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Breaking to Understand: DNA Repair in Response to Cancer Therapy

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Abstract

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Human DNA constantly faces endogenous and exogenous damage, with DNA double-strand breaks (DSBs) posing the greatest threat to genome integrity. However, DSBs can be leveraged to kill cancer cells, as many treatments act as DSB inducers. The dominant repair pathway, non-homologous end-joining (NHEJ), resolves the majority of DSBs. This thesis explores strategies to sensitize resistant cancer cells through combination therapy and investigates NHEJ's response to varying DSB complexities.

Paper I addresses cisplatin resistance in ovarian cancer. We found that combining cisplatin with the HSP90 inhibitor onalespib enhances sensitivity by increasing DSB levels, inducing apoptosis, and causing G2/M arrest, making it a promising strategy. **Paper II** focuses on glioblastoma (GBM), an aggressive brain tumor with limited treatment options. We demonstrated that onalespib enhances radiosensitivity in 2D and 3D GBM models by increasing DSB levels, promoting apoptosis, and altering protein expression, suggesting that HSP90 inhibition could improve radiotherapy outcomes. **Paper III** investigates the alpha emitter Ra-223, used in bone-metastatic prostate cancer. Our findings revealed that Ra-223 generates clustered DSBs, triggering NHEJ activation, growth inhibition, and apoptosis in prostate cancer cells, with no detectable cellular uptake. **Paper IV** explores pharmacological ascorbate (Asc) effect on NHEJ pathway. We found that Asc induces delayed DSBs, extensive pan-nuclear γH2AX formation, necrosis, and G2/M arrest in colorectal cancer cells, with stronger effects in XRCC4 KO cells. We concluded that Asc does not generate prompt DSBs, and the delayed DSBs are linked to necrotic nuclear degradation, with sensitivity influenced by cell cycle regulation rather than NHEJ deficiency. **Paper V** examines NHEJ's role in repairing DSBs of varying complexity in colorectal cancer cells. Wild-type cells exhibited both fast and slow repair kinetics, while NHEJ-deficient cells showed only a fast repair phase, followed by repair failure. Non-DSB clusters increased as the DSB:SSB ratio decreased (from calicheamicin to X-rays, bleomycin, etoposide, and temozolomide). These clusters were rapidly removed, independent of NHEJ, highlighting the impact of DSB type/complexity on repair efficiency.

In conclusion, this thesis presents strategies to overcome cisplatin resistance, enhance radiosensitivity in GBM, and elucidate Ra-223 toxicity mechanisms in prostate cancer. It also examines Asc's effects on DSB induction and repair and reveals NHEJ's role in processing complex DSBs. Our findings provide new insights into optimizing DSB repair and therapeutic strategies in cancer treatment.

Keywords: DSB, NHEJ, HSP90 inhibition, X-ray, alpha-particle, clustered DSB, ascorbate, XRCC4, DNA-PKcs, DSB complexity

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I dedicate this thesis to my parents, ***Ashraf Firoozeh*** *and* ***Jalal Hariri****; to my motherland,* ***Iran****, which nurtured me; and to my second home,* ***Sweden****, which gave me the opportunity to grow.*

A person is defined by the problems they solve and how well they solve them.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Mortensen, A., Mohajershojai, T., **Hariri**, **M**., Pettersson, M., Spiegelberg, D. (2020) Overcoming limitations of cisplatin therapy by additional treatment with the HSP90 inhibitor onalespib. *Frontiers in Oncology,* vol 10.

II. Uffenorde*, J., **Hariri, M***., Papalanis, E., Staffas, A., Berg, J., Stenerlöw, B., Berglund, H., Malmberg, C., Spiegelberg, D. (2025) Enhancing glioblastoma therapy: Unveiling synergistic anticancer effects of onalespib - radiotherapy combination therapy. *Frontiers in Oncology,* vol 15. (*Equal contribution)

III. Abramenkovs*, A., **Hariri, M***., Spiegelberg, D., Nilsson, S., Stenerlöw, B. (2020) Ra-223 induces clustered DNA damage and inhibits cell survival in several prostate cancer cell lines. *Translational Oncology,* 26, 10154. (*Equal contribution).

IV. **Hariri, M**., Papalanis, E., Spiegelberg, D., Plotnikov, E., Viktorsson, K., Stenerlöw, B. (2025) Ascorbate induces G2/M arrest and necrosis without generation of direct DNA double-strand breaks. Accepted in *Heliyon.*

V. **Hariri, M.,** Stenerlöw, B. The role of non-homologous end joining in the repair of different types of DNA double-strand breaks. *Manuscript.*

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List of publications not included in the thesis

VI. Mortensen, A., Berglung, B., **Hariri**, **M**., Pettersson, M., Papalanis, E., Malmberg, C., Spiegelberg, D. (2023) Combination therapy of tyrosine kinase inhibitor sorafenib with the HSP90 inhibitor onalespib as a novel treatment regimen for thyroid cancer. *Scientific Reports,* 6;13(1):16844

VII. Lundsten, S., Berglung, B., Jha, P., Krona, C., **Hariri, M**., Nelander, S., Lane, D. P., Nestor, M. (2021) p53-mediated radiosensitization of ^{177}Lu-DOTATATE in neuroblastoma tumor spheroids. *Biomolecules,* 11, 1965

