD51 polymerisation on the single-stranded DNA is required for searching of a homologous correct DNA sequence and promote the sensing of homologies between DNA strands. Apart from RAD proteins, the single-stranded DNA-binding protein RPA participates in exchange of DNA strands [148]. DNA polymerase and ligases then resynthesise and ligate the four strands which form the intact two DNA helices.

1.3.2.2 Non-homologous end joining (NHEJ)

The DNA end-joining, NHEJ, only needs limited homologies of DNA sequences to rejoin juxtaposed ends of broken DNA double strands and only the damaged helix is involved, allowing this repair pathway to be active in all phases of the cell cycle and, hence, to be of great importance for DNA DSB repair [149].

The religation/rejoining of the two broken DNA ends, which takes place, can, however, cause loss of nucleotides and/or chromosomal rearrangements [56], and, hence, NHEJ has less DNA repair fidelity than HR [150].

In NHEJ the presence of the DNA DSBs is detected by a protein heterodimer, consisting of Ku70/80, which rapidly upon break formation, binds to the free DNA ends within the break [151, 152]. It is through that the DNA/Ku acts as a platform or scaffold to which the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is recruited, resulting in the formation of the DNA-PK holoenzyme (**Figure 6**) [153-155]. At the DSB junction, two closely opposed molecules of this holoenzyme assemble into a synapse, which stabilise the free DNA ends in position and increase significantly the kinase activity of DNA-PKcs [136, 154-157]. In response to this, the DNA-PKcs also undergoes autophosphorylation on clusters of serine/threonine residues (e.g. S2056, T2609, T2647), which in turn causes large-scale conformational changes, allowing DNA-PKcs to direct the NHEJ DNA DSB processing and ligation [152, 158, 159]. In the subsequent steps of NHEJ nucleases and polymerases, such as Artemis and DNA ligase IV/XRCC4, are phosphorylated by DNA-PK and also incorporated into the complex, where they complete or take out the DNA single-stranded ends before rejoning of the broken strands (**Figure 6**) [143, 160-164].

Apart from the above mentioned classic NHEJ (C-NHEJ) pathway, a number of subpathways activated in certain cellular context have been identified, which all promote DNA DSB ligation [165, 166]. The complete constitution of alternative NHEJ subpathways still needs to be defined, but at present a number of overlapping networks has been outlined i.e. alternative NHEJ (A-NHEJ), microhomology-mediated end joining (MMEJ) and backup NHEJ (B-NHEJ) [167, 168]. Interestingly, B-NHEJ has been demonstrated to be more error prone and slower than C-NHEJ, resulting in even more chromosomal rearrangements to be generated during the repair process [165, 169, 170].

1.3.3 Fate of the cells with DNA damage

The cellular outcome of the DNA damage is in principal either correct or faulty repair or induction of cell death. Different genotoxic endpoints i.e. gene mutations, changes in chromosomes and, in consequence, often malignant transformation, are considered as negative consequences of the non- or misrepaired DNA damage. Multiple signalling networks are involved in the cellular decision to repair damage or to induce cell death, and these take into consideration the type of damage, the amount of damage, the cell cycle phase and the energy balance of the cell. The central coordinator in this aspect is the tumour suppressor p53, which controls both cell cycle progression and different cell death pathways [171-176].

DNA damage-induced cell death may proceed via different cell inactivation modes i.e. apoptosis, necrosis, mitotic catastrophe, senescence and autophagy [177, 178]. **Apoptosis** is a cell death type which is highly controlled and which does not elicit an inflammation response in the intact organism. It is characterised by cell rounding and volume reduction, membrane

blebbing, and condensation of chromatin, which is followed by fragmentation of the nucleus and in the intact organism phagocytosis of the apoptotic cell by macrophages [179]. **Necrosis** is, in contrast, associated with plasma membrane rupture resulting in cytoplasmic organelles dilution, cytoplasmic swelling, with modest chromatin condensation [180, 181]. In the intact organism it provokes an immune response, inflammation. **Mitotic catastrophe** with micro- or multinuclei formation appears during mitosis or afterwards [182-184]. **Autophagy** occurs without chromatin condensation, but with appearance of typical two-membrane autophagic vacuoles containing degenerating organelles and cytosol [185, 186]. Finally, **senescence**, is a permanent cell cycle arrest which occurs usually at the end of the lifespan of the cell, but which also is induced by stress signals response, such as those generated in response to extensive unrepaired DNA damage [68, 187, 188]. Apoptosis and necrosis are cell death mechanisms considered to be irreversible, while autophagy and senescence are reversible [189, 190]. Both autophagy and mitotic catastrophe can also under certain conditions promote survival [191-195]. Senescence is not considered as a typical cell death mode since senescent cells remain metabolically active despite being clonogenically dead [196].

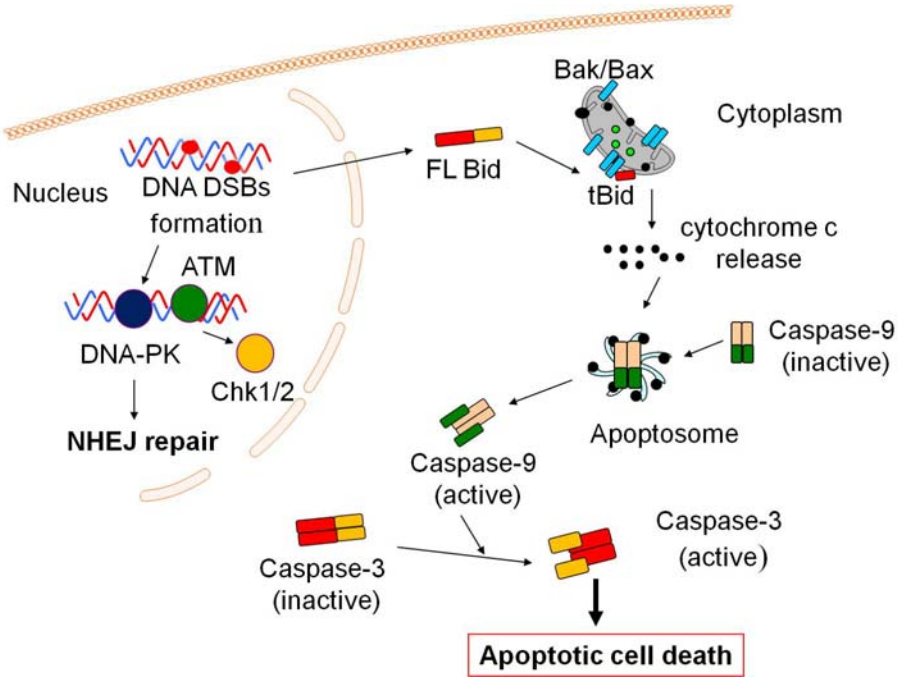

Figure 7. Basic apoptotic cell death signalling via the intrinsic pathway.

Apoptosis proceeds via either of two main mechanisms - extrinsic and intrinsic pathways, respectively [197]. The extrinsic (or receptor-mediated) pathway is controlled by the cell surface death receptors, e.g. Fas, which upon binding of its ligand, e.g. FasL, interacts with Fas-associated protein with death domain (FADD), allowing two pro-caspase-8 molecules to be brought in close proximity and cleaved into functional caspase-8 and further trigger activation of execution caspases. Caspase-8 may also amplify the apoptotic signals via the mitochondrial pathway in which cleaved Bid to tBid is the control molecule [198]. The intrinsic (or mitochondrial) apoptotic cascade starts when cytochrome c is released from mitochondria and forms, together with Apaf-1, the apoptosome, which after a conformational

change, in turn activates procaspase 9. Once autoactivated by cleavage, procaspase 9 becomes caspase 9. Within the apoptosome the effector caspases are activated, i.e. caspase-3, -6 and -7, and caspase-9, by cleavage of their prodomains (**Figure 7**) [199]. The final step of such caspase cascade is cleavage of signalling and structural proteins, such as the DNA repair protein PARP-1. The intrinsic pathway is mainly controlled by the Bcl-2 family which consists of proapoptotic (Bak, Bax, Bad, Bid, Bik, and Bim) and antiapoptotic (Bcl-2 and Bcl-XL) proteins [200, 201]. The proapoptotic proteins take action on the surface of the mitochondrial membrane where they stimulate pore channel formation, which, in turn, results in the mitochondrial transmembrane potential reduction and cytochrome c release and triggering of the apoptosome (see above) [202].

Autophagy can be triggered by starvation and deficiency of growth factors. It is a multi-step process, starting with the sequestration of the cytoplasm portions into double-membrane autophagic vacuoles, called the autophagosomes. Then, autophagosome undergoes fusion with a lysosome, and an autolysosome is formed, whose hydrolases cause a degradation of the cytoplasmic material (**Figure 8**). The free fatty acids and amino acids produced in this process, but also proteins retrieved from autophagosome, can be reused by the cell to maintain ATP levels and protein synthesis, supporting cell survival [203-207].

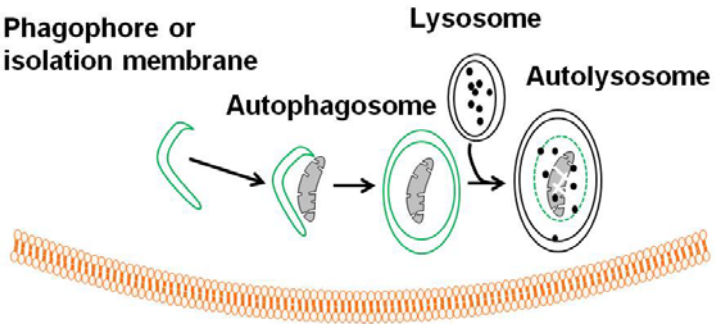

Figure 8. Basic stages of autophagy.

1.4 DDR SIGNALLING IN CANCER AND AS A TARGET FOR CANCER THERAPY

1.4.1 DDR as a barrier against cancer

During DDR cells make decision to repair inflicted DNA damage or to descend to death, hence, DDR is considered as first barrier against the malignant process [69, 208, 209]. Loss of genetic stability, which is a hallmark of tumorigenesis [16, 17, 210], is driven by DNA damage and errors that are incorporated during DNA repair [211]. In addition, tumours often harbor genetic/epigenetic defects that, consequently impair factors in DDR signalling pathways i.e. p53, ATM, Chk2, γH2AX, causing further activation of protooncogenes and inhibition of tumour suppressor genes, respectively [104, 212, 213]. Studies on DDR have revealed plenty of links between oncogenesis and inherited changes in the genome. Moreover, cells defective in DDR/DNA repair mechanisms generally present increased sensitivity towards DNA damaging agents and this leads to high probability of cancer.

The role of DDR in protection against cancer development is also supported by the reported genetic defects in some DDR components and the increased cancer incidence in individuals carrying these aberrations. One example is defects in the NER components in Xeroderma pigmentosum (XP) syndrome. The XP syndrome is associated with impaired capacity to repair point mutations such as those inflicted by UV and, accordingly, individuals with XP deficiency has an about 1000-fold higher probability of incidence of UV-induced skin cancer and also increased neurodegeneration and premature ageing [214]. Other inherited human syndromes linked to DDR, which are rare diseases, are chromosome abberations in ATM in Ataxia telangiectasia (AT), in MRE11 in AT-like disorder, or in NBS1 in Nijmegen breakage syndrome (NBS). Patients with these syndromes have higher predisposition to cancer (especially lymphomas), immunodeficiency, radiation hypersensitivity, and often also neurological complications and premature ageing [215-219]. Syndromes connected with defects in HR repair include hereditary breast/ovarian cancers caused by defects in BRCA1 and BRCA2 [220], but also cancer-prone chromosomal instabilities i.e. Werner (WRN), Bloom (BLM) and Rothmund Thomson (RECQL4) syndromes, with involved RecQ-like helicases [221, 222]. Moreover, mouse models with deficiency in HR and NHEJ give severe mutant phenotypes, further illustrating the importance of these two DNA DSB repair mechanisms [223].

1.4.2 DDR signalling as a target for cancer therapy

Overexpression or loss of specific factors in DNA repair machinery, result in altered functions of HR and NHEJ repair pathways in tumours [224]. Deregulations in DNA repair promote genomic instability and malignant progression, but still some tumour cells survive and, hence, they have acquired capacity to survive also DNA damage inflicted by chemo- and radiation therapy [223, 225]. Therefore, inhibition of DDR and/or DNA repair pathways has become an attractive strategy to overcome resistance to DNA damaging therapy and small DNA repair inhibitors has been developed to be used either as single-agent therapy or, more often, in tandem with DNA damaging treatments [226-232]. Some of the most common strategies are described below.

1.4.2.1 Cell cycle checkpoints abrogation

Inactivation of the tumour suppressor p53 [233], by chromosomal abberation (deletion), inactivating mutation or overexpression of p53 negative regulator, MDM2, results in impaired G1 checkpoint control of tumours. Accordingly, tumour cells with inactivation of p53 function rely on S and G2 phases checkpoints to repair DNA damage and survive [87, 234]. Based on these observations, one of the strategies to overcome altered function of DDR in tumours is an abrogation of the remaining, intact checkpoints and in this way enhance tumour cell death [92]. Thus, results from preclinical studies have shown that abrogation of the S and G2 checkpoints with small molecule inhibitors, specific enzymes, RNA interference towards Chk1 [235], can impair DNA repair response to DNA damaging chemotherapy in a tumour selective way [98, 100, 236, 237], which also have formed the basis for clinical trials with Chk1/2 inhibitors [238, 239].

1.4.2.2 PARP inhibitors

Pharmacological inhibition of the DNA repair pathways might significantly increase the cell death inducing capacity of DNA damaging agents and combinations of cytotoxic agents with DNA repair inhibitors are under preclinical investigations or have already been introduced to the clinic as ongoing clinical trials. However, some aspects need to be considered i.e. normal tissue reaction to inhibition of DNA repair and possible selectivity of the potential drugs towards target(s) in tumour cells, as usually many DNA repair pathways are involved in tumour cell response to therapy, but also tumour DNA repair pathway redundancy when exposed to a certain chemotherapeutic drugs [240].

One of the more advanced DNA repair inhibiting strategies is to attack PARP-1, a component of base-excision repair (BER) of apurinic sites [241-244]. The benefit of targeting PARP-1 to sensitise for DNA damaging treatments was already reported in 1980 [245], in which it was demonstrated that application of certain PARP-1 inhibitors prevented the rejoining of DNA strand breaks caused by dimethyl sulphate, leading to increased *in vitro* cytotoxicity. PARP-1 inhibitors (PARPi) in combination with temozolomide, platinum chemotherapy (cisplatin, carboplatin) are now in clinical trials, but concerns are raised because of toxicity of the combined treatment regimen towards normal cells [246]. PARPi have also been tested as monotherapy. The rationale for this use of PARPi, is based on synthetic lethality, a concept proposed by T. Helleday et al. [228], to be 'a genetic phenomenon in which the combination of two otherwise non-lethal mutations results in an inviable cell. Synthetic lethal phenotypes are indicative of an interaction between the products of the two mutant genes within the cell.'. This concept was introduced when PAPRi were used in patients with inherited breast and ovarian cancers which lacked wild type and carried mutated BRCA1 and/or BRCA2 genes [247, 248], resulting in impaired HR repair [249] and increased sensitivity to PARP inhibition. BRCA1- or BRCA2-deficient cells lose their ability to base excision repair (BER) with PARP-1 as component, which results in single-strand breaks (SSBs), which in consequence of stalled replication forks, can convert into DSBs [250]. Because of HR deficiency these DNA lesions cannot be repaired and consequently trigger cell death [248, 249]. The obtained preclinical results have given support for clinical trials with PARPi as monotherapy in breast and ovarian cancers patients carrying BRCA1 or BRCA2 mutations, but, unfortunately, not all patients with BRCA mutations respond to this new, targeted therapy and resistance to such treatment is also reported [241]. Interestingly, deficiency in other HR repair proteins than BRCA, presents also enhanced sensitivity to PARPi, suggesting broad spectrum of their utility, alone or even in combination with other inhibitors [251-254].

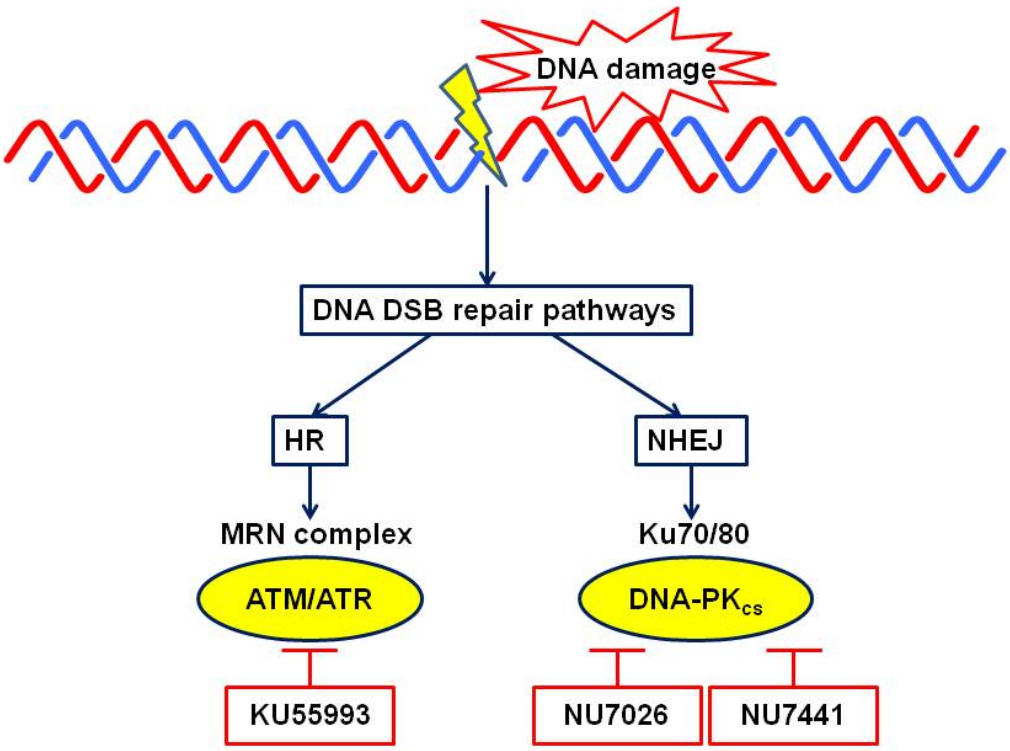

Figure 9. DNA repair inhibition strategies.

1.4.2.3 ATM and DNA-PK inhibitors

Inhibition of one of the main kinases of the phosphatidylinositol 3-kinase (PI3K)-related protein kinase (PIKKs) family, ATM, which play crucial role in repair of DNA DSBs, have also been tested. The rationale is that inhibition of ATM will result in lack of proper detection of the DNA DSB inflicted by the chemotherapy and, hence, they will accumulate to a level leading the tumour cells towards cell death. Attempts have so far generated small molecules which in preclinical settings been shown to inhibit ATM kinase activity, e.g. KU55933 (AstraZeneca) (**Figure 9**). DNA-PK has also given its critical role in NHEJ-mediated repair and has been in focus for small molecule inhibitor development [255, 256]. A number of candidates have been generated, among them NU7441 and NU7026 (**Figure 9**). These agents have shown some effect as monotherapy [257-261], but more importantly, they have also been demonstrated to sensitise tumour cells to DNA DSB-inducing treatments i.e. ionising radiation and etoposide, a topoisomerase II inhibitor, proving the concept of DNA-PK inhibition in tumour treatment [262-265]. Interestingly, induced hyperactivation of DNA-PK has also been shown to cause a chemosensitising effect in tumour cells [266, 267]. This support that perturbations of DNA-PK kinase activity i.e. hypo- or hyperactivation/phosphorylation, may also increase sensitivity of tumour to standard DNA damaging treatment. This concept was also explored in **Paper III** and **IV** of this thesis.