Contents

Abbreviations

AKT	Protein kinase B
Alt-NHEJ	Alternative non-homologous end Joining
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
BER	Base excision repair
BRCA1	Breast cancer-associated gene 1 protein
BRCA2	Breast cancer-associated gene 1 protein
CDKs	Cyclin-Dependent Kinases
CETN2	Centrin 2
CHK2	Checkpoint Kinase 2
DDR	DNA damage response
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
DSB	Double-strand breaks
EGFR	Epidermal growth factor receptor
HATs	Histone AcetylTransferases
HLS	Heat-Labile Sites
HMTs	Histone MethylTransferases
HP1	Heterochromatin Protein 1
HR	Homologous recombination
HRK	Activator of apoptosis harakiri protein
H2AX	H2A histone family member X
γ-H2AX	Phosphorylated H2AX
KAP1	KRAB-associated protein 1
LET	Linear energy transfer
MCL	Mantle cell lymphoma
MDC1	Mediator of DNA damage checkpoint protein 1
CHK2	Checkpoint Kinase 2
MMEJ	microhomology-mediated end joining
MMR	Mismatch Repair
MN	Micronucleus
MRE11	Meiotic recombination 11
MTIC	Methyl triazeno imidazole carboxamide
NAD+	Nicotinamide Adenine Dinucleotide
NBS1	Nijmegen breakage syndrome 1

NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining
PARP 1	Poly (ADP-Ribose) Polymerase 1
PFGE	Pulsed-field gel electrophoresis
PI3K	Phosphoinositide 3-kinase
PIKKs	Phosphoinositide 3kinase-like kinases
Pol θ	DNA polymerase θ
PTIP	Pax transactivation domain-interacting protein
RAD23B	UV excision repair protein RAD23 homologue B
RAD50	Radiation repair 50 protein
RIF1	RAP1-interacting factor 1
RIPK1	Receptor-Interacting Protein Kinase 1
PARP 1	Poly (ADP-Ribose) Polymerase 1
RNF8 protein	Ubiquitin-protein ligase ring finger 8
ROS	Reactive oxygen species
RPA	Replication protein A
SSA	Single-Strand Annealing
SSB	Single strand breaks
TFIIH	Transcription initiation factor IIH
TRP53BP1	Tumor suppressor p53 Binding Protein 1
TOP1	Topoisomerase 1
UV–DDB	Ultraviolet radiation DNA damage-binding protein
XRCC4	X-ray repair cross-complementing protein 4

Introduction

Almost all cancer therapy, except surgery, kills tumor cells by inducing DNA damage. DNA double-strand breaks (DSB) is the most lethal form of DNA damage, due to difficulty of correct repair. Almost all DSBs rejoin by non-homologous end joining (NHEJ).
However, the following problems related to NHEJ remain partially or completely unresolved:

- Increasing the efficiency of this pathway for tumor radio-chemosensitization
- The ratio of NHEJ involvement in the overall structure of DNA damage
- Exact nature of DSB structure which activates NHEJ
- NHEJ response to the DSB complexity levels and effect of these complexities on DSB repair pathway choice

This thesis addresses these problems, which are explained in the five included papers.

1.1. DNA macromolecule

The double-stranded helix DNA is considered the origin of life and cell data bank for protein synthesis. DNA consists of almost similar building blocks that repeat along a chain shaped double helix. The DNA helix backbone is a pentose sugar molecule of deoxyribose attached to a phosphate group and coupled by hydrogen bonds to four types of nitrogen-containing bases (adenine (A), guanine (G), cytosine (C), and thymine (T)). The A base specifically pairs with T and the G base always pairs with C. One strand of DNA duplex locates anti-parallel to another strand, this means the 5' end of a single strand always faces to 3' of another strand. Further, phosphate groups link each nucleotide to another vertically and the outer edges of DNA bases are capable of making a hydrogen bond with proteins e.g. during DNA replication (1).

As the cell's largest macromolecule DNA is packed and organized by wrapping around histones, making nucleosomes inside the nucleus. Histones regulate cells' epigenetic traits by chromatin remodeling modification and gene post-translation variations. Histone variants provide a platform for the

recruitment of chromatin-interacting kinases to modify a gene's post-translational variation.

Around 147 DNA base pairs wrap around the nucleosome core structure or histone octamer consisting of eight core particles, two each of H2A, H2B, H3, and H4. The nucleosomes are attached to the H1 linker histone which sits on the nucleosome. H1 histone connects each nucleosome to the other with linker DNA. The length of linker DNA is around 20-90 bp at the entry and exit parts of the nucleosome, forming the shape of beads and string (2–5).

1.2. DNA damage induction

DNA stability is the key player in cell survival and proliferation. This stability is under constant attack by endogenous and exogenous elements. Depending on the source and severity of DNA damage, cells face different fates such as genomic abnormal rearrangement, autophagy, apoptosis, DNA repair, cell cycle arrest, epigenomic alterations, and tumorigenesis. The internal sources can be transcription errors, replication stress, and reactive oxygen species (ROS) from metabolism (metabolic oxidation). Exogenous sources are considered strong elements of assault such as ionizing radiation (inducer of DSB, single-strand breaks (SSB), and base lesions), ultraviolet light (base damage and dimer generator), platinum-based chemotherapeutics (DNA cross-links and DNA adducts inducer), and oxidative agents (abasic sites and SSB generation) (6,7).

1.2.1. DNA Base lesions

Hydrolytic force, oxidative stress, alkylating agents, and ultraviolet light can generate base lesions. The higher molarity of water molecules inside the cell, causes the hydrolytic force to remove the amino group from DNA bases or deamination. The common form of this lesion is the deamination of cytosine and later conversion to uracil. Also, water-induced deamination of adenine produces hypoxanthine (8). It is noteworthy that deamination can happen during DNA replication by DNA polymerase as the most common form of base modification in human normal and cancer cells which happens by substitution of A with G or C to T (9). Oxidative stress from ROS family members (hydroxyl radicals, superoxide radicals, and hydrogen peroxide) is a common cause of oxidation of guanine and forming 8-oxoG, which promotes the substitution of G with T. Hydroxyl free radicals can damage DNA sugar moiety and form 8,5′-cyclopurine-2′-deoxynucleosides (10). Covalent binding of alkylating elements to DNA bases can increase the rate of DNA depurination and generation of 3-methyladenine and 7-methylguanine. Pyrimidine dimer generation from ultraviolet light is another source of base lesions (11).

1.2.2. SSB

The SSB occurs frequently, with up to tens of thousands of them happening in a single cell daily, because of both intracellular metabolites attack and DNA decay. Also, the SSB can occur at the site of ROS damage and DNA repair excision steps or faulty function of topoisomerase (TOP1) enzymes.

TOP1 relaxes supercoiled DNA by producing DNA nick cleavage during transcription. The collision of cleaved DNA with DNA polymerase, RNA, or DNA lesions at proximity can convert cleavage complexes into TOP1-linked SSBs and TOP1-linked DSBs (Fig. 1) (12,13).

Oxidative stress (ROS) from the internal metabolic reaction is the most common cause of SSB. ROS can directly generate SSB via attack and oxidize the sugar molecule of DNA. The indirect action of ROS is through affecting DNA abasic sites, damaged bases, or by interfering in base excision repair.

Figure 1. Different types of DNA damage.

1.2.3. DSB

The DSB is the most toxic type of DNA lesion. The source of DSB can be ionizing radiation, chemotherapeutic agents, ROS, and mechanical forces that break the chromatin structure. Also, replication fork collision with SSB, sugar lesion and base damage can generate DSB. The defective metabolism of telomeres also can lead to DSB formation. DSB is an important part of some vital biological processes inside the human cells such as the initial step at recombination of homologous chromosomes in meiosis, V(D)J recombination, and recombination in immunoglobulin class switching. The repair of DSB lesions is more challenging to the cell than other types of DNA damage and there is a possibility of the transition of non-repaired DSBs to the new generation of cells. G1/S checkpoint activation is slow and can pass unrepaired DSB to the S phase in 4-6 hours after irradiation. In contrast, the G2/M checkpoint is quickly activated by DSBs, but the activation threshold is 10-20 DSB. If the DSB number is below this threshold, then the G2/M checkpoint will not be activated and a cell can be divided carrying an unrepaired DSB (14). The cell's adoption to the low rate of non-repaired DSB can increase the possibility of mutation in tumor suppressor genes or activation of oncogenes. Also, errors in DSB rejoining can generate chromosomal translocation and tumorigenesis. This faulty chromosomal rearrangement in T cell receptor genes happens in

lymphoid cancers (15). Besides direct clustered damage by irradiation, processing of non-DSB clustered lesions can generate complex DSBs (7).

1.2.4. Clustered DNA damage

Clustered DNA damage happens when several DNA lesions are located in close proximity, within approximately 20 base pairs. Inside the damaged cluster there are different kinds of DNA damage such as SSB, DSB, oxidative base, and sugar damage. These lesions can locate on one strand or both strands of DNA close together. DNA clustered damage is associated with genomic instability, elevated mutation, and cancer (16). The basic form of clustered damage is a DSB consisting of two SSB in opposite DNA strands in close vicinity.

1.2.5. DSB complexity

Cancer treatment with X-rays and various cytotoxic drugs primarily kills cells by inducing DSBs, as the most harmful type of DNA damage (17). However, DSBs are highly heterogeneous, varying from simple enzymatically induced breaks to complex DSBs, such as those associated with additional DSBs, single-strand breaks (SSBs), or base lesions within 20-30 base pairs and clustered DSBs within chromatin, induced by X-rays or especially with high-linear energy transfer (LET) radiation (18).

The complexity of DSB can be classified into various levels predicated on the nature of the lesions, their quantity, and their spatial distribution relative to each other. Each DNA-damaging agent has its own DNA lesion signature. For instance, calicheamicin, an exceptionally potent inducer of DSBs, is classified as an enediyne antitumor antibiotic that cleaves DNA at sites rich in pyrimidines (19). Phleomycin, a glycopeptide antibiotic containing copper and derived from *Streptomyces verticillus*, is part of the bleomycin family and induces DNA adduct formation, by disrupting DNA polymerase activity and resulting in the generation of numerous SSBs as well as a limited number of DSBs (20). X-rays are distinguished by their ability to induce clustered DNA damage, encompassing interstrand crosslinks, oxidative base modifications, SSBs, and DSBs, through mechanisms involving both direct ionization and indirect pathways mediated by reactive oxygen species (ROS) (20,21).

Etoposide (ETP) is a topoisomerase II (topo II) inhibitor that transforms temporary DNA breaks into stable DSBs (21). At clinically relevant concentrations, the majority of DNA damage caused by ETP is SSBs. At higher concentrations, with saturation of topo II ligase pockets, etoposide can generate DSBs (22,23). Temozolomide (TMZ) is an alkylating agent that forms DNA cross-links, inhibiting DNA replication. The DNA damage induced by TMZ primarily consists of base pair mismatches and SSBs, with little to no generation of DSBs (24,25).

1.2.6. Ionizing radiation

Ionizing radiation is a high-frequency energy with a short wavelength from natural or synthetic sources which can produce ions when interacting with matter. It strips off the atom's electrons, producing a nucleus with a positive charge. Natural types of ionizing radiation are cosmic radiation and radon. The exposure from synthetic sources can be from medical imaging, treatment devices, and nuclear energy production wastes. There are different types of ionizing radiation such as photons and charged particles with different biological effects. A radiation's biological effect depends on the amount of energy absorbed by interacted biological matter or delivered dose per unit of biological target define by gray (Gy) or joules per kilogram (26). Further, the biological effect is also dependent on the amount of energy that the ionizing particle, or photon, delivers to the encountered material along its track, in each distance unit (keV/μm), defined as the ionizing radiation linear energy transfer (LET). High-LET particles like alpha-particles and heavy ions have typical LET of 70-200 keV/μm. In contrast the deposition from low-LET radiations like γ-rays and X-rays are in the range 0.1-2 keV/μm. (27).