1.4.2.4 Epigenetics as a new tool to target DDR signalling

Epigenetics is defined as altered patterns of gene expression mediated by changes different from alterations in the primary nucleotide sequence, e.g. DNA methylation, miRNAs, nucleosome remodeling and covalent histone modifications [268]. Such modifications influence gene regulation of several fundamental processes in tumours, including DNA repair and cell death signalling [269, 270]. Recently, a novel, promising approach has been introduced to cancer therapy and there are successful examples that targeting of alterations in epigenetic signalling in tumour cells may be used as therapy, as shown by the introduction of HDAC inhibitors (HDACi) in hematological malignancies [268, 271]. Epigenetic alterations have indeed been shown to be also involved in DDR signalling, e.g. the NAD+-dependent histone deacetylase, SIRT1 was reported to impair repair via the NHEJ pathway [272]; SIRT6 was found to stabilise DNA-PK associated with chromatin and in this way influence DNA DSB repair [273], and HDAC1 and HDAC2 were both reported to promote DSB repair [274]. Several studies also demonstrated that HDACi applied in tandem with DNA damaging agents caused increased cytotoxicity as a consequence of increased DNA damage and/or impaired DNA repair capacity [275]. One such example is decitabine (2′-deoxy-5-azacytidine), a DNA demethylating agent, which was combined in tests with platinum-based drugs (i.e. cisplatin or carboplatin) to reverse drug resistance in ovarian cancer patients in clinical trials [276]. This concept of connection between epigenetics and DDR was explored in **Paper IV** of the thesis.

1.5 PHENOTHIAZINES IN PSYCHIATRY AND ANTICANCER THERAPY

Phenothiazines were primarily designed as antipsychotic drugs. Yet, it was recognised that apart from their role as antipsychotic drugs, phenothiazines can regulate cellular functions also outside the central nervous system. For instance, perturbations of lysosomal functions, DNA repair and chromatin remodelling are also affected by phenothiazines and can result in anticancer activities, as illustrated in Papers II-IV of this thesis.

1.5.1 Development of phenothiazines as antipsychotic drugs and their targets

Phenothiazines are a class of heterocyclic compounds, which at present are classified as the first generation, so called 'conventional' or 'typical' antipsychotic agents (**Figure 10**). As such, phenothiazines are described to be dopamine antagonists used for treatment of psychiatric disorders, such as schizophrenia [277-281]. This development was proceed by their use in other conditions as summarised by C.L. Zirkle as: 'To tranquilizers and antidepressants: from antimalarials and antihistamines' (F.H Clarke (Ed.), How Modern Medicines Are Discovered, Futura, Mt. Kisco, NY (1973), p. 55077). Thus, the phenothiazine derivative, methylene blue, was observed to have anti-malarial capacity, but was also used in anti-emetic therapy to overcome chemotherapy-induced vomiting [280, 282]. It was subsequently noticed that phenothiazines possessed antihistaminergic properties. In 1951, the aliphatic phenothiazine, chlorpromazine (**Figure 10**), was administered as an anesthetic agents upon surgery. Almost at the same time, the use of phenothiazines was extended to psychiatry, where their

antipsychotic activity was discovered [277]. During the next twenty years, between 1954 and 1975, about 15 antipsychotic drugs based on the phenothiazine structure were introduced worldwide [280].

Until early 90's, phenothiazines were one of the most commonly used group of antipsychotic drugs. Then, another class of agents, so called second generation, 'atypical' antipsychotics were introduced, with clozapine and risperidone as the most common representatives, which in a more accurate way could target the dopamine pathway resulting in less detrimental effect on induction of the side effects, extrapyramidal symptoms (EPS), but also with improved efficacy on schizophrenia symptoms even, especially for clozapine, in patients previously found to be non-responsive group [283-289].

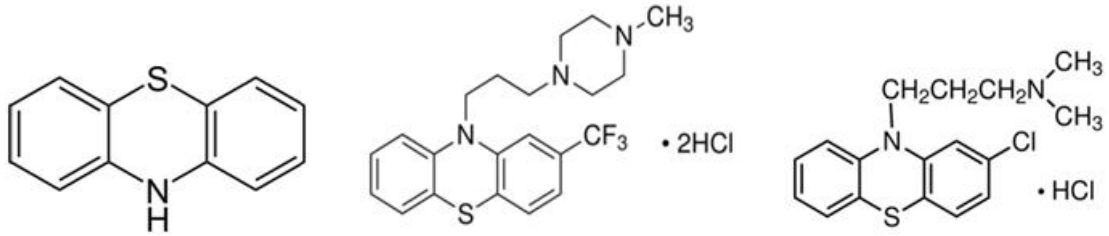

Figure 10. Chemical structures of the phenothiazine ring (*left*) and two of the phenothiazine compounds used in this thesis - trifluoperazine, TFP (*middle*) and chlorpromazine, CPZ (*right*).

1.5.2 Phenothiazines as dopamine antagonists and beyond

The antipsychotic effect of phenothiazines is governed by inhibition of neurotransmitter receptors, in particular the dopamine D2 receptor (D2R) [279]. Thus, phenothiazines and other first generation antipsychotics are considered as D2R antagonists and were found to reduce dopaminergic neurotransmission in the dopamine pathways in the brain (mainly in mesolimbic pathway) [290]. This is in contrast to newer antipsychotics which are dopamine-serotonin antagonists, and mainly has high affinity for 5HT2A receptors [291-294]. Phenothiazines also bind to other types of neurotransmitter receptors and channels involved in other dopamine pathways, what is thought to be responsible for observed side effects [294, 295]. Thus, the chronic administration of phenothiazines for antipsychotic treatment was found to be associated with side effects i.e. sedation as a consequence of anti-histaminergic effect, anti-cholinergic effect, weight gain as metabolic effect, photosensitivity [296, 297], but also more serious effects such as acute extrapyramidal symptoms (EPS) with parkinsonism (anti-dopaminergic effect), dystonias, and akathisia [280, 298], dyskinesia [299], and rare, but very dangerous, neuroleptic malignant syndrome (NMS) [300]. The group of phenothiazine drugs interferes with and modulates different cellular and metabolic processes [295]. Thus, the spectrum of side effects caused by phenothiazines, suggest that these compounds are also able to influence many physiological/metabolic processes, other than their actions in central nervous system (CNS).

1.5.3 Phenothiazines targets outside the neuronal system

A plethora of cellular targets of phenothiazines, apart from D2R in the neuronal system, have been characterised, i.e. from calmodulin (CaM) to lipid membranes, DNA, proteins and reversal of multi-drug resistance [301-304], suggesting other functions of these compounds, different from their role in nervous system and impact on neuroendocrine signalling [295, 305].

One of the more extensively studied cellular target of phenothiazines is CaM, a calcium-binding protein, responsible for many of the intracellular processes controlled by calcium i.e. cell motility, cell secretion, formation of the microtubular apparatus, cytoskeletal dynamics, cell cycle progression, cellular respiration and homeostatsis of mitochondria [306], cellular proliferation [307], nucleotide metabolism, phosphorylation and dephosphorylation of structural and functional proteins, inflammation or cell death [308-311]. CaM is considered as a potential intercellular target of phenothiazines and some of these, among them trifluoperazine (TFP) (**Figure 10**), show quite high affinity for CaM. Hence, after binding to CaM phenothiazines are reported to antagonise calcium–CaM interactions and block CaM dependent cellular events [312]. On the other hand, there is no clear evidence that CaM regulates synthesis and release of neurotransmitters, and if CaM antagonism by phenothiazines can be responsible for neuromodulation in psychiatric therapy. However, the phenothiazine class of antipsychotic drugs antagonises the action of CaM and disrupts its functions, which provided a way for elucidating the role of CaM in phenothiazine-induced cytotoxicity and their anti-proliferative effect [313]. Phenothiazines are also reported to compete with the CaM-targeted enzymes and in this way inhibit the stimulatory effect of CaM [312]. Moreover, phenothiazines can also interact with other calcium-binding proteins i.e. protein kinase C or troponin C [310, 314, 315].

Phenothiazines have also been demonstrated to influence plasma membrane fluidity and permeability by interacting with lipids in biological membranes, causing membrane-destabilizing effects [301, 316]. As a consequence, phenothiazines impact on cholesterol metabolism/homeostasis by modulation of genes involved in sterol biosynthesis [317], but phenothiazines are also reported to induce dissociation of membrane-bound signalling factors such as receptors/ligands [318]. The major effect of the phenothiazines on drug retention takes place in the late S-G2/M phases of the cell cycle, when CaM is believed to exert a major effect on cellular proliferation, which is connected with the fact that drug efflux pumps are also considered as one of the molecular targets of phenothiazines [319-321].

1.5.4 Phenothiazines as anticancer agents

The cytotoxic effects of phenothiazines in tumour cells and their ability to augment the cytotoxicity of chemotherapeutic agents have been quite extensively explored over the years. Thus, different studies have demonstrated antitumour activities of phenothiazines, especially *in vitro*, in different tumour cell lines [322-327]. However, the molecular mechanism(s) behind phenothiazine-induced effects on cell viability are still not understood, albeit various

hypotheses, i.e. CaM antagonism [309, 310], membrane fluidization [316] and disruption of mitosis [328], have been proposed.

The rationale for testing phenothiazines and anticancer drugs in combination, was in part based on reports showing phenothiazines to be CaM modulators, CaM antagonists, calcium channel blockers, and given the important role of CaM and calcium in cellular processes. Thus, obtained results with CaM antagonists showed enhanced cytostatic and cytotoxic effects when applied in tandem with certain anticancer chemotherapeutic agents i.e. adriamycin, doxorubicin, vincristine [329-331]. Given that phenothiazines were reported to act as CaM antagonist, it was also tested as to what extent they could potentiate cytotoxicity of chemotherapy. For instance, it was demonstrated that brief exposures to non-toxic concentrations of TFP enhanced the cytotoxicity of toxic agents (e.g. adriamycin), in a resistant leukemia P388 cell line [332]. TFP was also found to act synergistically with bleomycin, daunomycin or cisplatin in some mammalian tumour cell lines, and to improve therapeutic efficacy of these anticancer drugs [308, 333-336]. In more recent studies it was found that TFP and other phenothiazines induce apoptosis, alone and in combination with chemotherapy, in tumour cell lines of different origin [302, 337-342]. Despite these observations, the pharmacological properties of phenothiazines and molecular mechanism(s) underlying obtained results still remain unclear, but were further explored in **Papers II-IV** of this thesis.

2 AIMS

The studies presented in this thesis were focused on the analysis and characterization of therapy sensitising strategies in tumours using different radiation modalities or phenothiazines and chemotherapy to interact with DNA damage response.

The specific aims of each part of the PhD project were:

- To compare the effects of high LET ions with low LET photons irradiations on cell survival and to use mathematical modelling to determine whether the cellular response to low LET could predict the response to high LET radiation **(Paper I)**.

- To evaluate the potential utility of single phenothiazines treatment in human lung cancer and characterise effects of phenothiazines on lysosomal signalling pertubations **(Paper II)**.

- To investigate in detail the effects of phenothiazines on DDR signalling and reveal how they could be used to enhance therapeutic utility of chemotherapy and/or DNA repair inhibitors **(Paper III)**.

- To elucidate if phenothiazines could be used to treat tumour cells in which epigenetic signalling is deregulated, to analyse combination of phenothiazines with chromatin-modifying drugs and to study how such combined treatment affect DDR signalling **(Paper IV)**.

3 MATERIALS AND METHODS

Cell lines. To compare low and high LET IR effects, in **Paper I,** cell lines of different tumour origin were used: melanoma (AA), small cell lung cancer (SCLC) (U-1690) [343], head and neck squamous cell carcinoma (SCC) (FaDu) and two metastatic prostate cancer cell lines, PC-3 (from bone metastasis) and DU-145 (from brain metastasis). Cells were seeded at a density of 2-5 x 10^5 in 0.2 ml of medium, in the middle of 3 cm Petri dishes one day before the irradiation exposures and were in exponential growth when irradiated. The effects of phenothiazines in **Paper II** focused on lung cancer and the following SCLC and NSCLC cell lines were used: H69, H82, H592, U-1258, U-1568, U-1690, U-1906 and U-2020 (all SCLC); A549, H125, H1299, H157, H23, H661, U-1752 and U-1810 (all NSCLC). To ascertain if observed phenothiazine-induced effects were tumour specific the primary fetal human lung fibroblasts (WI-38) [344] were tested in parallel. In **Paper III** the effects of phenothiazines were studied in NSCLC (U-1810, H125, H1299, H23, A549, CL1-5), but also in breast carcinomas (MDA-MB-231, MCF7, T47D, BT474), ovarian carcinoma (A2780), as well as in glioblastoma cell lines proficient (M059K) and deficient (M059J) in DNA-PKcs expression, respectively, and also in osteosarcoma (U2OS-EJ5). Also here the tumour specificity for phenothiazines was compared with the non-cancerous cell lines WI-38 and RPE1 (hTERT-immortalized retinal epithelial cells). To study the connection between phenothiazine-induced effects on chromatin associated signalling and epigenetic deregulation, two tumour types with reported epigenetic deregulations - acute myeloid leukemia (AML) cells (KG1a, NB4 and Kasumi-1) and neuroblastoma (NB) cells (SH-SY5Y, SK-N-SY, SK-N-BE(2), SK-N-AS, Kelly and IMR32) were examined in **Paper IV**. In addition, a panel of SCLC cell lines were used as these were, in **Paper III,** found to be sensitive to phenothiazine single treatment (H69, H82, H592, U-1258, U-1568, U-1690 and U-1906).

Cell lines with the letter 'U' were established at the University of Uppsala, Sweden [343]. The others were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) or Coriell Cell Repositories (Camden, NJ, USA). CL1-5 was obtained from Dr Pan-Chyr Yang and U2OS-EJ5 cells, which harbor a chromosomally integrated GFP reporter construct [167], was a kind gift from Dr Jeremy Stark.

Irradiation procedures. The cells were irradiated with low (**Paper I** and **Paper III**) and high LET (**Paper I**). For the low LET exposure ^{60}Co or ^{137}Cs photons were used and cells were irradiated at room temperature with doses of 0-12 Gy either from a ^{60}Co therapy unit with a dose rate of <0.5 Gy/min and LET of 0.2 keV/µm or with a ^{137}Cs source with a dose rate of 0.5 Gy/min. For the high LET exposure doses, various ions in the range 0-4 Gy were used. Irradiations were performed at the Biomedical Unit at the Theodor Svedberg Laboratory (TSL), Uppsala, Sweden, with 33.7 MeV/u nitrogen ions (LET 85 keV/µm) and 24.8 MeV/u carbon ions (LET 80 keV/µm). Irradiations with 500 MeV/u argon ions (LET 90 keV/µm) and 290 MeV/u carbon ions (LET 80 keV/nm) were conducted at the Heavy Ion Medical

Accelerator (HIMAC), at the National Institute of Radiological Sciences (NIRS), Chiba, Japan.

Clonogenic survival assay. The colony formation assay is a long-term assay for assessment of cytotoxicity and allows to examine the ability of a single cells to produce colonies, usually over 1-2 weeks. In **Paper I** and **Paper III** colony formation assay was used, in which the cells were seeded as monolayer at a confluency of 70-80% allowing for an exponential growth and then exposed to the indicated doses of irradiation or drugs. In **Paper I**, cells were harvested immediately after the irradiation exposure, washed and reseeded as single cells with different cell numbers depending on the used dose and quality of radiation, in a colony formation assay, to test their sensitivity to radiation. The cell density in the dishes was also adjusted to compensate for differences in the plating efficiency (PE) of the cell lines used in the studies in **Paper I**. Similarly, in **Paper III**, after exposure of the cells to the indicated drug(s), the drug was removed by washing cells with PBS and fresh, drug-free medium was added. In both cases, in **Paper I** and **Paper III**, cells were cultured for 10-14 days, with medium change after 6-7 days. To test the ability of single cells to produce colonies cells were cultured for about 10 days allowing colonies of at least 50 cells to be formed to ascertain that reproductive cell death could be measured. The obtained colonies were fixed and stained with Giemsa stain (**Paper I**) or crystal violet (**Paper III**) for visualization and quantification of clonogenic potential. In **Paper I**, the surviving fraction for each absorbed dose was calculated as 'the ratio of the mean PE in dishes with irradiated cells over the PE in dishes with non-irradiated control cells. PE was calculated as the mean number of cell colonies over the number of plated cells.' [21].

Cell survival models. In **Paper I**, two mathematical models, the Repairable-Conditionally Repairable (RCR) model and the standard, linear quadratic (LQ) model, were used to fit the data obtained from clonogenic assay upon treatment with high and low LET radiation. In the RCR model the clonogenic survival (S) of the cells upon irradiation with a given dose (D) can be described by the formula:

$$S = e^{-aD} + bDe^{-cD}$$

with a, b and c as the parameters of the model, which describe type of response to irradiation, as presented previously in the Introduction (see section 1.2.3) [46]. Given the interpretation of the parameters of the model, the following constraints were used for fitting clonogenic cell survival data since the survival fractions cannot be greater than 1 and the total number of damage events has to be larger than or equal to the number of repairable damage events:

$$a \geq b$$
$$a \geq c$$

Since the first term in the RCR model equation means the non-damaged fraction of cells and the second term represents the cells that have been damaged and subsequently repaired, the

fraction of correctly repaired cells normalised to the fraction of cells that have been damaged, r, is described by the following equation:

$$r = \frac{bDe^{-cD}}{1 - e^{-aD}} \approx \frac{b}{a}$$

The mean inactivation dose, $\overline{D}$, for a cell population which responds to radiation according to a given function describing the clonogenic cell survival, was mathematically described as the area under the survival curve which could be calculated by integrating the cell survival function in response to dose. If cell survival is described by the RCR model, it has been previously shown that $\overline{D}$ can be calculated as [46]:

$$\frac{1}{a} + \frac{b}{c^2} = \overline{D}$$

The survival of the cells in **Paper I**, was also calculated using the LQ model [345]:

$$S = e^{-\alpha D - \beta D^2}$$

where α and β are the parameters of the model. Several explanations have been proposed for the α and β parameters, among the most common one being that β/α gives a measure of the repair capacity of the cells in the given population [346].

Relative biological effectiveness calculations. The relative biological effectiveness (RBE) values for the different tumor cells and irradiation exposures in **Paper I** were calculated in two ways: either by dividing the photon doses to ion doses giving 10% survival (D_{10}) or by dividing the corresponding $\overline{D}$ values using either of the RCR and LQ survival models, respectively.

Drugs, DNA damaging agents, chemicals and reagents. In **Paper II** a panel of structurally different phenothiazines were applied at a concentrations of 0–20 μM: trifluoperazine dihydrochloride (TFP), fluphenazine dihydrochloride (FPZ), chlorpromazine hydrochloride (CPZ), triflupromazine hydrochloride (TFPZ), promazine hydrochloride (PZ), cis-flupenthixol dihydrochloride (cis-FPX). Other substances used in the study were: tamoxifen (TMX), chloroquine diphosphate (CQ), bafilomycin A1 (BafA1), ammonium chloride (NH4Cl), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7, a calmodulin antagonist), L-741626 (a potent and selective antagonist for the dopamine receptor D2), acridine orange (AO), fluorescein di-b-D-galactopyranoside (FDG) and carboxyfluorescein diacetate N-succinimidyl ester (CFSE), the pan-caspase inhibitor (z-VAD-fmk), the lysosomal protease inhibitor (E-64d), cycloheximide (CHX), propidium iodide (PI), tetramethylrhodamine ethyl ester perchlorate (TMRE) and LysoTracker Green DND-26. In **Paper III** the potential of phenothiazines (described above) was tested for their capcacity to increase DNA damaging agent induced cytotoxicity. The DNA damaging agents tested were: bleomycin, an antibiotic compound which directly induces DNA DSBs [347]; cisplatin, which generates DNA interstrand cross-links (ICLs), resulting in DNA DSBs upon DNA replication and/or ICLs repair [348]; and the indirect-acting DNA damaging agents,

etoposide and gemcitabine. In **Paper III** the following inhibitors were used: the ATP-competitive DNA-PK inhibitor 2-(morpholin-4-yl)-benzo[h]chomen-4-one (NU7026), the PARP inhibitor Olaparib (AZD2281, KU0059436), the ATP-competitive ATM inhibitor 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (KU55933), and the inhibitor of PI3K, LY-294002 hydrochloride. In **Paper IV**, apart from previously mentioned phenothiazines, also clinically used chromatin-modifying drugs, the BRD4 antagonist JQ1 (BPS Biosciences, San Diego, CA, USA) and the pan-HDAC inhibitor panobinostat (Selleckchem, Munich, Germany), were used.