1.2.6.1. Biological effects

Both high and low-LET radiation can induce biological damage directly and indirectly by energy transfer, especially to the nucleus which makes it a unique tool for DNA damage induction. The direct effect comes from the radiation-delivered dose to the phosphodiester backbone. The indirect effect is through interaction with the surrounding environment of DNA such as water, oxygen, salts, and proteins (5). This indirect damage is mainly related to the production of hydroxyl radicals and oxygen atoms from water radiolysis. It is estimated that for low-LET radiation one-third of radiation damage on DNA is direct and the rest is indirect (28), whereas for high-LET 50-70% is direct.

1.2.6.2. Radiation-induced clustered DNA damage

Clustered DNA damage is a signature of ionizing radiation. Both high-LET and low-LET radiation can induce highly complex DNA damage or clustered DNA damage. The complexity of damage increases with elevated LET and the cluster formation is related to the activation of several electrons after the initial hit from the first activated electron (29). For high-LET, damage are clustered along and around the track of travel through the nucleus. In contrast, the low-LET radiation damage is more randomly distributed (30). For example, 1 Gy of low-LET γ-rays produces about 1000 ionization tracks in one mammalian cell nucleus compared to only 4-5 tracks produced by 1 Gy of α-particles.

All types of ionizing radiation initial insult can be followed with the help of induced radiation track structure simulation. The Monte Carlo simulation of the low-LET ionization track shows 30% complex DSBs in at least one

lesion together with base damages. This number is 90% for high-LET. The number of non-DSB clusters is around 4 times more than DSB at low-LET and 8 times more for high-LET (7). The clustered DNA lesions have the highest correlation with the final biological cell fate (31).

Metabolic oxidative stress cannot generate this type of damage. Also, reactive radicals from the indirect effect of ionizing radiation are not likely to produce clustered damage. Therefore, if the indirect oxidation rate of ionizing radiation and oxidative chemical become the same, irradiation is much more toxic because of extra direct damage induction in the form of clustered damage.

1.2.7. Chromatin structure and DNA damage

DNA is more protected from radiation when wrapped around histones than transcriptionally active open parts and linker DNA. The inner part of DNA that faces the histone is more protected than the outer DNA surface. This histone protection provides physical cover and less exposure face to irradiation and radiomimetic agents. However, ROS due to their very small size still can interact with a large part of nucleosome DNA (32). Nevertheless, histones removal from DNA using high salt increases the DSB induction 20-40 fold after removing H2A and H2B, and it elevates to around 70 fold by removing all core histones (5).

Chromatin packaging heterogeneity influences the binding sites of DNA-damaging cytotoxic drugs and the accessibility of ROS to DNA. This packaging will also influence both the distribution of DNA damage and the DNA repair proteins accessibility to initiate the repair (33). If DNA damage is complex, alternative repair pathways may process some DSBs before the activation of major pathways like NHEJ and HR (discussed below), leading to slower repair. Complex DSBs in compact chromatin may require chromatin-related kinases for modification and repair access (34,35).

1.3. DNA repair

DNA damage can transfer to the daughter cells after mitosis or meiosis to increase the probability of genetic defects. To protect DNA integrity through cell division and metabolism all eukaryote cells develop profound DNA repair machinery. This system includes three main parts. First, the sensing section identifies and recognizes the type of DNA lesion. After understanding the DNA damage occurrence, the second part of the DNA repair machinery becomes active by the initiation of a cascade of alarm messages via cell signaling. In the third part, the cell reacts to the DNA repair signals by programmed cell death or apoptosis, senescence, necrosis, cell cycle arrest, or repair of the damaged DNA lesions (36). This dynamic process is concomitant with

chromatin modulation and rearrangement to adjust the chromatin structure for accessibility and transcription by DNA repair modulators at the damage site and whole chromatin. These DNA repair strategies help multicellular organisms to hamper defective cells with heavily damaged DNA from propagation and reduce the possibility of cancer (37). In general view, the DNA repair system in its different forms is designed to cut the lesion out of the DNA and insert the correct sequence of nucleotides (38). Here the important DNA repair pathways of BER (Base Excision Repair), NER (Nucleotide excision repair), MMR (Mismatch repair), SSB repair, and DSB repair will be briefly discussed in the following section.

1.3.1. BER

Most abnormal bases and DNA lesions from oxidative and alkylating elements, do not distort the DNA and can be repaired fast (few minutes) via BER in the G1 phase. BER function is inside the bigger context of cell repair machinery and not just as an isolated pathway (39). The repair initiation starts with the recognition of the damaged base by DNA glycosylase which cleaves the N-glycosylic bond of a base to the DNA sugar backbone, leaving a baseless AP site. APE1 endonuclease processes the AP site and cleaves the phosphodiester bond producing a SSB. At this point, BER continues with the short (single nucleotide replacement) or long-patch repair (2-10 nucleotide resynthesis). In the classic BER or short-patch BER the Pol β recognizes the one nucleotide gap (3′OH and 5′ P ends) and removes the 5′ P end and activates XRCC1 and Lig3 to complete the repair by ligating the DNA ends. If the 5′ P end of SSB is blocked and not possible to process by SSB end-processing kinases (Pol β, APE1, PNKP, Aprataxin, TPD1) long-patch repair takes over. In this way, resynthesizing of 2-10 AP nucleotides starts with Pol δ which displaces the strand. Then flap endonuclease 1 cuts the flapped strand and DNA ligase I, ligates the DNA (40).

1.3.2. NER

NER has a more complicated process than BER with the detection of DNA distortion. It is in charge of fixing the DNA cross-link and DNA adducts. NER consists of two sub-pathways of transcription-coupled NER (TC-NER) and global genome NER (GG-NER).

GG-NER: protects DNA from duplex distorting damages. DNA distortion recognizes by the XPC-RAD23B-CETN2 complex with the help of UV-DDB. Then XPC binding to the damage site dissociates RAD23B.

TC-NER: During the stalling of RNA transcription, UVSSA, USP7, and CSB start interaction with RNA polymerase II and accumulate at the transcription site. Then after increasing the affinity of CSB to RNA pol II, CSA forms

a complex with CSB which leads to backtracking or reverse translocation of RNA Pol II to make the lesion accessible for repair.

From this point, the repair process is common for TC-NER and GG-NER. After lesion detection, the TFIIH complex binds to the damaged site, and CAK dissociates from the TFIIH complex. XPG endonuclease binds to the NER complex or TFIIH. Then TFIIH opens the DNA duplex around the damage site from 5′ to 3′. XPD verifies the lesion with the help of TFIIH XPB and XPA. Meanwhile, RPA protects the undamaged nucleotides. The XPA recruits the XPF-ERCC1 complex for lesion incision (No return point) and XPG cuts the damaged strand around 22-30 nucleotides long. Then PCNA recruits DNA Pol δ, DNA Pol ε, or DNA Pol κ to fill the gap. Finally, ligation happens by DNA ligase 1 or DNA ligase 3 (41).

1.3.3. MMR

MMR is responsible for fixing replication errors and single nucleotide mismatches from DNA polymerase action. First MSH2-MSH6 complex detects the mismatch and forms the sliding clamp. Then EXO 1 cut out the nucleotide. RPA protects the strand from degradation and EXO 1 action. DNA polymerase δ synthesizes the nucleotide and finally, DNA ligase seals the DNA (42).

1.3.4. SSB repair

SSB can be generated directly from ROS, abortive TOP1 function, or during the processing of closely located lesions. It can be induced indirectly during BER, NER, and MMR and has an extensive overlapping repair process with them. In the direct SSB repair process, PARP 1 (Poly (ADP-Ribose) Polymerase 1) activates by SSB and binds to the lesion site. It starts recruiting downstream DNA end-processing elements APE1, APTX, and PNKP. Then Pol β, Pol δ, or Pol ε fill the nucleotide gap. In the end, DNA ligates by DNA ligase 3 or DNA ligase 1 (12).

1.3.5. DSB Repair

DSB initiate by lesion sensing, followed by a cascade of intra-cellular interwoven signaling to stimulate DSB repair and cell cycle checkpoints activation. Two important chromosomal DSB mediators, 53BP1 and γH2AX (43), besides three members of the phosphoinositide 3-kinases (PI3Ks) family including ATM (Ataxia telangiectasia mutated), ATR (Ataxia telangiectasia and Rad3-related protein), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit), as transducer DSB repair signaling, launch the cascade of protein phosphorylation to activates DSB repair pathways (6,44). Here characteristics of these mediators, transducers, and non-homologous end joining

(NHEJ) and homologous recombination (HR) pathways with emphasis on NHEJ are discussed.

1.3.5.1. 53BP1

The phosphorylation of the 53BP1 protein is important for DSB repair activation. The p53-binding protein 1 known as p202, TDRD30, p53BP1 or TRP53BP1 protein with over 200 kDa and 1972 amino acid encodes by *TP53BP1* gene located on the human's chromosome number 15 (15q15.3). The expression of this gene in the brain is higher than in other organs (45,46). It is a multifunctional protein belonging to the Tudor domain protein family. The Tudor domain enables the protein to read histones by detecting and binding to methylated arginine and lysine residues to modify histone and accelerate DNA damage repair response (47). Besides the well-known role of 53BP1 in DNA damage response, it plays different roles in cellular processes, like apoptosis, autophagy, and aging (45).

In the DNA damage response chain, 53BP1 plays downstream of PIKKs, ATM, ATR, and MRN complex (MRE11-RAD50-NBS1). 53BP1 protein doesn't act as an enzyme per se, but it functions as a substrate to mediate other proteins' functions possible in a cell cycle-dependent manner. In the time of DNA damage, the histone recognition starts with the dimethylation of histone H4 at Lysine 20. 53BP1 through its Tandem Tudor domain has selective specificity to bind to this dimethylated site to make 53BP1/H4-K20me2 Complex (48). Also, 53BP1 assembly to the site of DSB facilitates by ubiquitylation of it through RNF8 and RNF 168 catalyzation and recruitment of p97 protein to the damage site. This reaction leads to the removal of L3MBTL1 protein from the methylated site of histone H3K4 lysin20 and accelerates more 53BP1 binding to the damage site (49). Moreover, the 53BP1 Tudor domain acts as a mediator of DNA damage response sensors to effectors of checkpoint kinase 1 (CHEK1), checkpoint kinase 2 (CHEK2), ATM, breast cancer-associated gene 1 (BRCA1), and topoisomerase II-binding protein 1 (TOPBP1) (45).

53BP1 has a crucial role in DSB repair pathway choice by providing a recruitment scaffold for sensors and effectors of DDR (DNA Damage Response). 53BP1 is involved in HR and NHEJ by activation of p53 and also can function independently of p53. It stimulates the DSB repair pathway in favor of NHEJ versus HR by competing against ubiquitination via BRCA1. ATM phosphorylates 53BP1 to recruit RIF1 and PTIP to the damaged site. This complex of proteins promotes NHEJ by protecting DSB ends from resection in the cell cycle G1 phase. But in S/G2 cell cycle phases, BRCA1 C Terminus (BRCT) domain inhibits and antagonizes phosphorylation of 53BP1 by ATM. As a consequence, HR becomes the dominant repair pathway in the S/G2 phases (Fig. 2).

The mechanistic resolution of 53BP1 during the S phase is not clear yet. The consequence of defective 53BP1 function is hypersensitivity to ionized irradiation with error in DNA damage checkpoint, abnormal cell cycle

regulation, and defective DNA damage repair (50,51). Imaging of 53BP1 accumulation into so called foci after e.g. radiation-exposure, serve as a common biomarker for DSB.