Cell viability analysis. In **Papers II - IV** cell viability was analysed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay [349], which is a short-term assay used to visualise cytotoxicity. The MTT reagent is cell-permeable and upon entrance to cells it is reduced by mitochondrial metabolic enzymes into purple coloured formazan crystals. The formazan crystals are solubilized in SDS/HCl-containing buffer and quantified by spectrophotometer with a 595 nm filter. Briefly, in **Paper II** and **IV**, 5.000 – 10.000 cells per well, and, in **Paper III**, 20.000 cells per well (for WI-38 fibroblasts only), were seeded in 96-well plates. Cells were, 24 hours after seeding, treated as monotherapy (**Paper II**), with different concentrations of phenothiazines or with cisplatin, gemcitabine or etoposide, and as combination treatment of those drugs (**Paper III**), or with phenothiazines and chromatin-modifying drugs (panobinostat or JQ1) (**Paper IV**). Viability of treated cells was given as % of untreated cells which were set to 1 (**Paper II**) or 100% (**Paper III** and **IV**). In **Paper IV** cell viability was also examined by staining with trypan blue and manual counting of cells in a Bürken chamber in which viable, non-stained, cells were distinguished from dead, blue-stained, cells. In **Paper IV** alterations in cell morphology upon treatment with phenothiazines, panobinostat or JQ1 were also examined with respect to induction of morphological changes, where cells after treatment for 72 h, were cytospun onto slides and stained with standard hematoxylin and eosin (HE) [350].

Immunofluorescence staining and apoptotic nuclear cell morphology analysis. Apoptosis is associated with characteristic changes in morphology, e.g. in the appearance of chromatin condensation and nuclear fragmentation, which both can be visualised with the nuclear staining dye 4',6-diamidino-2-phenylindole (DAPI) and assessment by fluorescent microscopy. In **Paper IV**, apoptotic nuclear morphology was analysed in SCLC H69 or NB SK-N-BE(2) cells after single or combined treatment with TFP, panobinostat or JQ1. Fluorescence microscopy was also used to detect DDR proteins with *in situ* immunofluorescence of untreated or TFP (10 μM, 24 h) treated SCLC H69 cells incubated with the following primary antibodies: phospho-DNA-PKcs (S2056), c-myc or histone H3 (K18) (all from Abcam, Cambridge, UK), and subsequently with secondary antibodies, Alexa Fluor 488 (green channel) or Alexa Fluor 561 (red channel). For nuclei visualisation also counterstaining with DAPI were used.

Flow cytometry assessment of lysosomal and apoptotic signalling and cell cycle perturbations.

Lysosomes are organelles that are involved in degradation of cellular waste material and maintain an acidic intraluminal pH by the protonation with vacuolar ATPases. This is crucial for lysosomes functions as the proteolytic enzymes gain optimal activity at low pH [351]. Designed fluorescent probes accumulate in lysosomes and their fluorescence correlates with the pH and the size of the cellular acidic compartments, and, hence, they are used to monitor functionality of these organelles. It has also been shown that compounds that are weak bases accumulate in acidic organelles and become protonated (lysosomotropism) [352, 353]. In **Paper II**, the influence of phenothiazines on lysosome integrity was analysed with the fluorescent lysosomotropic probe LysoTracker Green DND-26 (50 nM, 1 h). In addition functionality was studied by analysing lysosome-associated β-galactosidase activity in which the enzymatic cleavage of a substrate of β-gal, FDG (50 mM, 30 min), was measured by flow cytometry. In response to pro-apoptotic stimuli, the mitochondrial transmembrane potential increases transiently (hyperpolarization) as a result of an electron transport from ATP synthesis. Persistent apoptotic signalling will, if it works via the intrinsic pathway, lead to the activation of apoptotic proteins i.e. Bak and Bax which cause mitochondrial outer membrane permeabilization (MOMP) and loss of mitochondrial transmembrane potential (depolarization). In turn, MOMP results in release of apoptotic factors (e.g. cytochrome c) to the cytosol where they activate caspases and further apoptotic signalling [354-356]. In **Paper II**, transmembrane potential of mitochondria was measured by retention of the potentiometric dye TMRE (50 nM, 30 min), a fluorescent dye whose accumulation in or leakage from mitochondria can be used to monitor mitochondrial hyperpolarization and depolarization, respectively [357].

The influence of phenothiazine mono- and chemotherapy combined treatment on cell cycle progression was studied in **Paper II** using two different techniques. In the first one, cells were fixed with ethanol overnight, washed, permeabilized and then labeled with the DNA-binding dye PI, whose staining of cells is proportional to the content of the DNA, allowing to distinguish to what extent a certain treatment blocked cell cycle progression [358]. The second method allowed to assess the proliferative potential of cells and is based on the CFSE staining of cells. Labelling of cells with CFSE results in covalent linkages to intracellular molecules [359] and each time a labeled cell divides, the associated CFSE fluorescence is divided between daughter cells, what gives information about mitotic activity and checkpoint arrest.

In **Paper III**, antibody-based flow cytometric analysis of caspase-3 activity and KAP-1 phosphorylation was used. To examine caspase-3 activation a phycoerythrin (PE)-conjugated anti-active caspase-3 was applied and the activation of KAP-1 was studied using the phospho-KAP-1 (S824) antibody followed by goat anti mouse-Alexa488 secondary antibody.

All the above described fluorescence probe or antibody based methods for **Paper II** and **Paper III**, were evaluated using a Becton-Dickinson FACSCalibur flow cytometer. Subsequent data analyses were performed with the Cell Quest software and for the cell cycle distribution the ModFit software was also applied.

Immunoblotting. In **Papers II-IV**, lysosome-associated and DDR-associated events were detected by immunoblotting with commercially available antibodies. Whole-cell lysates (WCL) were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Na-deoxycholate, 1% NP-40), supplemented with protease and phosphatase inhibitor cocktail tablets (Roche Diagnostics AB, Stockholm, Sweden). 20-50 micrograms of WCL were resolved by SDS-PAGE (NuPAGE, Invitrogen, Stockholm, Sweden). In immunoblotting, the following primary antibodies were used recognising: **Paper II** - PARP, LAMP-1 (both Santa Cruz Biotechnology, Santa Cruz, CA, USA), LC3B (Cell Signaling Technology, Danvers, MA, USA), p62 (BD Transduction Laboratories, San Jose, CA, USA), D2R (Abcam, Cambridge, UK) and GAPDH (Trevigen, Gaithersburg, MD, USA); **Paper III** - phospho-DNA-PKcs (S2056), phospho- H2AX (S139), KAP-1, RPA32, H2AX (all from Abcam, Cambridge, UK), phospho-ATM (S1981), phospho-Chk2 (T68), LC3B (Cell Signaling Technology, Danvers, MA, USA), phospho-KAP-1 (S824), phospho-SMC1 (S966), phospho-RPA2 (S4/S8) (all from Bethyl Laboratories, Montgomery, TX, USA), SMC1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ATM (Epitomics, Burlingame, CA, USA), total DNA-PKcs (Millipore, Billerica, MA, USA), Ku80 (Abcam), α-tubulin (Cell Signaling Technology), β-tubulin (Sigma-Aldrich, Stockholm, Sweden) or GAPDH (Trevigen, Gaithersburg, MD, USA); **Paper IV** - apart from antibodies listed above, antibodies towards: n-myc and H3K18ac (both from Abcam, Cambridge, UK), were applied. To visualise bands on the Odyssey platform (LI-COR Biosciences) IR-Dye-linked secondary antibodies (LI-COR Biosciences, Bad Homburg, Germany) were used.

Fractionation. To test how phenothiazines influence chromatin-associated proteins, we, in **Paper IV**, examined how TFP influences the localization of phosphorylated and total DNA-PKcs in SCLC cells (H82) in chromatin-associated fractions of cell nuclei. The Subcellular Protein Fractionation Kit for Cultured Cells (Thermo-Fisher Scientific, Rockford, IL, USA) was applied for cells with or without TFP (10 μM, 4 h or 24 h) treatment. Soluble and chromatin bound nuclear fractions were isolated with the fractionation kit and were subsequently used for immunoblotting procedures in which the purity of fractionation was verified using an antibody towards the chromatin bound histone H3 acetylated on lysine 18 (H3K18ac).

Chromosomal DSB repair assay. It was previously reported that the phenothiazine TFP could suppress rejoining of restriction endonuclease-cleaved plasmids when added to cellular extract *in vitro*, indicating an inhibition of NHEJ by TFP [360]. In **Paper III**, the effect of TFP on DNA DSB repair in intact cells was further studied using a biochemically defined U2OS cell-based model (U2OS-EJ5) [167, 361], which was a kind gift from Dr. Jeremy Stark. These U2OS-EJ5 cells have been designed to express a GFP reporter construct via integration in their genome, which can be used to monitor DNA ligation capacity of cells. Thus, the GFP reporter has been separated from its promoter by the introduction of a puromycin (Puro) resistance gene and with a recognition site for the rare-cutting endonuclease I-SceI on both sides [167, 361]. By applying I-SceI via transfection into these cells the Puro encoded sequence is removed and DSBs are formed which will be repaired by

ligation if NHEJ is functional within the cells and the GFP coding sequences will be in order with its upstream promoter leading to GFP expression. The GFP expression of cells can then be used as surrogate marker for NHEJ activity in the cells. In **Paper III** the GFP-expressing U2OS-EJ5 cells were used to study the influence of TFP on NHEJ-mediated DSB repair, in which transient transfection of a I-SceI construct (pCBASce) was applied to generate DNA DSBs. Following overnight transfection, allowing I-SceI to generate DNA DSBs, cells were allowed to recover in complete medium without puromycin or were exposed to 10 μM TFP for 24 h. The percentage of GFP-positive cells was quantified by flow cytometry. With this system, we demonstrated that TFP impeded NHEJ activity as indicated by a reduction of GFP-positive U2OS-EJ5 cells.

In vitro kinase assays. DNA-PK kinase activity is central in the NHEJ repair, and in **Paper III** the effect of phenothiazines on this kinase was studied using an *in vitro* kinase assay [156]. For that purpose, DNA-PK was either isolated from cells upon phenothiazine treatment using fractionation or a purified DNA-PK (Promega, Madison, WI, USA) was used. *In vitro* kinase activity of DNA-PK was monitored either by the SignaTECT DNA-PK assay system (Promega), where incorporation of radio-labeled phosphate into a substrate peptide in the presence of activating calf thymus DNA is measured, or by the EasyLite luminescence ATP detection system (PerkinElmer, Waltham, MA, USA), where results were verified using a complementary assay in which DNA-PK kinase activity was measured by the amount of ATP remaining after DNA-PK activation i.e. ATP consumption.

In silico target prediction by assessment of gene expression with Connectivity Map (cmap). In order to get insight into phenothiazines mechanism(s) of action, the bioinformatics tool Connectivity Map (cmap), (http://www.broadinstitute.org/cmap/index.jsp), was applied to generate a hypothesis of putative cellular targets. Cmap is a bioinformatics database created by the Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA, USA. The database 'is a collection of genome-wide transcriptional expression data generated from human cell lines treated with active small molecule compounds (perturbagens) and pattern-matching algorithms that together enable the searching for possible functional connections between drugs, genes and diseases through the gene-expression changes, and allow users to compare the gene expression signature induced by perturbagen (query) to all collected instances' [317, 325, 362]. The cmap is used as a resource, which is based on the concept that the gene expression signature (an instance) induced by an agent (perturbagen) with unknown target and/or action mechanism is compared with gene expression signature induced by agents with known action mechanism, and if similarities in gene expression signature are obtained, they are taken as an assumption for similar mechanisms of action of molecules, drugs or diseases, and such analysis provides a hypothesis which subsequently can be validated [325, 363, 364]. The cmap database (build 01), used in **Paper IV**, is built upon about 6100 distinct gene expression profiles (instances) generated from a defined set of tumour cells - the breast cancer epithelial cell line MCF7, the prostate cancer epithelial cell line PC-3, the non-epithelial lines: HL-60 (leukemia) and SKMEL-5 (melanoma), corresponding to approximately 1000 different compounds

(perturbagens) in the cmap database. For any cmap analysis a query signature, consisting of up or down regulated genes, is compared with all instances in the database and similarity in gene expression pattern is provided by a score. The up score shows the extent of similarity where score 1 is given to the one showing the most similar pattern, and the down score means the same with down-regulated genes where -1 is assigned to the ones which are the most different. In the analyses performed in **Paper IV**, query signatures were obtained from previously published studies of phenothiazines [317, 365] and HDAC inhibitors [317, 366]. For visualization of data, a barview was applied which depicts the relative ranking of all instances corresponding to the indicated compound(s), and the top 5 or 10 instances (or all instances if n<5) are listed along with their respective scores.

4 RESULTS AND DISCUSSION

4.1 PAPER I

The successful clinical experience with accelerated ions has resulted in establishment of a number of ion facilities around Europe [367] and a high interest in radiobiological experiments comparing the relative efficiency in cell killing of ions and photons, as the photons are still the main radiation modality used in radiation therapy [368]. The treatment planning systems (TPSs) used at these ion therapy facilities (in Japan, HIMAC/NIRS, Chiba and in Germany, HIT, Heidelberg), although using different approaches, require as input data parameters describing the cellular response to photons and ions, determined *in vitro* [369]. Accordingly, radiobiological models with various complexity have been developed and compared with respect to their ability of accurate fitting experimental cell survival data with the linear quadratic (LQ) model for cell survival, initially developed for photons [46, 370]. There is still a need for more experimental data on the efficiency of cell killing in response to high LET ion radiation and in comparison with low LET photon radiation, as well as, to improve modelling not only able to accurately fit the data, but also to assess the relative biological efficiency in cell killing after different radiation qualities. For that purpose, in **Paper I** we tried to elucidate and compare effects of irradiation with different ions, with clinically relevant LET in different human tumour cell lines using a golden standard method in radiobiology, clonogenic survival assay. Obtained data were analysed by using two different mathematical models, i.e. LQ and RCR, with the aim to compare their ability to predict the sensitivity to high and low LET irradiations of human tumour cells.

Thus, we performed clonogenic cell survival experiments after low LET photons (^{60}Co or ^{137}Cs, at doses 0-12 Gy) and high LET ions (^{12}C or ^{14}N or ^{36}Ar, at does 0-4 Gy) irradiations of a panel of various human tumour cell lines: small cell lung cancer (SCLC) cell line (U-1690), head and neck squamous cell carcinoma (HNSCC) cell line (FaDu), melanoma cell line (AA) and two prostate cancer cell lines (DU-145 and PC-3). All five cell lines were exposed to photons and were also exposed to ^{12}C and ^{36}Ar ions while two cells lines, AA and U-1690, were in addition treated with ^{14}N ions. The generated clonogenic survival curves from these experiments were fitted using the traditional LQ model, but also the RCR model which differs from LQ model by having more parameters describing probability of DNA repair [46]. The aim of these analyses was to assess the ability of the RCR model to provide hypothesis whether the sensitivity to low LET irradiation of the tested cells could be used to predict the relative biological effectiveness (RBE) of ion therapy.

As expected, our results show an evident difference in radiosensitivity to photon irradiation among the cell lines, whereas high LET irradiation resulted in a more efficient and uniform cell kill even at lower doses. The corresponding RBE values for the different tumour cell lines were calculated either as the ratio of doses giving 10% survival (D_{10}), or by dividing the $\bar{D}$values using both RCR and LQ survival models. No significant differences were observed between the obtained values, showing that the choice of the model and method for the RBE

calculation between cell lines and radiation modality were not, at least in these cell systems, critical.

In order to assess the differences in response to different radiation qualities of the various cell lines, the RBE calculated as the ratio of $\overline{D}$ values, was plotted against the parameters of the cell survival models used for fitting the experimental data: the $\overline{D}$ ratio was used as function of the β/α parameters of the LQ model, where the α and β parameters applied correspond to the fitting of the cell survival data after photon irradiation; and the $\overline{D}$ ratio was applied as function of the b/a parameters of the RCR model fitting the cell survival after photon irradiation. For both models a linear relationship between the RBE and the cellular repair capacity was obtained. In the RCR model the parameter b is assumed to be connected with the repair capacity of the cells and the b/a value corresponds to the fraction of damaged cells that are repaired [46]. Using RCR model a correlation between the b/a ratio and RBE values was observed, indicating large capacity of tumour cells to repair the damage. The same plot for the LQ model shows almost no correlation between the β/α values and the $\overline{D}$ ratios/RBE. Results show that by using the RCR model, tumour cells which benefit the most from ion radiation therapy could be identified.

The studies presented in **Paper I**, analysing the cell survival curves for different tumour cell lines, showed a large variation in their response to photons, but a smaller variation when exposed to different ion beams. This reduced variability in sensitivity to high LET radiation is likely a consequence of more severe damage induced by high LET irradiation that previously have been reported i.e. complex, toxic DNA DSBs, DNA fragmentation, cluster damage [371, 372], and during such severe damage the intrinsic sensitivity of the different tumour cells to DNA damage becomes less critical. The biological effect, measured as RBE at a 10% survival level or $\overline{D}$ ratios, was almost independent of the methods of calculations i.e. the two survival models used in the studies in **Paper I**. However, it is of importance to notice that the RBE varies at different survival levels. Thus, the results in **Paper I** showed that there is no significant difference in the calculated RBE values from the ratio of the D_{10} doses and the $\overline{D}$ ratios, irrespective of whether the LQ and the RCR models were applied. Obtained results confirmed that the tumour cells with higher repair capacity are more resistant to low LET radiation, but are still sensitive to high LET irradiation [373, 374]. Thus, plotting the RBE against the b/a values for the photon response, an increased biological effect was observed for larger b/a values, which, when determined for low LET beams, may be used to predict the response to ion beams. The correlation between RBE and the b/a ratio of the RCR model indicates that tumour cells showing a larger capacity for repairing the inflicted DNA damage, will benefit the most from the radiation therapy with ion beams. The similar analysis performed using the LQ model showed that the β/α parameters did not retain the capacity of predicting the expected increased radiobiological effectiveness of ion therapy in resistant cell lines, which should be taken under consideration when parameters derived from a linear-quadratic dependence for cell killing are used in the TPSs. Thus, differences in approaches and capacity of different models, as shown in **Paper I**, illustrate the importance of further development of suitable methods for assessing the sensitivity of tumour cells, cell killing

effect and repair capacity, when considering choice between low and high LET radiation therapy for tumour treatment.

4.2 PAPER II

Phenothiazines are a class of heterocyclic compounds which initially were constructed as dopamine receptor antagonists to be used for treatment of psychiatric disorders such as schizophrenia [280, 294]. These compounds belong to the first generation of antipsychotics, but have in fact been shown to affect also non-CNS cells in various ways [295]. As these compounds possess anti-proliferative and cytotoxic effects, these can result in cell differentiation or death in a cell type dependent manner in tumour cells [305, 375]. Moreover, it has been demonstrated that phenothiazines may trigger apoptosis in tumour cells as part of an anti-tumour mechanism [323]. Albeit it has been shown that interference with CaM-controlled signalling as well as altered permeability of the cell membrane may be partly involved in the anti-tumor effects of phenothiazines [309, 316], the targets that are critical for the anti-tumour activity of phenothiazines remain poorly defined. Interestingly, phenothiazines have been shown to hold capacity to chemosensitise tumour cells in various ways i.e. enhance the cytotoxicity [332] or induce apoptosis [302]. Thus, it has been demonstrated that certain phenothiazines can chemosensitise tumour cells by suppressing DNA repair and thereby cause cell death [360, 376]. Also in this settings the key target of phenothiazines remains elusive, yet, it shows that compounds with a chemical structure similar to phenothiazines may be used as a valuable molecular template to create novel anti-tumour, sensitising compounds. In an attempt to elucidate the context-dependent utility of phenothiazines as monotherapy in tumours, we studied, in **Paper II**, their antitumour effect in *in vitro* system of non-small and small cell lung cancer, NSCLC and SCLC, respectively. The main aim was to reveal mechanism(s) of action and putative target(s) involved. First, the potential therapeutic utility of phenothiazines was evaluated in a panel of different SCLC and NSCLC cell lines, which were treated with doses ranging between concentrations 1 to 20 μM. These concentrations of phenothiazines have been shown to be possible to obtain in plasma of patients and in the treatment of psychiatric disorders such concentrations were not found to cause any serious side effects [377]. Obtained results of the screening showed that phenothiazines were more potent inducers of cytotoxicity in SCLC cell lines, whereas NSCLC cells were in general less sensitive, even if both subtypes of these LCs presented comparable response to the chemotherapeutic drugs, such as cisplatin, etoposide and gemcitabine, the standard treatments of LC. Moreover, we found that the sensitivity of SCLC to phenothiazines, was not dependent on growth pattern, isolation site, prior exposure to conventional cancer therapy and occurred regardless of the status of tumour suppressor p53. To further understand mechanism of action of phenothiazines in SCLC and NSCLC, two cell lines, H82 and U-1810, were studied in depth for their response to phenothiazines of different structures: trifluoperazine dihydrochloride (TFP), fluphenazine dihydrochloride (FPZ), triflupromazine hydrochloride (TFPZ) and promazine hydrochloride (PZ). We observed that SCLC cells were more sensitive to all these structurally different compounds than NSCLC cells. Importantly, results from treatment of normal primary fetal lung WI-38 fibroblasts

revealed that phenothiazine had less influence on their survival than observed in the studied tumour cells, suggesting a possible therapeutic window of phenothiazines in SCLC.