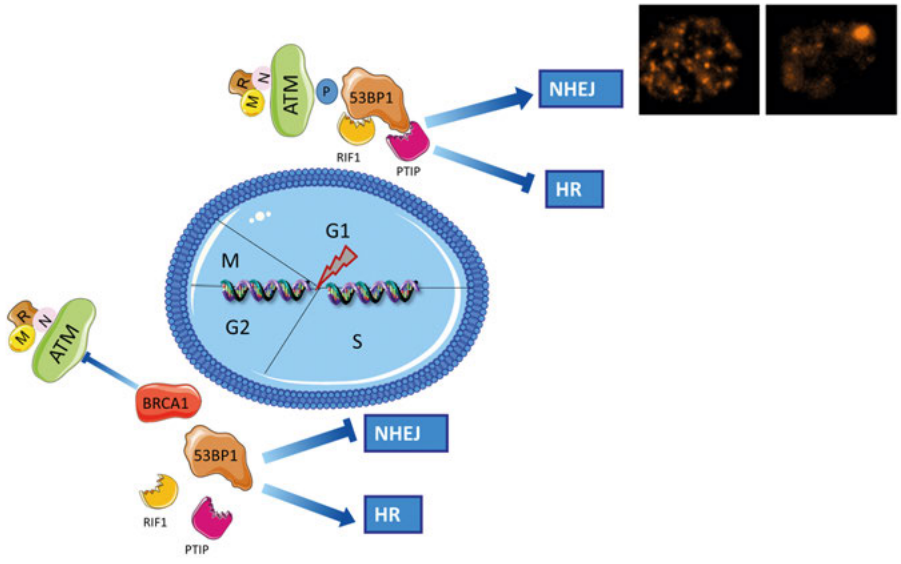

Figure 2. 53BP1 cell cycle-dependent function under the effect of BRCA1 at DSB repair. The immunofluorescence image of HCT116 cells shows 53BP1 biomarker in orange in S and G phases.

1.3.5.2. γH2AX

Out of all H2A histones in vertebrate cell, 2-25% are H2AX conserved variant encoded by the *H2AFX* gene on chromosome 11 (52,53). One of the initial DSB responses is the quick phosphorylation of histone H2AX at Ser-139 to form γH2AX(54). The role of γH2AX in DDR is to make DNA accessible for the recruitment of repair factors. It holds broken ends close together during repair to avoid losing chromosomal sites (55). Moreover, γH2AX reduces chromatin density at the site of damage and repositions the nucleosome for anchoring DNA broken ends. Also, γH2AX plays a role in checkpoint activation response for DNA repair (56). After DSB happens three main kinases of ATM, ATR, and DNA-PKcs are contributing to H2AX phosphorylation with overlapping parallel functions. However, ATM is the main direct activator of H2AX phosphorylation (6). The other activation way is indirectly from the MRN complex which directly interacts with the DSB site and then recruits ATM. Then ATM phosphorylates H2AX through auto-phosphorylation and monomerization. Also, phosphorylation of MDC1 can lead to H2AX phosphorylation (57).

γH2AX foci fluorescent imaging after irradiation revealed that up to 30 Mbp can be modified as the result of H2AX phosphorylation leading to huge chromatin relaxation. These foci are detectable after minutes of irradiation by reaching the peak in 30 minutes. After 1 h the number of foci starts to decline (58). After finishing the DSB repair, γH2AX foci disappear. However, the complete DSB repair stage that leads to foci clearance is not well understood. It is still elusive if this DSB repair completion happens after DSB rejoining or chromatin remodeling (53). Defective H2AX in mice model, make them extremely radiosensitive with genomic instability, defective G2/M cell cycle checkpoint, and retardant DNA repair (59).

Visualization of chromatin modification by immunofluorescence staining is a sensitive biomarker of DNA damage and repair. The γH2AX immunostaining is 100 times more sensitive than other DSB detection methods such as PFGE for detecting 1 focus in every 30 cells after receiving of 1mGy ionizing radiation dose without the need for lysing cells. The PFGE DSB detection threshold for ionizing radiation is 5 to 10 Gy which is much higher than γH2AX immunostaining. This high sensitivity makes γH2AX imaging a versatile marker for DNA damage and repair detection not only for various types of ionizing radiation but also for a wide variety of DNA-interacting chemical agents (59).

1.3.5.3. ATR

ATR is one of the DSB repair main kinases with many substrates in response to DNA replication stress, the vast range of genotoxic insults, and cell proliferation. Activated ATR, phosphorylates CHK1 for the regulation of the cell cycle, DNA repair, deoxynucleotide synthesis promotion, DNA replication fork restart, and prevention of DSBs formation. Single-stranded DNA coated by replication protein complex A (RPA) recruits ATR via ATRIP. This binding follows by adding a TopBP1 activator to ATRIP and C-terminal PRD in ATR to trigger ATR kinase action. A main function of ATR is the phosphorylation of protein kinase CHK1. Activated CHK1 promotes the degradation of CDC25A to end the inhibitory phosphorylation in CDKs for sake of cell cycle progression. Genotoxic stress leads to the inactivation of CHK1 followed by cell cycle arrest. This delay gives more time for the cell to fix the damage before entering mitosis. In case of extensive and not repairable damage, cells will enter senescence or apoptosis pathways. The second vital function of ATR is protecting replication forks by inhibiting CDKs to restrain replication origin firing. This inhibition safeguards the repair factor`s pool and prevents replication exhaustion (6,60). Many cancer cells' survival is dependent on the ATR signaling pathway which makes it a good candidate for tumor radio and chemo-sensitization (61).

1.3.5.4. ATM

ATM is one of the main DSB repair signal transducers which is activated by DSB, oxidative stress, and chromatin change. After DSB, ATM autophosphorylates at Ser198 which changes the close dimer of ATM to the active monomer state for the recruitment of downstream partners (62). However, the majority (80%) of DSB outside of the S phase, are repaired via NHEJ and independent of ATM, and the rest minority is repaired by ATM, DNA-PK, and MRN complex. It is suggested that ATM participate in NHEJ for bridging the DSB gap. In the deficiency of DNA-PKcs, ATM becomes hyperactive. The biological relevance of this interaction in normal cells is preventing the cell from entering the unwanted apoptosis phase. ATM is not necessary for HR initiation but it promotes DNA end resection process in favor of HR by phosphorylation of CtIP. In response to DSB, MRN recruits ATM to the DSB site and activates it. The mechanism of this activation is not fully understood. One of the interesting roles of ATM is in the positive feedback loop of γH2AX activation by MDC1 and MRN complex phosphorylation. Also, histone H2AX and MDC1 activation by ATM promotes RNF8 and RNF168 activation in favor of 53BP1 recruitment (6).

1.3.5.5. NHEJ

The human cell repairs DSBs by recruiting two staddle pathways of NHEJ as a less accurate quick way and HR as a slow precise way (63). The cell chooses between HR and NHEJ to repair DSBs based on the cell phase (37). NHEJ is active in all cell phases, especially in G0/G1 and early S phase by directly using the ends of DSBs for ligation (64). But HR is recruited more during the S/G2 phase to perform the error-free repair by using sister chromatid to restore genuine DNA integrity (65,66). Recent findings show that when DSBs occur in DNA transcriptional-associated regions, HR fixes it and NHEJ repairs more chromosome abnormalities in G2 (67).

The NHEJ initiation starts by loading Ku 70/80 heterodimer with strong binding affinity to DNA broken ends. The binding of Ku, protects two DNA ends from nucleases (68). Ku recruits DNA-PKcs to bridge the gap. Another critical function of DNA-PKcs is recruiting Artemis to initiate DNA-end processing (69). KU and DNA-PKcs complex is responsible for DNA-end tethering followed by recruitment of other repair factors for NHEJ like XRCC4, XLF, DNA ligase IV, PAXX, and Artemis in an orchestrated fashion. Each of these recruitment processes facilitates part of NHEJ. XRCC4 is necessary to stabilize LIG4 to make a complex with XLF. This complex establishes the final step of DNA ends ligation. The main elements for DNA end-ligation are Ku heterodimer, XRCC4, LIG4, and XLF (68) (Fig. 3).

1.3.5.5.1. KU

KU is a heterodimer protein with Ku70 and Ku 80 subunits. Ku subunits have a vonWillbrand antigen domain (vWA), C-terminal domain, and core or central domain. The last domain is used for direct binding to DNA and making dimers by forming a ring shape to encompass two ends of DSB. Ku has a strong affinity (K_d =10-9 M) for DSB and binds to it in a sequence-independent manner by loading Ku70 close to the DSB end and Ku 80 away from the DNA end. Ku provides a scaffold for loading all DSB repair elements onto the lesion site. Besides DSB end bridging, Ku stabilizes DNA end ligation at the end of DSB repair. Ku has an indirect role in DSB repair by involvement in cell cycle checkpoint activation and apoptosis signaling. It protects the telomere's end from DSB repair trimming and excision (70). A deficiency of Ku70/80 in humans is fatal and disrupts telomere protection and maintenance (71).

1.3.5.5.2. DNA-PKcs

DNA-PKcs is a large (460 KDa) versatile serine-threonine protein kinase with playing different roles from, NHEJ and apoptosis to nonnuclear pathways (72). After DSB, the C terminus of Ku activates DNA-PKcs for DSB bridging to provide a scaffold for recruiting other partners of NHEJ (73). However, the interesting regulatory function of DNA-PKcs laid into its possibility of autophosphorylation. It can be autophosphorylated at S2056 and T2609 HEAT-repeat sites for DNA repair. This autophosphorylation changes the assembly formation of it to the DSB site for disassociation and DSB end ligation. The T3950 autophosphorylation at the kinase domain shuts down the DNA-PKcs kinase activity.

Besides the pivotal role in DSB repair, DNA-PKcs involves in several cancer-related processes. DNA-PKcs interaction with Snail1 (E-box binding transcription factor) drives genomic instability and tumor malignancy. DNA-PKcs phosphorylates Snail1 followed by DNA damage which reciprocally blocks DNA-PKcs activity. Additionally, DNA-PKcs activation is associated with spindle apparatus stability during mitosis by regulation of microtubule dynamics and chromosomal integrity. Recent studies revealed the possibility of functional change in DNA-PKcs performance in cancer by losing its protective activity by tumor progression. Another out of DSBs repair role of DNA-PKcs is its activation by hypoxia through the HIF family in cancer which affects the cell`s survival. Also, DNA-Pkcs plays a significant role in energy regulation of tumor environment metabolism by phosphorylation of AMPK in glucose deprivation. The other role of DNA-PKcs is its ability to activate tumor-associated immunity response by binding to foreign DNA. This pleiotropic characteristic of DNA-PKcs makes it an attractive target to approach human malignancies through multiple pathways using inhibitors (74,75).

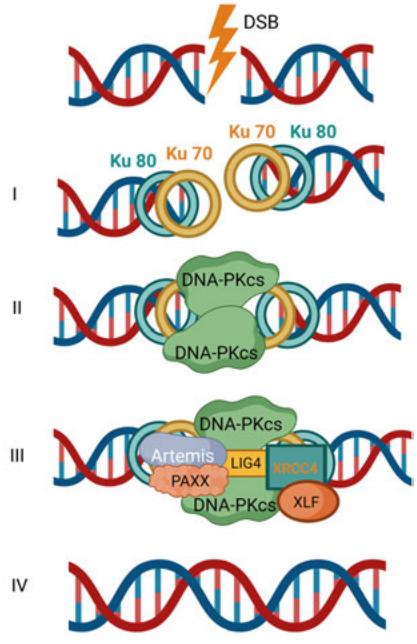

Figure 3. NHEJ pathway steps in repair of a DSB (Created with BioRender.com)

1.3.5.6. HR

HR as a dominant DSB repair pathway at S and G2 phases starts by recruiting MRN complex to flank the DSB for DNA end resection (from 5′end). If DSB ends are not clean (hairpins and proteins), DSB end resection accomplishes with the help of CtIP endonuclease leading to the generation of single strand 3′ tail. RPA binds quickly to this single-stranded DNA for protection. Then it replaces by RAD51 and RAD54 which activates the complementary DNA search of sister chromatid and D-loop formation. In the next step, D-loop dissociates and repair continues by non-crossover repair or double Holliday junction structure repair through non-crossover or crossover recombination. The DNA polymerase synthesis the new DNA on invading strand and finally DNA ligase 1 seal the DNA (5,76).