In the next step, we explored in depth how one of the phenothiazines, TFP, triggered cell death in the SCLC and NSCLC cells, with the aim to understand why SCLC were more sensitive to phenothiazines. Obtained results clearly demonstrated that the observed increased phenothiazine sensitivity of SCLC cells was attributed to both growth arrest and increased cell death response. Thus, cell cycle kinetics analysis showed that SCLC responded to TFP with an arrest in both late S and in G2/M phases of the cell cycle. As previous studies also showed that phenothiazines may trigger apoptosis in tumor cells [323], we also examined if SCLC responded to phenothiazines by induction of apoptosis. In contrast to the reported results we found that only around 10% of SCLC cells exhibited chromatin condensation and fragmentation, nuclear morphologic changes typical for apoptosis. Instead, SCLC treated with TFP had shrunken cell nuclei with no or little condensation of the chromatin. Moreover, TFP caused only minor caspase-mediated cleavage of PARP in response to doses which triggered extensive cytotoxicity, indicating that other mechanisms than apoptosis likely were involved. Further support for this statement were the results showing that the pan-caspase inhibitor (z-VAD-fmk) could not block TFP-induced cell death efficiently.

Phenothiazines are basic lipophilic drugs which show acidotropism, and may accumulate in various cellular compartments and tissues which are acidic, such as in lysosomes, but also endosomes, acidosomes and secretory granules [353, 378]. Lysosomes, small membrane-bound organelles with over 30 acid hydrolases, are the biggest acidic compartments of the cell and play a critical function in digestion and autophagy. These organelles also have high pharmacokinetic significance, as efficiency of the drug depends on their properties and types of tissue/cell, where the phospholipid pattern and lysosomal density are crucial features [353, 379]. Therefore, next we investigated if phenothiazines influenced lysosomal functions in SCLC and NSCLC cells, respectively. Interestingly, results revealed that TFP or other phenothiazines, i.e. cis-flupenthixol dihydrochloride (cis-FPX), PZ, chlorpromazine hydrochloride (CPZ), TFPZ and FPZ, all lead to an increase expression of the autophagy marker, light chain 3 (LC3)-II. This was observed in both SCLC and NSCLC cells and a further increase in LC3 conversion was evident in response to both higher phenothiazine dose and after prolonged exposure time. Such aggregation of LC3-II can either be a result of increased autophagic flux or happens in response to autophagic perturbation [380]. In order to find which of these two alternatives were operative in SCLC and NSCLC cells, both these processes were manipulated after which phenothiazines were applied and response on LC3 conversion was analysed. The autophagic degradation of LC3-II was, thus, inhibited using the lysosomal protease inhibitor E-64d, which did not impair TFP-triggered LC3-II formation, as accumulation of LC3-II was still observed, indicating an enhanced autophagic flux. In contrast, when BafA1, a blocker of the vacuolar adenosine triphosphatase ATPase (V-ATPase) which impairs the lysosomal pH gradient, was used, intra-lumenal entrapment of lysosomotropic compounds and/or signalling molecules and TFP-induced cytotoxicity were abrogated. Thus, these results show that lysosomal targeting of TFP-induced signalling is

crucial for its cytotoxic activity, whereas the lysosomal proteases *per se* are not essential for this effect. Overall, these data show that phenothiazine treatment is able to disturb the homeostatis of lysosomes i.e. to induce changes in the amount of endogenous LC3-II and conversion of LC3-I into LC3-II (hallmark of autophagy), and to cause formation of autophagic vesicles more promptly in SCLC relative to NSCLC cells. Targeting lysosome-initiated cell death has recently attracted anti-tumour therapeutic interest as it can function in a p53 and/or caspases independent manner, both which frequently are impaired in human tumors [381, 382]. Our results of phenothiazine effect in SCLC show no relation to p53 status and can occur even in tumour cells resistant to conventional chemotherapy. Given that SCLC is often refractory to chemotherapy upon relapse, our results on phenothiazines may open up for a new treatment regimen.

We show that the lysosome is critical for the execution effect of the phenothiazine-induced cell death. It has been shown that, if phenothiazines enter the acidic lumen of lysosomes, their amino groups are protonated resulting in a positively charged molecules, which then are trapped in these organelles [352]. Phenothiazines, including trifluoperazine and chlorpromazine, have been also identified as stimulators of autophagy degradation, because of disturbed calcium-calmodulin signalling [383-385]. Next, we therefore compared if SCLC and NSCLC displayed differences in lysosomal mass and to what extent the lysosomal pH was altered in SCLC as compared to NSCLC cells and if this could be linked to the observed differences in phenothiazine sensitivity among the two types of tumour cells. Retention of Lyso-Tracker, which requires acidic pH to accumulate, was used for these assessments. Albeit a heterogenous loss in LysoTracker retention was evident upon TFP treatment within the examined SCLC and NSCLC groups, we found a correlation between loss in LysoTracker induced by TFP and cytotoxicity. Moreover, lower concentrations of TFP in SCLC caused the loss of LysoTracker retention than in NSCLC and further increase of dose or exposure time resulted in permeabilization of lysosomal membrane along with mitochondrial depolarization. In conclusion, our data show that the prolonged disruption of lysosomal functions induced by phenothiazines in SCLC cells, whose lysosomes have lower buffer capacity, leads to disruption of lysosomal functions and induction of cytotoxicity. Our results also indicate that sensitivity to phenothiazines in SCLC and NSCLC cells can be partly predicted by monitoring their lysosomal mass and/or pH buffer capacity. These findings are in line with a previous study where TFP was identified as an autophagy inducer in human glioblastoma cells [383]. Moreover, our findings that SCLC cells are more sensitive to lysosome disrupting agents was further supported using various well-known lysosomotropic agents/lysosome disrupting agents, i.e. tamoxifen (TMX) and chloroquine (CQ), which both caused a higher magnitude of changes in LysoTracker retention than observed in NSCLC cells.

In summary, in the **Paper II** we uncovered a potential new treatment strategy for SCLC based on phenothiazines. Given that, this tumour type often shows a chemotherapy refractory phenotype, novel agents are urgently needed and in this context phenothiazines may hold potential if results are confirmed in *in vivo* studies in animal xenografts of this tumour

disease. From a mechanistic point of view, we found perturbations of lysosomal functions are pivotal for phenothiazine-induced cytotoxicity in SCLC. Yet, it remains to be confirmed if this effect is a consequence of lysosomotrophic accumulation of the phenothiazine *per se*, or if it is a consequence of second messenger generated from the effect on DNA damage and repair signalling.

4.3 PAPER III

The DNA damage response (DDR) is a barrier against tumorigenesis [69], however DNA DSB repair pathways in cancer cells often show altered functions as compared to normal cells, as exemplified by HR or NHEJ repair factors that are overexpressed (e.g. RAD51 in breast or pancreas cancers or in hematological malignancies) [386-388], or lost (e.g. BRCA1/2 in breast and ovarian cancers) [389]. Targeting DNA repair systems in tumours have become an attractive novel anticancer strategy with examples such as agents that abolish DNA checkpoints and/or repair, respectively. Along this line, we, in **Paper III**, explored if and how phenothiazines could interact with DDR signalling and whether it may constitute a novel treatment strategy for tumours.

The basis for **Paper III** was an earlier study from the group which demonstrated that the phenothiazines, i.e. trifluoperazine (TFP), inhibit DNA DSBs repair [360, 390-392]. To further explore this phenomenon, in this work we used a cellular system by which NHEJ activity could be examined in intact cells. The system consists of U2OS-EJ5 cells in which a chromosomally integrated GFP reporter construct is separated by the puromycin resistance gene sequence from its upstream promoter, with a recognition site for the endonuclease I-SceI on either side [167]. By transfecting the cells with a construct encoding ISceI, DNA DSB is generated as consequence of puromycin excision, resulting in cleavage of the GFP reporter. Hence, NHEJ activity will be required in order to restore the GFP sequence and GFP expression which accordingly was used as a surrogate marker for functional NHEJ. Using this system, we showed that the model phenothiazine compound, TFP, indeed suppresses NHEJ-mediated DNA DSB repair. Next, we demonstrated that this inhibitory effect of TFP on NHEJ was associated with increased DNA damage-induced S2056 autophosphorylation of the key NHEJ enzyme, DNA-PKcs, and also phosphorylation of its substrate RPA32 (S4/S8). This effect on phosphorylation of DNA-PKcs and RPA32 was even more evident when tumour cells were pretreated with bleomycin, a *bona fide* potent DSB-inducing agent [347]. Moreover, the observed effect of TFP on DNA-PKcs S2056 phosphorylation was recapitulated in both NSCLC and breast cancer cells in response to several structurally related phenothiazines, but was not observed in normal diploid fibroblasts, indicating a tumour specific action of phenothiazines on DDR signalling. By using DNA-PK *in vitro* kinase assay we demonstrated that this effect of phenothiazines on DNA-PKcs phosphorylation occurred despite the lack of inhibitory effect on DNA-PK kinase activity. ATM is another key component of the DDR, instrumental for recognition of the DNA damage lesion(s) to control checkpoint and repair factors associated with the DNA damage [393]. Interestingly, we found that TFP and related phenothiazines, in addition to

hyperphosphorylation of DNA-PK on S2056, also increased ATM phosphorylation (S1981) upon treatment with DNA DSB inducing agents, and phenothiazines also potentiated the phosphorylation of the ATM chromatin-localised downstream substrates, H2AX (S139), KAP-1 (S824), SMC1 (S966) and NBS1 (S343). This effect of phenothiazines was, as seen for DNA-PKcs S2056, also specific for tumour cells. It has previously been reported that the ATM-mediated phosphorylation of KAP-1 is connected to slower DSB repair kinetics as a result of histone modifications [394]. Hence, with respect to our data we can speculate that phenothiazines likely stimulate DNA damage signalling by increasing the persistence of non-rejoined DSBs, which then activate both DNA-PK and ATM [136, 140], and most likely impair DNA DSB repair. Our results are also in line with earlier reports showing that phenothiazines can interact with DNA directly, cause local changes in the DNA and in this way influence replication and repair processes [336, 395].

Phenothiazines are reported to effect a plethora of cellular processes, ranging from antagonism of CaM and D2R signalling to fluidization of biological membrane and disruption of autophagy [279, 312, 316, 396]. Given this, we, in **Paper III**, tested if any of these molecular interactions could explain the observed impact of phenothiazines on DDR signalling. In this respect, we found that neither the CaM antagonist, W7, nor the D2R antagonist, L-741626, caused augmentation or inhibition of the bleomycin-induced DNA-PKcs autophosphorylation. This suggests that the observed effect of phenothiazines on DDR is distinct from other, well-known cellular activities of phenothiazines. In conclusion, results obtained in **Paper III** suggest that phenothiazines can antagonise DSB repair most likely independently of their best characterised pharmacological effects i.e. on neurotransmitter signalling, D2R antagonism [279], inhibition of the Ca2+/CaM pathway [312], and perturbation of lysosomal homeostasis [352, 353, 397].

Phenothiazines have previously been shown to potentiate the cytoxocity of different chemotherapy agents, including both, direct DNA DSB inducing agents i.e. bleomycin [360] and the ICL inducing agent i.e. cisplatin [376], in tumour cells. Along this line, in **Paper III**, we tested different tumour cell lines and agents i.e. bleomycin and cisplatin, with respect to autophosphorylation of DNA-PK, upon combined treatment. Interestingly, increased DNA-PKcs S2056 autophosphorylation was observed among tumour cells of different histologies after combination treatment, but, again, this chemosensitization effect was not observed in normal fibroblasts. Phenothiazines, e.g. TFP, did not affect DNA-PKcs autophosphorylation when used together with etoposide, gemcitabine or ionising radiation, and accordingly, no sensitisation was observed in NSCLC, breast and ovarian carcinomas after such combinations.

To further explore therapeutic utility of the sustained DNA-PK/ATM hyper-activation by phenothiazines and reveal possible therapeutic combinations, we tested if small molecule chemical inhibitors of ATM or against PARP, could further increase chemotherapy response. While ATM inhibitor, KU55993, or the clinically used PARP-1 inhibitor, olaparib, individually enhanced the sensitivity of NSCLC cells towards bleomycin/cisplatin, an

additive effect was evident when they were used in combination with TFP. These findings, thus, suggest that the adaptive hyperactivation of DNA-PK/ATM by TFP-mediated disruption of DSB repair can be targeted by other DNA repair inhibitors to maximise the efficacy of DNA DSB inducing chemotherapeutic drugs, and, hence, provide a novel therapeutic opportunity.

In summary, results obtained in **Paper III** present a new activity of phenothiazines with respect to DNA DSB repair inhibition, that can be used to specifically enhance the chemosensitivity of tumour cells, either when used alone or in tandem with DNA DSB inducing chemotherapeutic agents to increase treatment efficacy without affecting normal cells.

4.4 PAPER IV

Alteration of epigenetic signalling influence gene regulation and, hence, many fundamental processes in tumours [269, 270]. Accordingly, targeting chromatin-modifying factors i.e. the BET bromodomains or histone deacetylation (HDAC), have become a promising therapeutic concept in oncology [271, 398, 399], as illustrated by results in hematological malignancies, where the downregulation of myc-mediated transcription following BET inhibition is evident [398]. Interestingly, promising results of tandem use of chromatin-modifying drugs together with DNA damaging agents are reported, with the increased efficiency in cell death as a consequence of higher DNA damage formation and impaired DNA repair mechanisms [273, 275]. Thus, epigenetic alterations which influence DDR signalling open up a development of new therapeutic strategies. Previous findings from us and others have shown that phenothiazines have a potential to inhibit DNA DSB repair and sensitise to chemotherapy [360, 391, 400]. Moreover, structure-activity relationships and cytotoxicity of phenothiazines, provide further evidence that nuclear/chromatin localization is required for some of the cellular processes controlled by phenothiazines [336]. In **Paper III** we also showed that phenothiazines cause hyperactivation of DDR proteins i.e. DNA-PKcs and ATM, and their chromatin associated substrates, KAP-1 and RPA32, resulting in inhibition of DNA DSB repair. In **Paper IV**, we tried to elucidate and gain further insight into molecular interactions relevant to the modulation of DDR signalling caused by phenothiazines in the context of epigenetic signalling.

First, a bioinformatic tool, the Connectivity Map [317], was used to compare the phenothiazine-induced gene expression signatures obtained from different sources [365, 366, 401], with those elicited by bioactive small molecule compounds collected in the database for which action mechanisms were known. Interestingly, the results from cmap *in silico* analysis showed that human tumour cells treated with phenothiazines had a similar gene expression patterns as cells exposed to chromatin modulators, HDAC inhibitors, i.e. trichostatin A (TSA) and vorinostat (SAHA). This finding supported our hypothesis that phenothiazines may act as chromatin-active compounds. Moreover, recently reported findings further support this as HDAC inhibitors have also anti-depressant potential [402-405]. This finding suggested us a possible, yet unknown role of phenothiazines in the regulation of chromatin-related processes

in response to DNA damage, which we further studied in neuroblastoma (NB) and acute myeloid leukemia (AML), two tumour types in which myc activation and/or deregulation of other epigenetic signalling events are reported, and in which some therapeutic strategies attacking these signalling have been introduced [271, 406-408]. Additionally, the small cell lung cancer (SCLC) cells were also added to these analyses, as this tumour type show increased sensitivity to phenothiazine treatment as monotherapy, previously described in **Paper II** of this thesis.

Along with our hypothesis, tumour cells in consequence of altered transcription may have high level of DNA DSB and present hyperactivated DDR signalling. In such context, the DDR activation additionally triggered by phenothiazines would cause DNA DSB repair inhibition resulting in an increase cell death. Indeed, analysis of γH2AX (S139), a marker of DNA DSBs, revealed activation in all the NB cell lines, and most of SCLC and AML cells at basal level, indicating that endogenous DNA DSBs are evident in these tumour types. Accordingly, our initial results showed DNA-PKcs to be phosphorylated on S2056, the site previously found to be hyperactivated in response to phenothiazine treatment (**Paper III**), at basal level in some, but not all of the cell lines of the three tumour types examined. Moreover, the downstream target of DNA-PKcs, RPA32, was, indeed, found to be phosphorylated in the majority of the SCLC cells, suggesting hyperactivated NHEJ repair at basal level, whereas in NB and AML cell lines this effect was not so pronounced. Obtained results, thus, supported our hypothesis and next, we tested NB, SCLC and AML cell lines for their sensitivity to phenothiazine treatment either alone or in combination with the pan-HDACi panobinostat or the BRD4 antagonist JQ1. Importantly, we found that the two tested phenothiazines caused significant cytotoxicity in the cell lines from all three tumour types. The phenothiazine-induced cytotoxicity was comparable in terms of magnitude to those induced by HDACi, panobinostat, or the bromodomain-containing protein 4 (BRD4) antagonist, JQ1. Moreover, also increased phosphorylation, acetylation or expression of some of the DDR factors were also observed with Western blots and immunofluorescence analyses, in NB and AML cells, but less pronounced in SCLC cells, after either phenothiazines or chromatin-modifying drugs' treatment, which is in line with previously reported connections between HDACi/epigenetics and DNA repair [409].

It has previously been shown that disturbances of epigenetic signalling may influence chromatin-associated proteins by e.g. histone modifications [410]. Accordingly, we analysed if DNA-PKcs may have another localization pattern rendering it more susceptible for manipulation with phenothiazines. In a preliminary set of experiments with SCLC cells we, therefore, analysed the influence of the phenothiazine, TFP, on DNA-PKcs dynamics on the chromatin. Results showed that TFP, indeed, caused increased DNA-PKcs S0256 phosphorylation in the chromatin-bound fraction, whereas total DNA-PK in these fractions remained unchanged. Obtained results may suggest that phenothiazines delay repair of endogenous DNA DSBs, prolong the retention of DSBs repair/DDR factors on damaged chromatin and delay recovery, what is in line with our findings in **Paper III**. The possible underlying mechanisms and interactions in chromatin-bound fractions of tumour cells altered

by phenothiazines need to be further studied to find critical signalling factors responsible for the observed effect. We also tested the impact of toxicity of combined regimen of phenothiazines and panobinostat or JQ1. A pronounced decrease in cell viability and increased cell death signalling were evident when the phenothiazine, TFP, was used in combination with JQ1 in NB cells or with panobinostat in SCLC cells, respectively. Thus, increased apoptotic signalling was observed after combined treatment as illustrated by increased PARP-1 cleavage. After TFP and JQ1 or panobinostat treatment an increased lysosomal conversion of the autophagosomal marker, light chain 3 (LC3-II), was also evident, suggesting possible involvement of lysosomal signalling in the observed cytotoxic effects, which is in line with our previous studies, where we observed lysosomal dysfunctions as a consequence of phenothiazines treatment in SCLC cells (**Paper II**).

Taken together, the results presented in **Paper IV** revealed new features of phenothiazines and may offer a therapeutic avenue, especially when used in tandem with drugs targeting epigenome. However, further studies are needed to delineate and understand mechanism(s) in DDR signalling affected by phenothiazines, responsible for the observed increased cytotoxic effects.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

Successful clinical experience with accelerated ions and in consequence increasing importance of particle therapy require further understanding of radiobiological basis of the observed effect. Mathematical modelling is one of the tools used to describe the radiation effects and, therefore, also used to compare the relative efficiency in cell killing of ions and, conventional radiation i.e. photons. In **Paper I**, we compared two mathematical models, LQ and RCR, for the effects of high LET ion irradiation and low LET photons. We demonstrated that radiobiological parameters of the response to the low LET irradiation, assessed with the RCR model, was useful in prediction of the response to high LET ions. Moreover, results achieved with RCR model suggested that tumour cells with high DNA repair capacity and which were more resistant to low LET photons, still could benefit from radiation therapy with ion beams, as an increased biological effect was observed. The LQ model, when used for similar analysis, could not predict the expected increased radiobiological effectiveness of ion therapy. Hence, our results illustrate the need for further work on establishing reliable dose-response models including different endpoints for high LET radiation. Moreover, our results also indicate that differences among radiobiological models should be considered alongside with differences in RBE in TPS and evaluation of accelerated particle therapy in the clinical setting.

Despite continuous development of new combination treatments of chemotherapy and biological targeting agents, there is still urgent need for new strategies including compounds with capacity to potentiate anticancer agents in a tumour selective manner. Phenothiazines have previously been reported to have anti-proliferative activity and were proposed to have potential as an adjuvant to chemotherapy [302, 323, 375, 411]. In **Papers II-IV** of this thesis, we focused on elucidating phenothiazines as mono- or chemotherapy combined treatments in tumours cells with the aim to understand action mechanism and how such drugs should be further refined and used with improved therapeutic utility. It was previously demonstrated that phenothiazines, as lysosomotropic agents, accumulate in acidic compartments within cells, mainly within lysosomes [353]. In **Paper II**, we demonstrated that monotherapy with phenothiazines offered a novel treatment approach for SCLC, a tumour type which initially is sensitive to conventional chemotherapy, but rapidly develop resistance. We found that phenothiazines reduced cell viability and showed that this effect in part was due to phenothiazine-induced lysosomal dysfunction. Thus, we found that lysosomal/autophagic degradation in response to anticancer therapy can suppress viability of SCLC cells. Our results open up for a clinical re-utility of phenothiazines as a lysosome-targeted treatment, a strategy which may also be used in chemotherapy refractory cases. There is so far only rudimentary evidence for beneficial role of phenothiazines as anti-cancer agents *in vivo* [333, 377, 412-414], and in none of these studies SCLC was the tumour type tested. Hence, for development of phenothiazines as a treatment for SCLC, it is critical with further *in vivo* assessment involving both toxicity studies in mice, but also *in vivo* efficacy studies using SCLC cell line xenografts or preferentially patient-derived xenografts from such patients as they better recapitulate the *in vivo* complexity of the tumour. Such *in vivo* efficacy studies of

phenothiazines in SCLC xenografts should also involve analysis of autophagic degradation/perturbations as to evaluate their potential as biomarker of response.

Previous results showed that phenothiazines may impede DNA strand break repair [360, 391, 400]. In **Paper III**, our mechanistic studies further confirmed a blocking effect on NHEJ-mediated repair. Importantly, we also uncovered that phenothiazine-mediated inhibition of chemotherapy-induced DNA DSBs repair was associated with enhance chromatin-centered DNA-PK/ATM signalling, with augmented substrate phosphorylation, and protracted checkpoint arrest. Moreover, to approach therapeutic utility of prolonged DNA-PK/ATM activation caused by phenothiazines, we show an additive effect of combination of phenothiazines, DNA damaging agents and small molecule chemical DNA repair inhibitors of ATM or against PARP. Here, further characterization of phenothiazine interaction with DDR signalling components in tumour cells in an unbiased way, such as global proteomic analyses, is the way ahead to reveal information about putative components involved. Such information could in turn allow construction of a phenothiazine-like chemical library and further screening to reveal even more potent phenothiazine-derived compounds to be used in combination with chemotherapy.

Recent findings on phenothiazines suggest these compounds to have antimetastatic action [415], inhibit growth of cancer stem cells (CSC), overcoming drug resistance in *in vitro* and *in vivo* settings [416, 417], but also utility for combination with small molecule inhibitors against mutant EGFR [418]. Our results presented in **Paper IV** revealed another function of these drugs, as potential chromatin-active compounds which can be considered for treatment of epigenetically deregulated tumours i.e. neuroblastoma (NB) and acute myeloid leukemia (AML), but also SCLC. Interestingly, we found that phenothiazines are able to prolong phosphorylation of DNA-PKcs in chromatin fractions of SCLC cells, what could be followed by further analysis of potential target(s) of phenothiazines at this site. Here, chromatin binding proteome studies in the presence and absence of phenothiazines is one step ahead that may lead to discovery of key signalling components. As epigenetics play significant role in tumour cells, our findings suggest a novel possible therapeutic utility for these compounds. Interestingly, supporting this hypothesis, a novel phenothiazine-derived compound i.e. inauhzin (INZ), was recently described and shown to be a potent SIRT1 inhibitor, that activates p53 by inducing epigenetic regulations, inhibiting its deacetylation, thereby suppressing tumour growth and enhancing chemosensitivity [419-421]. These findings are in line with our observation about phenothiazines in NB, AML and SCLC cells. Moreover, even results of the studies on cancer incidence among patients with schizophrenia, treated with phenothiazines, show their protective effect against cancer and possible antitumour activity of phenothiazines apart from their functions in CNS [422-425].

In summary, phenothiazines have a plethora of cellular targets and functions, some new also uncovered in this thesis. These new functions of phenothiazines i.e. targeting lysosomal perturbations of SCLC when used in monotherapy, or interfering with chromatin-associated DDR signalling when combined with chemotherapy or chromatin targeting drugs warrants

further development and studies on clinical applicability to allow them to be used as novel treatment approaches for LC and other tumour malignancies in the clinical settings.

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7 REFERENCES

1. Torre, L.A., et al., *Global cancer statistics, 2012.* CA Cancer J Clin, 2015. **65**(2): p. 87-108.
2. http://www.cancer.gov/statistics.
3. https://www.cancerfonden.se.
4. Chen, Z., et al., *Non-small-cell lung cancers: a heterogeneous set of diseases.* Nat Rev Cancer, 2014. **14**(8): p. 535-46.
5. Morgensztern, D., et al., *Molecularly targeted therapies in non-small-cell lung cancer annual update 2014.* J Thorac Oncol, 2015. **10**(1 Suppl 1): p. S1-63.
6. Viktorsson, K., L. De Petris, and R. Lewensohn, *The role of p53 in treatment responses of lung cancer.* Biochem Biophys Res Commun, 2005. **331**(3): p. 868-80.
7. Viktorsson, K., R. Lewensohn, and B. Zhivotovsky, *Systems biology approaches to develop innovative strategies for lung cancer therapy.* Cell Death Dis, 2014. **5**: p. e1260.
8. Stovold, R., et al., *Biomarkers for small cell lung cancer: neuroendocrine, epithelial and circulating tumour cells.* Lung Cancer, 2012. **76**(3): p. 263-8.
9. Stovold, R., et al., *Neuroendocrine and epithelial phenotypes in small-cell lung cancer: implications for metastasis and survival in patients.* Br J Cancer, 2013. **108**(8): p. 1704-11.
10. Lawrence, M.S., et al., *Mutational heterogeneity in cancer and the search for new cancer-associated genes.* Nature, 2013. **499**(7457): p. 214-8.
11. Peifer, M., et al., *Integrative genome analyses identify key somatic driver mutations of small-cell lung cancer.* Nat Genet, 2012. **44**(10): p. 1104-10.
12. Byers, L.A., et al., *Proteomic profiling identifies dysregulated pathways in small cell lung cancer and novel therapeutic targets including PARP1.* Cancer Discov, 2012. **2**(9): p. 798-811.
13. Pietanza, M.C. and M. Ladanyi, *Bringing the genomic landscape of small-cell lung cancer into focus.* Nat Genet, 2012. **44**(10): p. 1074-5.
14. Rudin, C.M., et al., *Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer.* Nat Genet, 2012. **44**(10): p. 1111-6.
15. Puglisi, M., et al., *Treatment options for small cell lung cancer - do we have more choice?* Br J Cancer, 2010. **102**(4): p. 629-38.
16. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
17. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
18. Weinberg, R.A., *The Biology of Cancer*2007.

19. De Palma, M. and D. Hanahan, *The biology of personalized cancer medicine: facing individual complexities underlying hallmark capabilities.* Mol Oncol, 2012. **6**(2): p. 111-27.

20. Hanahan, D., *Rethinking the war on cancer.* Lancet, 2014. **383**(9916): p. 558-63.

21. Hall, E.J.G., A.J., *Radiobiology for the Radiologist*2006.

22. Hall, E.J.G., A.J., *Radiobiology for the Radiologist*2012.

23. Skarsgard, L.D., *Radiobiology with heavy charged particles: a historical review.* Phys Med, 1998. **14 Suppl 1**: p. 1-19.

24. Kraft, G., *Tumor Therapy with Heavy Charged Particles.* Progress in Particle and Nuclear Physics, 2000.

25. Blakely, E.A. and P.Y. Chang, *Biology of charged particles.* Cancer J, 2009. **15**(4): p. 271-84.

26. Durante, M. and J.S. Loeffler, *Charged particles in radiation oncology.* Nat Rev Clin Oncol, 2010. **7**(1): p. 37-43.

27. http://www.ptcog.ch.

28. Tsujii, H. and T. Kamada, *A review of update clinical results of carbon ion radiotherapy.* Jpn J Clin Oncol, 2012. **42**(8): p. 670-85.

29. Kamada, T., *Clinical evidence of particle beam therapy (carbon).* Int J Clin Oncol, 2012. **17**(2): p. 85-8.

30. Pouget, J.P. and S.J. Mather, *General aspects of the cellular response to low- and high-LET radiation.* Eur J Nucl Med, 2001. **28**(4): p. 541-61.

31. Kamada, T., et al., *Carbon ion radiotherapy in Japan: an assessment of 20 years of clinical experience.* Lancet Oncol, 2015. **16**(2): p. e93-e100.

32. Okada, T., et al., *Carbon ion radiotherapy: clinical experiences at National Institute of Radiological Science (NIRS).* J Radiat Res, 2010. **51**(4): p. 355-64.

33. Schulz-Ertner, D. and H. Tsujii, *Particle radiation therapy using proton and heavier ion beams.* J Clin Oncol, 2007. **25**(8): p. 953-64.

34. Loeffler, J.S. and M. Durante, *Charged particle therapy--optimization, challenges and future directions.* Nat Rev Clin Oncol, 2013. **10**(7): p. 411-24.

35. Minohara, S., et al., *Recent innovations in carbon-ion radiotherapy.* J Radiat Res, 2010. **51**(4): p. 385-92.

36. Jensen, A.D., M.W. Munter, and J. Debus, *Review of clinical experience with ion beam radiotherapy.* Br J Radiol, 2011. **84 Spec No 1**: p. S35-47.

37. Tsujii, H., et al., *Clinical Results of Carbon Ion Radiotherapy at NIRS.* J Radiat Res, 2007. **48 Suppl A**: p. A1-A13.

38. Goodhead, D.T., *Mechanisms for the biological effectiveness of high-LET radiations.* J Radiat Res, 1999. **40 Suppl**: p. 1-13.

39. Weyrather, W.K. and J. Debus, *Particle beams for cancer therapy.* Clin Oncol (R Coll Radiol), 2003. **15**(1): p. S23-8.

40. Sorensen, B.S., J. Overgaard, and N. Bassler, *In vitro RBE-LET dependence for multiple particle types.* Acta Oncol, 2011. **50**(6): p. 757-62.

41. Durante, M., *New challenges in high-energy particle radiobiology.* Br J Radiol, 2014. **87**(1035): p. 20130626.

42. Friedrich, T., et al., *Systematic analysis of RBE and related quantities using a database of cell survival experiments with ion beam irradiation.* J Radiat Res, 2013. **54**(3): p. 494-514.

43. Vijayakumar, S., et al., *Mean inactivation dose (D). A critical analysis of a neglected parameter in radiotherapy.* Acta Oncol, 1990. **29**(1): p. 65-72.

44. Puck, T.T. and P.I. Marcus, *Action of x-rays on mammalian cells.* J Exp Med, 1956. **103**(5): p. 653-66.

45. Pomp, J., et al., *Cell density dependent plating efficiency affects outcome and interpretation of colony forming assays.* Radiother Oncol, 1996. **40**(2): p. 121-5.

46. Lind, B.K., et al., *Repairable-conditionally repairable damage model based on dual Poisson processes.* Radiat Res, 2003. **160**(3): p. 366-75.

47. Kase, Y., et al., *Microdosimetric approach to NIRS-defined biological dose measurement for carbon-ion treatment beam.* J Radiat Res, 2011. **52**(1): p. 59-68.

48. Elsasser, T., M. Kramer, and M. Scholz, *Accuracy of the local effect model for the prediction of biologic effects of carbon ion beams in vitro and in vivo.* Int J Radiat Oncol Biol Phys, 2008. **71**(3): p. 866-72.

49. Kramer, M. and M. Scholz, *Treatment planning for heavy-ion radiotherapy: calculation and optimization of biologically effective dose.* Phys Med Biol, 2000. **45**(11): p. 3319-30.

50. Inaniwa, T., et al., *Treatment planning for a scanned carbon beam with a modified microdosimetric kinetic model.* Phys Med Biol, 2010. **55**(22): p. 6721-37.

51. Yaes, R.J., P. Patel, and Y. Maruyama, *On using the linear-quadratic model in daily clinical practice.* Int J Radiat Oncol Biol Phys, 1991. **20**(6): p. 1353-62.

52. Hill, A.A. and L.D. Skarsgard, *Cell-age heterogeneity and deviations from the LQ model in the radiation survival responses of human tumour cells.* Int J Radiat Biol, 1999. **75**(11): p. 1409-20.

53. Jones, L., P. Hoban, and P. Metcalfe, *The use of the linear quadratic model in radiotherapy: a review.* Australas Phys Eng Sci Med, 2001. **24**(3): p. 132-46.

54. Wouters, B.G., A.M. Sy, and L.D. Skarsgard, *Low-dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity.* Radiat Res, 1996. **146**(4): p. 399-413.

55. Iwata, H., et al., *Compatibility of the repairable-conditionally repairable, multi-target and linear-quadratic models in converting hypofractionated radiation doses to single doses.* J Radiat Res, 2013. **54**(2): p. 367-73.

56. Jackson, S.P., *Detecting, signalling and repairing DNA double-strand breaks.* Biochem Soc Trans, 2001. **29**(Pt 6): p. 655-61.

57. Jackson, S.P. and J. Bartek, *The DNA-damage response in human biology and disease.* Nature, 2009. **461**(7267): p. 1071-8.

58. Rajagopalan, H. and C. Lengauer, *Aneuploidy and cancer.* Nature, 2004. **432**(7015): p. 338-41.

59. Hoeijmakers, J.H., *Genome maintenance mechanisms for preventing cancer.* Nature, 2001. **411**(6835): p. 366-74.

60. Friedberg, E.C., L.D. McDaniel, and R.A. Schultz, *The role of endogenous and exogenous DNA damage and mutagenesis.* Curr Opin Genet Dev, 2004. **14**(1): p. 5-10.

61. Kastan, M.B., *DNA damage responses: mechanisms and roles in human disease: 2007 G.H.A. Clowes Memorial Award Lecture.* Mol Cancer Res, 2008. **6**(4): p. 517-24.

62. Bensimon, A., R. Aebersold, and Y. Shiloh, *Beyond ATM: the protein kinase landscape of the DNA damage response.* FEBS Lett, 2011. **585**(11): p. 1625-39.

63. Khanna, K.K. and S.P. Jackson, *DNA double-strand breaks: signaling, repair and the cancer connection.* Nat Genet, 2001. **27**(3): p. 247-54.

64. Ciccia, A. and S.J. Elledge, *The DNA damage response: making it safe to play with knives.* Mol Cell, 2010. **40**(2): p. 179-204.

65. Giglia-Mari, G., A. Zotter, and W. Vermeulen, *DNA damage response.* Cold Spring Harb Perspect Biol, 2011. **3**(1): p. a000745.

66. Harper, J.W. and S.J. Elledge, *The DNA damage response: ten years after.* Mol Cell, 2007. **28**(5): p. 739-45.

67. Ljungman, M., *The DNA damage response--repair or despair?* Environ Mol Mutagen, 2010. **51**(8-9): p. 879-89.

68. Campisi, J. and F. d'Adda di Fagagna, *Cellular senescence: when bad things happen to good cells.* Nat Rev Mol Cell Biol, 2007. **8**(9): p. 729-40.

69. Halazonetis, T.D., V.G. Gorgoulis, and J. Bartek, *An oncogene-induced DNA damage model for cancer development.* Science, 2008. **319**(5868): p. 1352-5.

70. de Jager, M., et al., *Human Rad50/Mre11 is a flexible complex that can tether DNA ends.* Mol Cell, 2001. **8**(5): p. 1129-35.

71. D'Silva, I., et al., *Relative affinities of poly(ADP-ribose) polymerase and DNA-dependent protein kinase for DNA strand interruptions.* Biochim Biophys Acta, 1999. **1430**(1): p. 119-26.

72. Gottlieb, T.M. and S.P. Jackson, *The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen.* Cell, 1993. **72**(1): p. 131-42.

73. Tomimatsu, N., et al., *Ku70/80 modulates ATM and ATR signaling pathways in response to DNA double strand breaks.* J Biol Chem, 2007. **282**(14): p. 10138-45.

74. Cheng, Q., et al., *Ku counteracts mobilization of PARP1 and MRN in chromatin damaged with DNA double-strand breaks.* Nucleic Acids Res, 2011. **39**(22): p. 9605-19.

75. Kastan, M.B. and D.S. Lim, *The many substrates and functions of ATM.* Nat Rev Mol Cell Biol, 2000. **1**(3): p. 179-86.

76. Shiloh, Y., *ATM and ATR: networking cellular responses to DNA damage.* Curr Opin Genet Dev, 2001. **11**(1): p. 71-7.

77. Shiloh, Y. and Y. Ziv, *The ATM protein kinase: regulating the cellular response to genotoxic stress, and more.* Nat Rev Mol Cell Biol, 2013. **14**(4): p. 197-210.

78. Falck, J., J. Coates, and S.P. Jackson, *Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage.* Nature, 2005. **434**(7033): p. 605-11.

79. Dupre, A., L. Boyer-Chatenet, and J. Gautier, *Two-step activation of ATM by DNA and the Mre11-Rad50-Nbs1 complex.* Nat Struct Mol Biol, 2006. **13**(5): p. 451-7.

80. Shiloh, Y., *ATM and related protein kinases: safeguarding genome integrity.* Nat Rev Cancer, 2003. **3**(3): p. 155-68.

81. Bartek, J. and J. Lukas, *DNA damage checkpoints: from initiation to recovery or adaptation.* Curr Opin Cell Biol, 2007. **19**(2): p. 238-45.

82. Cimprich, K.A. and D. Cortez, *ATR: an essential regulator of genome integrity.* Nat Rev Mol Cell Biol, 2008. **9**(8): p. 616-27.

83. Andreassen, P.R., G.P. Ho, and A.D. D'Andrea, *DNA damage responses and their many interactions with the replication fork.* Carcinogenesis, 2006. **27**(5): p. 883-92.

84. Jazayeri, A., et al., *ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks.* Nat Cell Biol, 2006. **8**(1): p. 37-45.

85. Smith, J., et al., *The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer.* Adv Cancer Res, 2010. **108**: p. 73-112.

86. Zhou, B.B. and S.J. Elledge, *The DNA damage response: putting checkpoints in perspective.* Nature, 2000. **408**(6811): p. 433-9.

87. Bartek, J. and J. Lukas, *Mammalian G1- and S-phase checkpoints in response to DNA damage.* Curr Opin Cell Biol, 2001. **13**(6): p. 738-47.

88. Kastan, M.B. and J. Bartek, *Cell-cycle checkpoints and cancer.* Nature, 2004. **432**(7015): p. 316-23.

89. Niida, H. and M. Nakanishi, *DNA damage checkpoints in mammals.* Mutagenesis, 2006. **21**(1): p. 3-9.

90. Xiao, Z., et al., *Chk1 mediates S and G2 arrests through Cdc25A degradation in response to DNA-damaging agents.* J Biol Chem, 2003. **278**(24): p. 21767-73.

91. Massague, J., *G1 cell-cycle control and cancer.* Nature, 2004. **432**(7015): p. 298-306.

92. Asghar, U., et al., *The history and future of targeting cyclin-dependent kinases in cancer therapy.* Nat Rev Drug Discov, 2015. **14**(2): p. 130-46.

93. Sorensen, C.S., et al., *Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A.* Cancer Cell, 2003. **3**(3): p. 247-58.

94. Mailand, N., et al., *Regulation of G(2)/M events by Cdc25A through phosphorylation-dependent modulation of its stability.* EMBO J, 2002. **21**(21): p. 5911-20.

95. Branzei, D. and M. Foiani, *Regulation of DNA repair throughout the cell cycle.* Nat Rev Mol Cell Biol, 2008. **9**(4): p. 297-308.

96. Bartek, J. and J. Lukas, *Chk1 and Chk2 kinases in checkpoint control and cancer.* Cancer Cell, 2003. **3**(5): p. 421-9.

97. Ahn, J., M. Urist, and C. Prives, *The Chk2 protein kinase.* DNA Repair (Amst), 2004. **3**(8-9): p. 1039-47.

98. Luo, Y., et al., *Blocking Chk1 expression induces apoptosis and abrogates the G2 checkpoint mechanism.* Neoplasia, 2001. **3**(5): p. 411-9.

99. Koniaras, K., et al., *Inhibition of Chk1-dependent G2 DNA damage checkpoint radiosensitizes p53 mutant human cells.* Oncogene, 2001. **20**(51): p. 7453-63.

100. Ashwell, S. and S. Zabludoff, *DNA damage detection and repair pathways--recent advances with inhibitors of checkpoint kinases in cancer therapy.* Clin Cancer Res, 2008. **14**(13): p. 4032-7.

101. Wolffe, A.P., *Chromatin remodeling: why it is important in cancer.* Oncogene, 2001. **20**(24): p. 2988-90.

102. Huen, M.S. and J. Chen, *The DNA damage response pathways: at the crossroad of protein modifications.* Cell Res, 2008. **18**(1): p. 8-16.

103. Ziv, Y., et al., *Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway.* Nat Cell Biol, 2006. **8**(8): p. 870-6.

104. Bartek, J., J. Lukas, and J. Bartkova, *DNA damage response as an anti-cancer barrier: damage threshold and the concept of 'conditional haploinsufficiency'.* Cell Cycle, 2007. **6**(19): p. 2344-7.

105. Misteli, T. and E. Soutoglou, *The emerging role of nuclear architecture in DNA repair and genome maintenance.* Nat Rev Mol Cell Biol, 2009. **10**(4): p. 243-54.

106. Sulli, G., R. Di Micco, and F. d'Adda di Fagagna, *Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer.* Nat Rev Cancer, 2012. **12**(10): p. 709-20.

107. Lukas, J., C. Lukas, and J. Bartek, *More than just a focus: The chromatin response to DNA damage and its role in genome integrity maintenance.* Nat Cell Biol, 2011. **13**(10): p. 1161-9.

108. Goodarzi, A.A., et al., *ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin.* Mol Cell, 2008. **31**(2): p. 167-77.

109. Polo, S.E. and S.P. Jackson, *Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications.* Genes Dev, 2011. **25**(5): p. 409-33.

110. Shi, L. and P. Oberdoerffer, *Chromatin dynamics in DNA double-strand break repair.* Biochim Biophys Acta, 2012. **1819**(7): p. 811-9.

111. Becker, A., et al., *ATM alters the otherwise robust chromatin mobility at sites of DNA double-strand breaks (DSBs) in human cells.* PLoS One, 2014. **9**(3): p. e92640.

112. Costelloe, T., et al., *Chromatin modulation and the DNA damage response.* Exp Cell Res, 2006. **312**(14): p. 2677-86.

113. Kinner, A., et al., *Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin.* Nucleic Acids Res, 2008. **36**(17): p. 5678-94.

114. Mah, L.J., A. El-Osta, and T.C. Karagiannis, *gammaH2AX: a sensitive molecular marker of DNA damage and repair.* Leukemia, 2010. **24**(4): p. 679-86.

115. Cann, K.L. and G. Dellaire, *Heterochromatin and the DNA damage response: the need to relax.* Biochem Cell Biol, 2011. **89**(1): p. 45-60.

116. Miller, K.M. and S.P. Jackson, *Histone marks: repairing DNA breaks within the context of chromatin.* Biochem Soc Trans, 2012. **40**(2): p. 370-6.

117. Fernandez-Capetillo, O., et al., *H2AX: the histone guardian of the genome.* DNA Repair (Amst), 2004. **3**(8-9): p. 959-67.

118. Kuo, L.J. and L.X. Yang, *Gamma-H2AX - a novel biomarker for DNA double-strand breaks.* In Vivo, 2008. **22**(3): p. 305-9.

119. Dickey, J.S., et al., *H2AX: functional roles and potential applications.* Chromosoma, 2009. **118**(6): p. 683-92.

120. Kwon, S.J., et al., *ATM-mediated phosphorylation of the chromatin remodeling enzyme BRG1 modulates DNA double-strand break repair.* Oncogene, 2015. **34**(3): p. 303-13.

121. Ataian, Y. and J.E. Krebs, *Five repair pathways in one context: chromatin modification during DNA repair.* Biochem Cell Biol, 2006. **84**(4): p. 490-504.

122. Murr, R., et al., *Histone acetylation by Trrap-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks.* Nat Cell Biol, 2006. **8**(1): p. 91-9.

123. Morrison, A.J. and X. Shen, *Chromatin remodelling beyond transcription: the INO80 and SWR1 complexes.* Nat Rev Mol Cell Biol, 2009. **10**(6): p. 373-84.

124. van Attikum, H. and S.M. Gasser, *Crosstalk between histone modifications during the DNA damage response.* Trends Cell Biol, 2009. **19**(5): p. 207-17.

125. Goodarzi, A.A., A.T. Noon, and P.A. Jeggo, *The impact of heterochromatin on DSB repair.* Biochem Soc Trans, 2009. **37**(Pt 3): p. 569-76.

126. White, D.E., et al., *KAP1, a novel substrate for PIKK family members, colocalizes with numerous damage response factors at DNA lesions.* Cancer Res, 2006. **66**(24): p. 11594-9.

127. Noon, A.T., et al., *53BP1-dependent robust localized KAP-1 phosphorylation is essential for heterochromatic DNA double-strand break repair.* Nat Cell Biol, 2010. **12**(2): p. 177-84.

128. Sun, Y., X. Jiang, and B.D. Price, *Tip60: connecting chromatin to DNA damage signaling.* Cell Cycle, 2010. **9**(5): p. 930-6.

129. Humpal, S.E., D.A. Robinson, and J.E. Krebs, *Marks to stop the clock: histone modifications and checkpoint regulation in the DNA damage response.* Biochem Cell Biol, 2009. **87**(1): p. 243-53.

130. Iyengar, S. and P.J. Farnham, *KAP1 protein: an enigmatic master regulator of the genome.* J Biol Chem, 2011. **286**(30): p. 26267-76.

131. Kim, M.S., et al., *Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA.* Cancer Res, 2003. **63**(21): p. 7291-300.

132. Fortini, P., et al., *The base excision repair: mechanisms and its relevance for cancer susceptibility.* Biochimie, 2003. **85**(11): p. 1053-71.

133. Costa, R.M., et al., *The eukaryotic nucleotide excision repair pathway.* Biochimie, 2003. **85**(11): p. 1083-99.

134. Karran, P., J. Offman, and M. Bignami, *Human mismatch repair, drug-induced DNA damage, and secondary cancer.* Biochimie, 2003. **85**(11): p. 1149-60.

135. Czornak, K., S. Chughtai, and K.H. Chrzanowska, *Mystery of DNA repair: the role of the MRN complex and ATM kinase in DNA damage repair.* J Appl Genet, 2008. **49**(4): p. 383-96.

136. Lieber, M.R., *The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway.* Annu Rev Biochem, 2010. **79**: p. 181-211.

137. Kakarougkas, A. and P.A. Jeggo, *DNA DSB repair pathway choice: an orchestrated handover mechanism.* Br J Radiol, 2014. **87**(1035): p. 20130685.

138. Shiloh, Y., *ATM (ataxia telangiectasia mutated): expanding roles in the DNA damage response and cellular homeostasis.* Biochem Soc Trans, 2001. **29**(Pt 6): p. 661-6.

139. Davies, O.R., et al., *CtIP tetramer assembly is required for DNA-end resection and repair.* Nat Struct Mol Biol, 2015. **22**(2): p. 150-7.

140. Burma, S., et al., *ATM phosphorylates histone H2AX in response to DNA double-strand breaks.* J Biol Chem, 2001. **276**(45): p. 42462-7.

141. Kitagawa, R. and M.B. Kastan, *The ATM-dependent DNA damage signaling pathway.* Cold Spring Harb Symp Quant Biol, 2005. **70**: p. 99-109.

142. Matsuoka, S., et al., *ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage.* Science, 2007. **316**(5828): p. 1160-6.

143. Cann, K.L. and G.G. Hicks, *Regulation of the cellular DNA double-strand break response.* Biochem Cell Biol, 2007. **85**(6): p. 663-74.

144. Li, X. and W.D. Heyer, *Homologous recombination in DNA repair and DNA damage tolerance.* Cell Res, 2008. **18**(1): p. 99-113.

145. Powell, S.N. and L.A. Kachnic, *Roles of BRCA1 and BRCA2 in homologous recombination, DNA replication fidelity and the cellular response to ionizing radiation.* Oncogene, 2003. **22**(37): p. 5784-91.

146. Narod, S.A. and W.D. Foulkes, *BRCA1 and BRCA2: 1994 and beyond.* Nat Rev Cancer, 2004. **4**(9): p. 665-76.

147. Gudmundsdottir, K. and A. Ashworth, *The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability.* Oncogene, 2006. **25**(43): p. 5864-74.

148. Binz, S.K., A.M. Sheehan, and M.S. Wold, *Replication protein A phosphorylation and the cellular response to DNA damage.* DNA Repair (Amst), 2004. **3**(8-9): p. 1015-24.

149. Lees-Miller, S.P. and K. Meek, *Repair of DNA double strand breaks by non-homologous end joining.* Biochimie, 2003. **85**(11): p. 1161-73.

150. Pastwa, E. and J. Blasiak, *Non-homologous DNA end joining.* Acta Biochim Pol, 2003. **50**(4): p. 891-908.

151. Mari, P.O., et al., *Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4.* Proc Natl Acad Sci U S A, 2006. **103**(49): p. 18597-602.

152. Uematsu, N., et al., *Autophosphorylation of DNA-PKCS regulates its dynamics at DNA double-strand breaks.* J Cell Biol, 2007. **177**(2): p. 219-29.

153. Collis, S.J., et al., *The life and death of DNA-PK.* Oncogene, 2005. **24**(6): p. 949-61.

154. Meek, K., V. Dang, and S.P. Lees-Miller, *DNA-PK: the means to justify the ends?* Adv Immunol, 2008. **99**: p. 33-58.

155. Neal, J.A., et al., *Unraveling the complexities of DNA-dependent protein kinase autophosphorylation.* Mol Cell Biol, 2014. **34**(12): p. 2162-75.

156. Kurimasa, A., et al., *Requirement for the kinase activity of human DNA-dependent protein kinase catalytic subunit in DNA strand break rejoining.* Mol Cell Biol, 1999. **19**(5): p. 3877-84.

157. Weterings, E., et al., *The role of DNA dependent protein kinase in synapsis of DNA ends.* Nucleic Acids Res, 2003. **31**(24): p. 7238-46.

158. Dobbs, T.A., J.A. Tainer, and S.P. Lees-Miller, *A structural model for regulation of NHEJ by DNA-PKcs autophosphorylation.* DNA Repair (Amst), 2010. **9**(12): p. 1307-14.

159. Neal, J.A. and K. Meek, *Choosing the right path: does DNA-PK help make the decision?* Mutat Res, 2011. **711**(1-2): p. 73-86.

160. Koch, C.A., et al., *Xrcc4 physically links DNA end processing by polynucleotide kinase to DNA ligation by DNA ligase IV.* EMBO J, 2004. **23**(19): p. 3874-85.

161. Drouet, J., et al., *DNA-dependent protein kinase and XRCC4-DNA ligase IV mobilization in the cell in response to DNA double strand breaks.* J Biol Chem, 2005. **280**(8): p. 7060-9.

162. Weterings, E. and D.J. Chen, *The endless tale of non-homologous end-joining.* Cell Res, 2008. **18**(1): p. 114-24.

163. Weterings, E., et al., *The Ku80 carboxy terminus stimulates joining and artemis-mediated processing of DNA ends.* Mol Cell Biol, 2009. **29**(5): p. 1134-42.

164. Yano, K., et al., *Ku recruits XLF to DNA double-strand breaks.* EMBO Rep, 2008. **9**(1): p. 91-6.

165. Wang, H., et al., *Biochemical evidence for Ku-independent backup pathways of NHEJ.* Nucleic Acids Res, 2003. **31**(18): p. 5377-88.

166. Iliakis, G., *Backup pathways of NHEJ in cells of higher eukaryotes: cell cycle dependence.* Radiother Oncol, 2009. **92**(3): p. 310-5.

167. Bennardo, N., et al., *Alternative-NHEJ is a mechanistically distinct pathway of mammalian chromosome break repair.* PLoS Genet, 2008. **4**(6): p. e1000110.

168. McVey, M. and S.E. Lee, *MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings.* Trends Genet, 2008. **24**(11): p. 529-38.

169. Lieber, M.R., *NHEJ and its backup pathways in chromosomal translocations.* Nat Struct Mol Biol, 2010. **17**(4): p. 393-5.

170. Nussenzweig, A. and M.C. Nussenzweig, *A backup DNA repair pathway moves to the forefront.* Cell, 2007. **131**(2): p. 223-5.

171. Lakin, N.D. and S.P. Jackson, *Regulation of p53 in response to DNA damage.* Oncogene, 1999. **18**(53): p. 7644-55.

172. El-Deiry, W.S., *The role of p53 in chemosensitivity and radiosensitivity.* Oncogene, 2003. **22**(47): p. 7486-95.

173. Norbury, C.J. and B. Zhivotovsky, *DNA damage-induced apoptosis.* Oncogene, 2004. **23**(16): p. 2797-808.

174. Chipuk, J.E. and D.R. Green, *Dissecting p53-dependent apoptosis.* Cell Death Differ, 2006. **13**(6): p. 994-1002.

175. Meek, D.W., *Tumour suppression by p53: a role for the DNA damage response?* Nat Rev Cancer, 2009. **9**(10): p. 714-23.

176. Bieging, K.T., S.S. Mello, and L.D. Attardi, *Unravelling mechanisms of p53-mediated tumour suppression.* Nat Rev Cancer, 2014. **14**(5): p. 359-70.

177. Bursch, W., *Multiple cell death programs: Charon's lifts to Hades.* FEMS Yeast Res, 2004. **5**(2): p. 101-10.

178. Galluzzi, L., et al., *Essential versus accessory aspects of cell death: recommendations of the NCCD 2015.* Cell Death Differ, 2015. **22**(1): p. 58-73.

179. Roos, W.P. and B. Kaina, *DNA damage-induced cell death by apoptosis.* Trends Mol Med, 2006. **12**(9): p. 440-50.

180. Zong, W.X., et al., *Alkylating DNA damage stimulates a regulated form of necrotic cell death.* Genes Dev, 2004. **18**(11): p. 1272-82.

181. Golstein, P. and G. Kroemer, *Cell death by necrosis: towards a molecular definition.* Trends Biochem Sci, 2007. **32**(1): p. 37-43.

182. Castedo, M., et al., *Cell death by mitotic catastrophe: a molecular definition.* Oncogene, 2004. **23**(16): p. 2825-37.

183. Vakifahmetoglu, H., M. Olsson, and B. Zhivotovsky, *Death through a tragedy: mitotic catastrophe.* Cell Death Differ, 2008. **15**(7): p. 1153-62.

184. Vitale, I., et al., *Mitotic catastrophe: a mechanism for avoiding genomic instability.* Nat Rev Mol Cell Biol, 2011. **12**(6): p. 385-92.

185. Kroemer, G. and M. Jaattela, *Lysosomes and autophagy in cell death control.* Nat Rev Cancer, 2005. **5**(11): p. 886-97.

186. Rodriguez-Rocha, H., et al., *DNA damage and autophagy.* Mutat Res, 2011. **711**(1-2): p. 158-66.

187. Campisi, J., *Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors.* Cell, 2005. **120**(4): p. 513-22.

188. Acosta, J.C. and J. Gil, *Senescence: a new weapon for cancer therapy.* Trends Cell Biol, 2012. **22**(4): p. 211-9.

189. Amaravadi, R.K. and C.B. Thompson, *The roles of therapy-induced autophagy and necrosis in cancer treatment.* Clin Cancer Res, 2007. **13**(24): p. 7271-9.

190. Beausejour, C.M., et al., *Reversal of human cellular senescence: roles of the p53 and p16 pathways.* EMBO J, 2003. **22**(16): p. 4212-22.

191. Abedin, M.J., et al., *Autophagy delays apoptotic death in breast cancer cells following DNA damage.* Cell Death Differ, 2007. **14**(3): p. 500-10.

192. Illidge, T.M., et al., *Polyploid giant cells provide a survival mechanism for p53 mutant cells after DNA damage.* Cell Biol Int, 2000. **24**(9): p. 621-33.

193. Bitomsky, N. and T.G. Hofmann, *Apoptosis and autophagy: Regulation of apoptosis by DNA damage signalling - roles of p53, p73 and HIPK2.* FEBS J, 2009. **276**(21): p. 6074-83.

194. Hait, W.N., S. Jin, and J.M. Yang, *A matter of life or death (or both): understanding autophagy in cancer.* Clin Cancer Res, 2006. **12**(7 Pt 1): p. 1961-5.

195. Mathew, R., et al., *Autophagy suppresses tumor progression by limiting chromosomal instability.* Genes Dev, 2007. **21**(11): p. 1367-81.

196. Shay, J.W. and W.E. Wright, *Role of telomeres and telomerase in cancer.* Semin Cancer Biol, 2011. **21**(6): p. 349-53.

197. Viktorsson, K., R. Lewensohn, and B. Zhivotovsky, *Apoptotic pathways and therapy resistance in human malignancies.* Adv Cancer Res, 2005. **94**: p. 143-96.

198. Dragovich, T., C.M. Rudin, and C.B. Thompson, *Signal transduction pathways that regulate cell survival and cell death.* Oncogene, 1998. **17**(25): p. 3207-13.

199. Creagh, E.M. and S.J. Martin, *Caspases: cellular demolition experts.* Biochem Soc Trans, 2001. **29**(Pt 6): p. 696-702.

200. Danial, N.N., *BCL-2 family proteins: critical checkpoints of apoptotic cell death.* Clin Cancer Res, 2007. **13**(24): p. 7254-63.

201. Youle, R.J. and A. Strasser, *The BCL-2 protein family: opposing activities that mediate cell death.* Nat Rev Mol Cell Biol, 2008. **9**(1): p. 47-59.

202. Garrido, C., et al., *Mechanisms of cytochrome c release from mitochondria.* Cell Death Differ, 2006. **13**(9): p. 1423-33.

203. Shintani, T. and D.J. Klionsky, *Autophagy in health and disease: a double-edged sword.* Science, 2004. **306**(5698): p. 990-5.

204. Gozuacik, D. and A. Kimchi, *Autophagy as a cell death and tumor suppressor mechanism.* Oncogene, 2004. **23**(16): p. 2891-906.

205. Klionsky, D.J., *The molecular machinery of autophagy: unanswered questions.* J Cell Sci, 2005. **118**(Pt 1): p. 7-18.

206. Glick, D., S. Barth, and K.F. Macleod, *Autophagy: cellular and molecular mechanisms.* J Pathol, 2010. **221**(1): p. 3-12.

207. Wirawan, E., et al., *Autophagy: for better or for worse.* Cell Res, 2012. **22**(1): p. 43-61.

208. Bartkova, J., et al., *DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis.* Nature, 2005. **434**(7035): p. 864-70.

209. Aparicio, T., R. Baer, and J. Gautier, *DNA double-strand break repair pathway choice and cancer.* DNA Repair (Amst), 2014. **19**: p. 169-75.

210. Negrini, S., V.G. Gorgoulis, and T.D. Halazonetis, *Genomic instability--an evolving hallmark of cancer.* Nat Rev Mol Cell Biol, 2010. **11**(3): p. 220-8.

211. Aguilera, A. and B. Gomez-Gonzalez, *Genome instability: a mechanistic view of its causes and consequences.* Nat Rev Genet, 2008. **9**(3): p. 204-17.

212. Bartek, J., J. Bartkova, and J. Lukas, *DNA damage signalling guards against activated oncogenes and tumour progression.* Oncogene, 2007. **26**(56): p. 7773-9.

213. Nuciforo, P.G., et al., *Complex engagement of DNA damage response pathways in human cancer and in lung tumor progression.* Carcinogenesis, 2007. **28**(10): p. 2082-8.

214. Lehmann, A.R., *DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy.* Biochimie, 2003. **85**(11): p. 1101-11.

215. Rotman, G. and Y. Shiloh, *ATM: a mediator of multiple responses to genotoxic stress.* Oncogene, 1999. **18**(45): p. 6135-44.

216. Tauchi, H., et al., *Nijmegen breakage syndrome gene, NBS1, and molecular links to factors for genome stability.* Oncogene, 2002. **21**(58): p. 8967-80.

217. Lavin, M.F., *Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer.* Nat Rev Mol Cell Biol, 2008. **9**(10): p. 759-69.

218. Antoccia, A., et al., *Nijmegen breakage syndrome and functions of the responsible protein, NBS1.* Genome Dyn, 2006. **1**: p. 191-205.

219. Taylor, A.M., A. Groom, and P.J. Byrd, *Ataxia-telangiectasia-like disorder (ATLD)-its clinical presentation and molecular basis.* DNA Repair (Amst), 2004. **3**(8-9): p. 1219-25.

220. Cerbinskaite, A., et al., *Defective homologous recombination in human cancers.* Cancer Treat Rev, 2012. **38**(2): p. 89-100.

221. Shen, J. and L.A. Loeb, *Unwinding the molecular basis of the Werner syndrome.* Mech Ageing Dev, 2001. **122**(9): p. 921-44.

222. Bernstein, K.A., S. Gangloff, and R. Rothstein, *The RecQ DNA helicases in DNA repair.* Annu Rev Genet, 2010. **44**: p. 393-417.

223. Al-Ejeh, F., et al., *Harnessing the complexity of DNA-damage response pathways to improve cancer treatment outcomes.* Oncogene, 2010. **29**(46): p. 6085-98.

224. Curtin, N.J., *DNA repair dysregulation from cancer driver to therapeutic target.* Nat Rev Cancer, 2012. **12**(12): p. 801-17.

225. Darzynkiewicz, Z., F. Traganos, and D. Wlodkowic, *Impaired DNA damage response--an Achilles' heel sensitizing cancer to chemotherapy and radiotherapy.* Eur J Pharmacol, 2009. **625**(1-3): p. 143-50.

226. Damia, G. and M. D'Incalci, *Targeting DNA repair as a promising approach in cancer therapy.* Eur J Cancer, 2007. **43**(12): p. 1791-801.

227. O'Connor, M.J., N.M. Martin, and G.C. Smith, *Targeted cancer therapies based on the inhibition of DNA strand break repair.* Oncogene, 2007. **26**(56): p. 7816-24.

228. Helleday, T., et al., *DNA repair pathways as targets for cancer therapy.* Nat Rev Cancer, 2008. **8**(3): p. 193-204.

229. Ljungman, M., *Targeting the DNA damage response in cancer.* Chem Rev, 2009. **109**(7): p. 2929-50.

230. Aziz, K., et al., *Targeting DNA damage and repair: embracing the pharmacological era for successful cancer therapy.* Pharmacol Ther, 2012. **133**(3): p. 334-50.

231. Furgason, J.M. and M. Bahassi el, *Targeting DNA repair mechanisms in cancer.* Pharmacol Ther, 2013. **137**(3): p. 298-308.

232. Pearl, L.H., et al., *Therapeutic opportunities within the DNA damage response.* Nat Rev Cancer, 2015. **15**(3): p. 166-80.

233. Soussi, T., *p53 alterations in human cancer: more questions than answers.* Oncogene, 2007. **26**(15): p. 2145-56.

234. Bartek, J. and J. Lukas, *Pathways governing G1/S transition and their response to DNA damage.* FEBS Lett, 2001. **490**(3): p. 117-22.

235. Brooks, K., et al., *A potent Chk1 inhibitor is selectively cytotoxic in melanomas with high levels of replicative stress.* Oncogene, 2013. **32**(6): p. 788-96.

236. Tibbetts, R.S., et al., *A role for ATR in the DNA damage-induced phosphorylation of p53.* Genes Dev, 1999. **13**(2): p. 152-7.

237. Sarkaria, J.N., et al., *Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine.* Cancer Res, 1999. **59**(17): p. 4375-82.

238. Garrett, M.D. and I. Collins, *Anticancer therapy with checkpoint inhibitors: what, where and when?* Trends Pharmacol Sci, 2011. **32**(5): p. 308-16.

239. Chen, T., et al., *Targeting the S and G2 checkpoint to treat cancer.* Drug Discov Today, 2012. **17**(5-6): p. 194-202.

240. Chabner, B.A. and T.G. Roberts, Jr., *Timeline: Chemotherapy and the war on cancer.* Nat Rev Cancer, 2005. **5**(1): p. 65-72.

241. Haince, J.F., et al., *Targeting poly(ADP-ribosyl)ation: a promising approach in cancer therapy.* Trends Mol Med, 2005. **11**(10): p. 456-63.

242. Jagtap, P. and C. Szabo, *Poly(ADP-ribose) polymerase and the therapeutic effects of its inhibitors.* Nat Rev Drug Discov, 2005. **4**(5): p. 421-40.

243. Javle, M. and N.J. Curtin, *The role of PARP in DNA repair and its therapeutic exploitation.* Br J Cancer, 2011. **105**(8): p. 1114-22.

244. Lord, C.J. and A. Ashworth, *The DNA damage response and cancer therapy.* Nature, 2012. **481**(7381): p. 287-94.

245. Durkacz, B.W., et al., *(ADP-ribose)n participates in DNA excision repair.* Nature, 1980. **283**(5747): p. 593-6.

246. Sandhu, S.K., T.A. Yap, and J.S. de Bono, *Poly(ADP-ribose) polymerase inhibitors in cancer treatment: a clinical perspective.* Eur J Cancer, 2010. **46**(1): p. 9-20.

247. Farmer, H., et al., *Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy.* Nature, 2005. **434**(7035): p. 917-21.

248. Bryant, H.E., et al., *Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase.* Nature, 2005. **434**(7035): p. 913-7.

249. Helleday, T., H.E. Bryant, and N. Schultz, *Poly(ADP-ribose) polymerase (PARP-1) in homologous recombination and as a target for cancer therapy.* Cell Cycle, 2005. **4**(9): p. 1176-8.

250. Plummer, E.R. and H. Calvert, *Targeting poly(ADP-ribose) polymerase: a two-armed strategy for cancer therapy.* Clin Cancer Res, 2007. **13**(21): p. 6252-6.

251. Turner, N.C., et al., *A synthetic lethal siRNA screen identifying genes mediating sensitivity to a PARP inhibitor.* EMBO J, 2008. **27**(9): p. 1368-77.

252. Boulton, S., S. Kyle, and B.W. Durkacz, *Interactive effects of inhibitors of poly(ADP-ribose) polymerase and DNA-dependent protein kinase on cellular responses to DNA damage.* Carcinogenesis, 1999. **20**(2): p. 199-203.

253. Rehman, F.L., C.J. Lord, and A. Ashworth, *The promise of combining inhibition of PI3K and PARP as cancer therapy.* Cancer Discov, 2012. **2**(11): p. 982-4.

254. Znojek, P., E. Willmore, and N.J. Curtin, *Preferential potentiation of topoisomerase I poison cytotoxicity by PARP inhibition in S phase.* Br J Cancer, 2014. **111**(7): p. 1319-26.

255. Shinohara, E.T., et al., *DNA-dependent protein kinase is a molecular target for the development of noncytotoxic radiation-sensitizing drugs.* Cancer Res, 2005. **65**(12): p. 4987-92.

256. Davidson, D., et al., *Small Molecules, Inhibitors of DNA-PK, Targeting DNA Repair, and Beyond.* Front Pharmacol, 2013. **4**: p. 5.

257. Salles, B., et al., *The DNA repair complex DNA-PK, a pharmacological target in cancer chemotherapy and radiotherapy.* Pathol Biol (Paris), 2006. **54**(4): p. 185-93.

258. Kashishian, A., et al., *DNA-dependent protein kinase inhibitors as drug candidates for the treatment of cancer.* Mol Cancer Ther, 2003. **2**(12): p. 1257-64.

259. Veuger, S.J., et al., *Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-ribose) polymerase-1.* Cancer Res, 2003. **63**(18): p. 6008-15.

260. Veuger, S.J., et al., *Effects of novel inhibitors of poly(ADP-ribose) polymerase-1 and the DNA-dependent protein kinase on enzyme activities and DNA repair.* Oncogene, 2004. **23**(44): p. 7322-9.

261. Tavecchio, M., et al., *Further characterisation of the cellular activity of the DNA-PK inhibitor, NU7441, reveals potential cross-talk with homologous recombination.* Cancer Chemother Pharmacol, 2012. **69**(1): p. 155-64.

262. Leahy, J.J., et al., *Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromenone libraries.* Bioorg Med Chem Lett, 2004. **14**(24): p. 6083-7.

263. Willmore, E., et al., *A novel DNA-dependent protein kinase inhibitor, NU7026, potentiates the cytotoxicity of topoisomerase II poisons used in the treatment of leukemia.* Blood, 2004. **103**(12): p. 4659-65.

264. Nutley, B.P., et al., *Preclinical pharmacokinetics and metabolism of a novel prototype DNA-PK inhibitor NU7026.* Br J Cancer, 2005. **93**(9): p. 1011-8.

265. Zhao, Y., et al., *Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441.* Cancer Res, 2006. **66**(10): p. 5354-62.

266. Quanz, M., et al., *Small-molecule drugs mimicking DNA damage: a new strategy for sensitizing tumors to radiotherapy.* Clin Cancer Res, 2009. **15**(4): p. 1308-16.

267. Quanz, M., et al., *Hyperactivation of DNA-PK by double-strand break mimicking molecules disorganizes DNA damage response.* PLoS One, 2009. **4**(7): p. e6298.

268. Sharma, S., T.K. Kelly, and P.A. Jones, *Epigenetics in cancer.* Carcinogenesis, 2010. **31**(1): p. 27-36.

269. Dawson, M.A. and T. Kouzarides, *Cancer epigenetics: from mechanism to therapy.* Cell, 2012. **150**(1): p. 12-27.

270. Bolden, J.E., et al., *HDAC inhibitors induce tumor-cell-selective pro-apoptotic transcriptional responses.* Cell Death Dis, 2013. **4**: p. e519.

271. Rodriguez-Paredes, M. and M. Esteller, *Cancer epigenetics reaches mainstream oncology.* Nat Med, 2011. **17**(3): p. 330-9.

272. Dobbin, M.M., et al., *SIRT1 collaborates with ATM and HDAC1 to maintain genomic stability in neurons.* Nat Neurosci, 2013. **16**(8): p. 1008-15.

273. McCord, R.A., et al., *SIRT6 stabilizes DNA-dependent protein kinase at chromatin for DNA double-strand break repair.* Aging (Albany NY), 2009. **1**(1): p. 109-21.

274. Miller, K.M., et al., *Human HDAC1 and HDAC2 function in the DNA-damage response to promote DNA nonhomologous end-joining.* Nat Struct Mol Biol, 2010. **17**(9): p. 1144-51.

275. Thurn, K.T., et al., *Rational therapeutic combinations with histone deacetylase inhibitors for the treatment of cancer.* Future Oncol, 2011. **7**(2): p. 263-83.

276. Zeller, C. and R. Brown, *Therapeutic modulation of epigenetic drivers of drug resistance in ovarian cancer.* Ther Adv Med Oncol, 2010. **2**(5): p. 319-29.

277. Winkelman, N.W., Jr., *Chlorpromazine in the treatment of neuropsychiatric disorders.* J Am Med Assoc, 1954. **155**(1): p. 18-21.

278. Casey, J.F., et al., *Treatment of schizophrenic reactions with phenothiazine derivatives. A comparative study of chlorpromazine, triflupromazine, mepazine, prochlorperazine, perphenazine, and phenobarbital.* Am J Psychiatry, 1960. **117**: p. 97-105.

279. Feinberg, A.P. and S.H. Snyder, *Phenothiazine drugs: structure-activity relationships explained by a conformation that mimics dopamine.* Proc Natl Acad Sci U S A, 1975. **72**(5): p. 1899-903.

280. Shen, W.W., *A history of antipsychotic drug development.* Compr Psychiatry, 1999. **40**(6): p. 407-14.

281. Freedman, R., *Schizophrenia.* N Engl J Med, 2003. **349**(18): p. 1738-49.

282. Allan, S.G., *Mechanisms and management of chemotherapy-induced nausea and vomiting.* Blood Rev, 1987. **1**(1): p. 50-7.

283. Kane, J., et al., *Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine.* Arch Gen Psychiatry, 1988. **45**(9): p. 789-96.

284. Pickar, D., *Prospects for pharmacotherapy of schizophrenia.* Lancet, 1995. **345**(8949): p. 557-62.

285. Arnt, J. and T. Skarsfeldt, *Do novel antipsychotics have similar pharmacological characteristics? A review of the evidence.* Neuropsychopharmacology, 1998. **18**(2): p. 63-101.

286. Jibson, M.D. and R. Tandon, *New atypical antipsychotic medications.* J Psychiatr Res, 1998. **32**(3-4): p. 215-28.

287. Lieberman, J.A., et al., *Atypical and conventional antipsychotic drugs in treatment-naive first-episode schizophrenia: a 52-week randomized trial of clozapine vs chlorpromazine.* Neuropsychopharmacology, 2003. **28**(5): p. 995-1003.

288. Reynolds, G.P., L.A. Templeman, and B.R. Godlewska, *Pharmacogenetics of schizophrenia.* Expert Opin Pharmacother, 2006. **7**(11): p. 1429-40.

289. Kishimoto, T., et al., *Relapse prevention in schizophrenia: a systematic review and meta-analysis of second-generation antipsychotics versus first-generation antipsychotics.* Mol Psychiatry, 2013. **18**(1): p. 53-66.

290. Bergson, C., et al., *Dopamine receptor-interacting proteins: the Ca(2+) connection in dopamine signaling.* Trends Pharmacol Sci, 2003. **24**(9): p. 486-92.

291. Fleischhacker, W.W., *New drugs for the treatment of schizophrenic patients.* Acta Psychiatr Scand Suppl, 1995. **388**: p. 24-30.

292. He, H. and J.S. Richardson, *A pharmacological, pharmacokinetic and clinical overview of risperidone, a new antipsychotic that blocks serotonin 5-HT2 and dopamine D2 receptors.* Int Clin Psychopharmacol, 1995. **10**(1): p. 19-30.

293. Coyle, J.T. and R.S. Duman, *Finding the intracellular signaling pathways affected by mood disorder treatments.* Neuron, 2003. **38**(2): p. 157-60.

294. Roth, B.L., D.J. Sheffler, and W.K. Kroeze, *Magic shotguns versus magic bullets: selectively non-selective drugs for mood disorders and schizophrenia.* Nat Rev Drug Discov, 2004. **3**(4): p. 353-9.

295. Sudeshna, G. and K. Parimal, *Multiple non-psychiatric effects of phenothiazines: a review.* Eur J Pharmacol, 2010. **648**(1-3): p. 6-14.

296. Kato, M.M. and P.J. Goodnick, *Antipsychotic medication: effects on regulation of glucose and lipids.* Expert Opin Pharmacother, 2001. **2**(10): p. 1571-82.

297. Viola, G. and F. Dall'Acqua, *Photosensitization of biomolecules by phenothiazine derivatives.* Curr Drug Targets, 2006. **7**(9): p. 1135-54.

298. Gil-ad, I., et al., *Evaluation of the neurotoxic activity of typical and atypical neuroleptics: relevance to iatrogenic extrapyramidal symptoms.* Cell Mol Neurobiol, 2001. **21**(6): p. 705-16.

299. Wirshing, W.C., *Movement disorders associated with neuroleptic treatment.* J Clin Psychiatry, 2001. **62 Suppl 21**: p. 15-8.

300. Pelonero, A.L., J.L. Levenson, and A.K. Pandurangi, *Neuroleptic malignant syndrome: a review.* Psychiatr Serv, 1998. **49**(9): p. 1163-72.

301. Michalak, K., et al., *Interactions of phenothiazines with lipid bilayer and their role in multidrug resistance reversal.* Curr Drug Targets, 2006. **7**(9): p. 1095-105.

302. Bisi, A., et al., *Multidrug resistance reverting activity and antitumor profile of new phenothiazine derivatives.* Bioorg Med Chem, 2008. **16**(13): p. 6474-82.

303. Pluta, K., B. Morak-Mlodawska, and M. Jelen, *Recent progress in biological activities of synthesized phenothiazines.* Eur J Med Chem, 2011. **46**(8): p. 3179-89.

304. Spengler, G., et al., *Multidrug resistance reversing activity of newly developed phenothiazines on P-glycoprotein (ABCB1)-related resistance of mouse T-lymphoma cells.* Anticancer Res, 2014. **34**(4): p. 1737-41.

305. Ford, J.M., W.C. Prozialeck, and W.N. Hait, *Structural features determining activity of phenothiazines and related drugs for inhibition of cell growth and reversal of multidrug resistance.* Mol Pharmacol, 1989. **35**(1): p. 105-15.

306. Chaudhuri, S., et al., *Effect of trifluoperazine on toxicity, HIF-1alpha induction and hepatocyte regeneration in acetaminophen toxicity in mice.* Toxicol Appl Pharmacol, 2012. **264**(2): p. 192-201.

307. Lee, G.L. and W.N. Hait, *Inhibition of growth of C6 astrocytoma cells by inhibitors of calmodulin.* Life Sci, 1985. **36**(4): p. 347-54.

308. Hait, W.N., et al., *Inhibition of growth of leukemic cells by inhibitors of calmodulin: phenothiazines and melittin.* Cancer Chemother Pharmacol, 1985. **14**(3): p. 202-5.

309. Hait, W.N. and G.L. Lee, *Characteristics of the cytotoxic effects of the phenothiazine class of calmodulin antagonists.* Biochem Pharmacol, 1985. **34**(22): p. 3973-8.

310. Hait, W.N. and J.S. Lazo, *Calmodulin: a potential target for cancer chemotherapeutic agents.* J Clin Oncol, 1986. **4**(6): p. 994-1012.

311. Chin, D. and A.R. Means, *Calmodulin: a prototypical calcium sensor.* Trends Cell Biol, 2000. **10**(8): p. 322-8.

312. Prozialeck, W.C. and B. Weiss, *Inhibition of calmodulin by phenothiazines and related drugs: structure-activity relationships.* J Pharmacol Exp Ther, 1982. **222**(3): p. 509-16.

313. Jaszczyszyn, A., et al., *Chemical structure of phenothiazines and their biological activity.* Pharmacol Rep, 2012. **64**(1): p. 16-23.

314. Marshak, D.R., D.M. Watterson, and L.J. Van Eldik, *Calcium-dependent interaction of S100b, troponin C, and calmodulin with an immobilized phenothiazine.* Proc Natl Acad Sci U S A, 1981. **78**(11): p. 6793-7.

315. Marshak, D.R., T.J. Lukas, and D.M. Watterson, *Drug-protein interactions: binding of chlorpromazine to calmodulin, calmodulin fragments, and related calcium binding proteins.* Biochemistry, 1985. **24**(1): p. 144-50.

316. Drori, S., G.D. Eytan, and Y.G. Assaraf, *Potentiation of anticancer-drug cytotoxicity by multidrug-resistance chemosensitizers involves alterations in membrane fluidity leading to increased membrane permeability.* Eur J Biochem, 1995. **228**(3): p. 1020-9.

317. Lamb, J., et al., *The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease.* Science, 2006. **313**(5795): p. 1929-35.

318. Sengupta, P., et al., *Membrane-permeable calmodulin inhibitors (e.g. W-7/W-13) bind to membranes, changing the electrostatic surface potential: dual effect of W-13 on epidermal growth factor receptor activation.* J Biol Chem, 2007. **282**(11): p. 8474-86.

319. Osborn, M. and K. Weber, *Damage of cellular functions by trifluoperazine, a calmodulin-specific drug.* Exp Cell Res, 1980. **130**(2): p. 484-8.

320. Krishan, A. and L.Y. Bourguignon, *Cell cycle related phenothiazine effects on adriamycin transport.* Cell Biol Int Rep, 1984. **8**(6): p. 449-56.

321. Krishan, A., A. Sauerteig, and L.L. Wellham, *Flow cytometric studies on modulation of cellular adriamycin retention by phenothiazines.* Cancer Res, 1985. **45**(3): p. 1046-51.

322. Strobl, J.S., et al., *Inhibition of human breast cancer cell proliferation in tissue culture by the neuroleptic agents pimozide and thioridazine.* Cancer Res, 1990. **50**(17): p. 5399-405.

323. Motohashi, N., et al., *Cytotoxic potential of phenothiazines.* Curr Drug Targets, 2006. **7**(9): p. 1055-66.

324. Pluta, K., et al., *Anticancer activity of newly synthesized azaphenothiazines from NCI's anticancer screening bank.* Pharmacol Rep, 2010. **62**(2): p. 319-32.

325. Rho, S.B., B.R. Kim, and S. Kang, *A gene signature-based approach identifies thioridazine as an inhibitor of phosphatidylinositol-3'-kinase (PI3K)/AKT pathway in ovarian cancer cells.* Gynecol Oncol, 2011. **120**(1): p. 121-7.

326. Qi, L. and Y. Ding, *Potential antitumor mechanisms of phenothiazine drugs.* Sci China Life Sci, 2013. **56**(11): p. 1020-7.

327. Jelen, M., et al., *6-Substituted 9-fluoroquino[3,2-b]benzo[1,4]thiazines display strong antiproliferative and antitumor properties.* Eur J Med Chem, 2015. **89**: p. 411-20.

328. Lee, M.S., et al., *The novel combination of chlorpromazine and pentamidine exerts synergistic antiproliferative effects through dual mitotic action.* Cancer Res, 2007. **67**(23): p. 11359-67.

329. Tsuruo, T., et al., *Increased accumulation of vincristine and adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors.* Cancer Res, 1982. **42**(11): p. 4730-3.

330. Tsuruo, T., et al., *Potentiation of vincristine and Adriamycin effects in human hemopoietic tumor cell lines by calcium antagonists and calmodulin inhibitors.* Cancer Res, 1983. **43**(5): p. 2267-72.

331. Kuzma-Richeret, A., et al., *The influence of phenothiazine derivatives on doxorubicin treatment in sensitive and resistant human breast adenocarcinoma cells.* Folia Biol (Praha), 2011. **57**(6): p. 261-7.

332. Ganapathi, R. and D. Grabowski, *Enhancement of sensitivity to adriamycin in resistant P388 leukemia by the calmodulin inhibitor trifluoperazine.* Cancer Res, 1983. **43**(8): p. 3696-9.

333. Hait, W.N., et al., *Phase I trial of combined therapy with bleomycin and the calmodulin antagonist, trifluoperazine.* Cancer Chemother Pharmacol, 1989. **23**(6): p. 358-62.

334. Kennedy, K.A., W.N. Hait, and J.S. Lazo, *Chemical modulation of bleomycin induced toxicity.* Int J Radiat Oncol Biol Phys, 1986. **12**(8): p. 1367-70.

335. Lazo, J.S., et al., *Enhanced bleomycin-induced DNA damage and cytotoxicity with calmodulin antagonists.* Mol Pharmacol, 1985. **27**(3): p. 387-93.

336. Smith, P.J., J. Mircheva, and N.M. Bleehen, *Interaction of bleomycin, hyperthermia and a calmodulin inhibitor (trifluoperazine) in mouse tumour cells: II. DNA damage, repair and chromatin changes.* Br J Cancer, 1986. **53**(1): p. 105-14.

337. Ray, S.D., et al., *Ca(2+)-calmodulin antagonist chlorpromazine and poly(ADP-ribose) polymerase modulators 4-aminobenzamide and nicotinamide influence hepatic expression of BCL-XL and P53 and protect against acetaminophen-induced programmed and unprogrammed cell death in mice.* Free Radic Biol Med, 2001. **31**(3): p. 277-91.

338. Zhelev, Z., et al., *Phenothiazines suppress proliferation and induce apoptosis in cultured leukemic cells without any influence on the viability of normal lymphocytes. Phenothiazines and leukemia.* Cancer Chemother Pharmacol, 2004. **53**(3): p. 267-75.

339. Gil-Ad, I., et al., *Characterization of phenothiazine-induced apoptosis in neuroblastoma and glioma cell lines: clinical relevance and possible application for brain-derived tumors.* J Mol Neurosci, 2004. **22**(3): p. 189-98.

340. Gil-Ad, I., et al., *Phenothiazines induce apoptosis in a B16 mouse melanoma cell line and attenuate in vivo melanoma tumor growth.* Oncol Rep, 2006. **15**(1): p. 107-12.

341. Gutierrez, A., et al., *Phenothiazines induce PP2A-mediated apoptosis in T cell acute lymphoblastic leukemia.* J Clin Invest, 2014. **124**(2): p. 644-55.

342. Pan, D., et al., *Trifluoperazine regulation of calmodulin binding to Fas: a computational study.* Proteins, 2011. **79**(8): p. 2543-56.

343. Bergh, J., et al., *Establishment and characterization of cell lines from human small cell and large cell carcinomas of the lung.* Acta Pathol Microbiol Immunol Scand A, 1985. **93**(3): p. 133-47.

344. Hayflick, L. and P.S. Moorhead, *The serial cultivation of human diploid cell strains.* Exp Cell Res, 1961. **25**: p. 585-621.

345. Chadwick, K.H. and H.P. Leenhouts, *A molecular theory of cell survival.* Phys Med Biol, 1973. **18**(1): p. 78-87.

346. Fowler, J.F. and B.E. Stern, *Dose-rate effects: some theoretical and practical considerations.* Br J Radiol, 1960. **33**: p. 389-95.

347. Chen, J. and J. Stubbe, *Bleomycins: towards better therapeutics.* Nat Rev Cancer, 2005. **5**(2): p. 102-12.

348. Hanada, K., et al., *The structure-specific endonuclease Mus81-Eme1 promotes conversion of interstrand DNA crosslinks into double-strands breaks.* EMBO J, 2006. **25**(20): p. 4921-32.

349. Abate, G., R.N. Mshana, and H. Miorner, *Evaluation of a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for rapid detection of rifampicin resistance in Mycobacterium tuberculosis.* Int J Tuberc Lung Dis, 1998. **2**(12): p. 1011-6.

350. Fischer, A.H., et al., *Hematoxylin and eosin staining of tissue and cell sections.* CSH Protoc, 2008. **2008**: p. pdb prot4986.

351. Schroder, B.A., et al., *The proteome of lysosomes.* Proteomics, 2010. **10**(22): p. 4053-76.

352. Kaufmann, A.M. and J.P. Krise, *Lysosomal sequestration of amine-containing drugs: analysis and therapeutic implications.* J Pharm Sci, 2007. **96**(4): p. 729-46.

353. Daniel, W.A., *Mechanisms of cellular distribution of psychotropic drugs. Significance for drug action and interactions.* Prog Neuropsychopharmacol Biol Psychiatry, 2003. **27**(1): p. 65-73.

354. Kroemer, G., L. Galluzzi, and C. Brenner, *Mitochondrial membrane permeabilization in cell death.* Physiol Rev, 2007. **87**(1): p. 99-163.

355. Parsons, M.J. and D.R. Green, *Mitochondria in cell death.* Essays Biochem, 2010. **47**: p. 99-114.

356. Tait, S.W. and D.R. Green, *Mitochondria and cell death: outer membrane permeabilization and beyond.* Nat Rev Mol Cell Biol, 2010. **11**(9): p. 621-32.

357. Galluzzi, L., et al., *Methods for the assessment of mitochondrial membrane permeabilization in apoptosis.* Apoptosis, 2007. **12**(5): p. 803-13.

358. Darzynkiewicz, Z. and X. Huang, *Analysis of cellular DNA content by flow cytometry.* Curr Protoc Immunol, 2004. **Chapter 5**: p. Unit 5 7.

359. Quah, B.J., H.S. Warren, and C.R. Parish, *Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester.* Nat Protoc, 2007. **2**(9): p. 2049-56.

360. Polischouk, A.G., et al., *The antipsychotic drug trifluoperazine inhibits DNA repair and sensitizes non small cell lung carcinoma cells to DNA double-strand break induced cell death.* Mol Cancer Ther, 2007. **6**(8): p. 2303-9.

361. Gunn, A. and J.M. Stark, *I-SceI-based assays to examine distinct repair outcomes of mammalian chromosomal double strand breaks.* Methods Mol Biol, 2012. **920**: p. 379-91.

362. Lamb, J., *The Connectivity Map: a new tool for biomedical research.* Nat Rev Cancer, 2007. **7**(1): p. 54-60.

363. Hassane, D.C., et al., *Discovery of agents that eradicate leukemia stem cells using an in silico screen of public gene expression data.* Blood, 2008. **111**(12): p. 5654-62.

364. Li, J., X. Zhu, and J.Y. Chen, *Building disease-specific drug-protein connectivity maps from molecular interaction networks and PubMed abstracts.* PLoS Comput Biol, 2009. **5**(7): p. e1000450.

365. Choi, K.H., et al., *Effects of typical and atypical antipsychotic drugs on gene expression profiles in the liver of schizophrenia subjects.* BMC Psychiatry, 2009. **9**: p. 57.

366. Ellis, L., et al., *Histone deacetylase inhibitor panobinostat induces clinical responses with associated alterations in gene expression profiles in cutaneous T-cell lymphoma.* Clin Cancer Res, 2008. **14**(14): p. 4500-10.

367. Schlaff, C.D., et al., *Bringing the heavy: carbon ion therapy in the radiobiological and clinical context.* Radiat Oncol, 2014. **9**(1): p. 88.

368. Allen, C., et al., *Heavy charged particle radiobiology: using enhanced biological effectiveness and improved beam focusing to advance cancer therapy.* Mutat Res, 2011. **711**(1-2): p. 150-7.

369. Jakel, O., C.P. Karger, and J. Debus, *The future of heavy ion radiotherapy.* Med Phys, 2008. **35**(12): p. 5653-63.

370. Scholz, M.E., T., *Biophysical models in ion beam radiotherapy.* Advances in Space Research, 2007.

371. Campa, A., et al., *DNA DSB induced in human cells by charged particles and gamma rays: experimental results and theoretical approaches.* Int J Radiat Biol, 2005. **81**(11): p. 841-54.

372. Fakir, H., et al., *Clusters of DNA double-strand breaks induced by different doses of nitrogen ions for various LETs: experimental measurements and theoretical analyses.* Radiat Res, 2006. **166**(6): p. 917-27.

373. Hendry, J.H., *Repair of cellular damage after high LET irradiation.* J Radiat Res, 1999. **40 Suppl**: p. 60-5.

374. Okayasu, R., et al., *Repair of DNA damage induced by accelerated heavy ions in mammalian cells proficient and deficient in the non-homologous end-joining pathway.* Radiat Res, 2006. **165**(1): p. 59-67.

375. Nordenberg, J., et al., *Effects of psychotropic drugs on cell proliferation and differentiation.* Biochem Pharmacol, 1999. **58**(8): p. 1229-36.

376. Zong, D., et al., *Chemosensitization by phenothiazines in human lung cancer cells: impaired resolution of gammaH2AX and increased oxidative stress elicit apoptosis associated with lysosomal expansion and intense vacuolation.* Cell Death Dis, 2011. **2**: p. e181.

377. Sridhar, K.S., et al., *Prochlorperazine as a doxorubicin-efflux blocker: phase I clinical and pharmacokinetics studies.* Cancer Chemother Pharmacol, 1993. **31**(6): p. 423-30.

378. de Duve, C., et al., *Commentary. Lysosomotropic agents.* Biochem Pharmacol, 1974. **23**(18): p. 2495-531.

379. Castino, R., M. Demoz, and C. Isidoro, *Destination 'lysosome': a target organelle for tumour cell killing?* J Mol Recognit, 2003. **16**(5): p. 337-48.

380. Ashoor, R., et al., *The contribution of lysosomotropism to autophagy perturbation.* PLoS One, 2013. **8**(11): p. e82481.

381. Joseph, B., R. Lewensohn, and B. Zhivotovsky, *Role of apoptosis in the response of lung carcinomas to anti-cancer treatment.* Ann N Y Acad Sci, 2000. **926**: p. 204-16.

382. Erdal, H., et al., *Induction of lysosomal membrane permeabilization by compounds that activate p53-independent apoptosis.* Proc Natl Acad Sci U S A, 2005. **102**(1): p. 192-7.

383. Zhang, L., et al., *Small molecule regulators of autophagy identified by an image-based high-throughput screen.* Proc Natl Acad Sci U S A, 2007. **104**(48): p. 19023-8.

384. Indelicato, M., et al., *Role of hypoxia and autophagy in MDA-MB-231 invasiveness.* J Cell Physiol, 2010. **223**(2): p. 359-68.

385. Shin, S.Y., et al., *The antipsychotic agent chlorpromazine induces autophagic cell death by inhibiting the Akt/mTOR pathway in human U-87MG glioma cells.* Carcinogenesis, 2013. **34**(9): p. 2080-9.

386. Klein, H.L., *The consequences of Rad51 overexpression for normal and tumor cells.* DNA Repair (Amst), 2008. **7**(5): p. 686-93.

387. Reliene, R., A.J. Bishop, and R.H. Schiestl, *Involvement of homologous recombination in carcinogenesis.* Adv Genet, 2007. **58**: p. 67-87.

388. Salles, D., et al., *BCR-ABL stimulates mutagenic homologous DNA double-strand break repair via the DNA-end-processing factor CtIP.* Carcinogenesis, 2011. **32**(1): p. 27-34.

389. Tutt, A., et al., *Mutation in Brca2 stimulates error-prone homology-directed repair of DNA double-strand breaks occurring between repeated sequences.* EMBO J, 2001. **20**(17): p. 4704-16.

390. Rosenthal, S.A. and W.N. Hait, *Potentiation of DNA damage and cytotoxicity by calmodulin antagonists.* Yale J Biol Med, 1988. **61**(1): p. 39-49.

391. Gangopadhyay, S., et al., *Trifluoperazine stimulates ionizing radiation induced cell killing through inhibition of DNA repair.* Mutat Res, 2007. **633**(2): p. 117-25.

392. Ronald, S., et al., *Phenothiazine Inhibitors of TLKs Affect Double-Strand Break Repair and DNA Damage Response Recovery and Potentiate Tumor Killing with Radiomimetic Therapy.* Genes Cancer, 2013. **4**(1-2): p. 39-53.

393. Sancar, A., et al., *Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints.* Annu Rev Biochem, 2004. **73**: p. 39-85.

394. Gudjonsson, T., et al., *TRIP12 and UBR5 suppress spreading of chromatin ubiquitylation at damaged chromosomes.* Cell, 2012. **150**(4): p. 697-709.

395. Lialiaris, T., et al., *Chlorpromazine-induced damage on nucleic acids: a combined cytogenetic and biochemical study.* Mutat Res, 1992. **265**(2): p. 155-63.

396. Ruben, L. and H. Rasmussen, *Phenothiazines and related compounds disrupt mitochondrial energy production by a calmodulin-independent reaction.* Biochim Biophys Acta, 1981. **637**(3): p. 415-22.

397. Zong, D., et al., *Harnessing the lysosome-dependent antitumor activity of phenothiazines in human small cell lung cancer.* Cell Death Dis, 2014. **5**: p. e1111.

398. Dawson, M.A., et al., *Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia.* Nature, 2011. **478**(7370): p. 529-33.

399. Fiskus, W., et al., *Highly active combination of BRD4 antagonist and histone deacetylase inhibitor against human acute myelogenous leukemia cells.* Mol Cancer Ther, 2014. **13**(5): p. 1142-54.

400. Charp, P.A. and J.D. Regan, *Inhibition of DNA repair by trifluoperazine.* Biochim Biophys Acta, 1985. **824**(1): p. 34-9.

401. Glaser, K.B., et al., *Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines.* Mol Cancer Ther, 2003. **2**(2): p. 151-63.

402. Schroeder, F.A., et al., *Antidepressant-like effects of the histone deacetylase inhibitor, sodium butyrate, in the mouse.* Biol Psychiatry, 2007. **62**(1): p. 55-64.

403. Covington, H.E., 3rd, et al., *Antidepressant actions of histone deacetylase inhibitors.* J Neurosci, 2009. **29**(37): p. 11451-60.

404. Grayson, D.R., M. Kundakovic, and R.P. Sharma, *Is there a future for histone deacetylase inhibitors in the pharmacotherapy of psychiatric disorders?* Mol Pharmacol, 2010. **77**(2): p. 126-35.

405. Liu, R., et al., *SIRT2 is involved in the modulation of depressive behaviors.* Sci Rep, 2015. **5**: p. 8415.

406. Cueto, A., et al., *Neuroendocrine tumors of the lung: hystological classification, diagnosis, traditional and new therapeutic approaches.* Curr Med Chem, 2014. **21**(9): p. 1107-16.

407. Decock, A., et al., *Neuroblastoma epigenetics: from candidate gene approaches to genome-wide screenings.* Epigenetics, 2011. **6**(8): p. 962-70.

408. Kobayashi, K., L.M. Jakt, and S.I. Nishikawa, *Epigenetic regulation of the neuroblastoma genes, Arid3b and Mycn.* Oncogene, 2013. **32**(21): p. 2640-8.

409. Chen, C.S., et al., *Histone deacetylase inhibitors sensitize prostate cancer cells to agents that produce DNA double-strand breaks by targeting Ku70 acetylation.* Cancer Res, 2007. **67**(11): p. 5318-27.

410. Feinberg, A.P., *Phenotypic plasticity and the epigenetics of human disease.* Nature, 2007. **447**(7143): p. 433-40.

411. Ikediobi, O.N., et al., *In vitro differential sensitivity of melanomas to phenothiazines is based on the presence of codon 600 BRAF mutation.* Mol Cancer Ther, 2008. **7**(6): p. 1337-46.

412. Csatary, L.K., *Chlorpromazines and cancer.* Lancet, 1972. **2**(7772): p. 338-9.

413. Hercbergs, A., *Thioridazine: a radiation enhancer in advanced cervical cancer?* Lancet, 1988. **2**(8613): p. 737.

414. Jones, G.R., *Successful cancer therapy with promethazine: the rationale.* Med Hypotheses, 1996. **46**(1): p. 25-9.

415. Pulkoski-Gross, A., et al., *Repurposing the antipsychotic trifluoperazine as an antimetastasis agent.* Mol Pharmacol, 2015. **87**(3): p. 501-12.

416. Yeh, C.T., et al., *Trifluoperazine, an antipsychotic agent, inhibits cancer stem cell growth and overcomes drug resistance of lung cancer.* Am J Respir Crit Care Med, 2012. **186**(11): p. 1180-8.

417. Sachlos, E., et al., *Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells.* Cell, 2012. **149**(6): p. 1284-97.

418. Sangodkar, J., et al., *Targeting the FOXO1/KLF6 axis regulates EGFR signaling and treatment response.* J Clin Invest, 2012. **122**(7): p. 2637-51.

419. Zhang, Q., et al., *A small molecule Inauhzin inhibits SIRT1 activity and suppresses tumour growth through activation of p53.* EMBO Mol Med, 2012. **4**(4): p. 298-312.

420. Zhang, Q., et al., *Structure and activity analysis of Inauhzin analogs as novel antitumor compounds that induce p53 and inhibit cell growth.* PLoS One, 2012. **7**(10): p. e46294.

421. Zhang, Y., et al., *Inauhzin sensitizes p53-dependent cytotoxicity and tumor suppression of chemotherapeutic agents.* Neoplasia, 2013. **15**(5): p. 523-34.

422. Cohen, M., B. Dembling, and J. Schorling, *The association between schizophrenia and cancer: a population-based mortality study.* Schizophr Res, 2002. **57**(2-3): p. 139-46.

423. Hodgson, R., H.J. Wildgust, and C.J. Bushe, *Cancer and schizophrenia: is there a paradox?* J Psychopharmacol, 2010. **24**(4 Suppl): p. 51-60.

424. Chou, F.H., et al., *The incidence and relative risk factors for developing cancer among patients with schizophrenia: a nine-year follow-up study.* Schizophr Res, 2011. **129**(2-3): p. 97-103.

425. Fond, G., et al., *Antipsychotic drugs: pro-cancer or anti-cancer? A systematic review.* Med Hypotheses, 2012. **79**(1): p. 38-42.