1.3.5.7. Alternative Pathways for DSB repair

If the main DSB repair pathways are compromised or inhibited, alternative repair pathways can be activated. NHEJ mutations is rare in human cells. For this reason, it is still unclear if components of alternative DSB repair pathways have other functions in DSB processing beyond being distinct NHEJ back up pathway (77,78). However, it is known that DSB repair pathway choice between NHEJ, HR, Alt-NHEJ (Alternative Non-Homologous End Joining) or

SSA (Single-Strand Annealing) is based on difference in DNA end resection activity. Protecting DSB ends with KU70-KU80 allows NHEJ to avoid extensive resection. While Artemis and the DNA-PKcs complex can perform resection, it is minimal, involving fewer than 20 bp. In contrast CtIP and MRN complex carry out extensive 5′ to 3′ resection to generate 3′ overhang for HR, Alt-NHEJ, and SSA. SSA typically needs more homology (>20 pb) base pairs than Alt-NHEJ (4-6 bp). HR requires a homologous template with homology typically greater than 50 bp for efficient repair following DNA end resection (79).

1.3.5.7. 1. Alt-NHEJ

Alt-NHEJ also known as MMEJ (microhomology-mediated end joining) is a backup repair pathway that can be activated when NHEJ is defective. This pathway uses short microhomologies at the break site to rejoin the broken DNA ends. Alt-NHEJ typically operates on less than 20 bp microhomology, most often 4–6 bp. Alt-NHEJ promotes by CtIP and MRN complex resection, followed by PARP 1 and Pol θ (DNA polymerase θ) recruitment as central kinases for Alt-NHEJ. Alt-NHEJ is error-prone and can lead to deletions or insertions at the break site (79,80)

1.3.5.7. 2. SSA

Similar to HR, SSA pathway is mostly active in S and G2 cell cycle phase due to availability of sister chromatid. Some studies suggest that HR and SSA compete for DSB repair is S phase (81,82). SSA uses more than 20 bp of homology (83). BLM and EXO 1 perform extensive resection. Then RPA binds to ssDNA to activate SSA or HR. In this point RAD52, mediate annealing of large homologies in favor of promoting SSA, leading to F (XPF)–ERCC1 complex cutting the residual 3′ overhangs before ligation. Finally, XRCC1/Ligase III ligates the DNA ends. In contrast, after BLM and EXO 1 resection and RPA binding, RAD51 mediate strand exchange associated with BRCA1, BRCA2 and RAD54 to promote HR pathway (79–81).

1.3.6. DSB Repair Phases (kinetics)

DSB repair occurs in two distinct phases: a fast phase and a slow phase. The fast repair phase, occurring within the first two hours, primarily involves DSBs located in euchromatin, where the open and accessible chromatin structure allows for the rapid recruitment of NHEJ and HR components. During this phase, NHEJ repairs approximately 80% of DSBs. The slow repair phase, lasting 2 to 24 hours, is associated with DSBs in heterochromatin, where tightly packed chromatin must first undergo modifications and relaxation to permit access for repair components. In this phase, HR plays a more significant role, as it requires extensive DNA end resection and the presence of a

sister chromatid, making it more active in G2 and S phases of the cell cycle (Fig. 4).

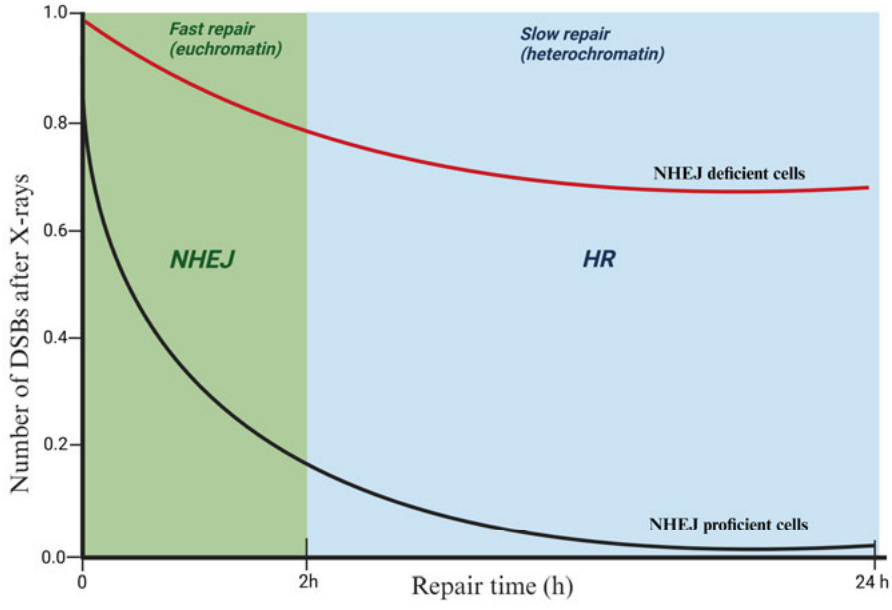

Figure 4. Schematic model of DSB repair kinetics in asynchronous cells. In NHEJ-proficient cells exposed to 40 Gy X-rays, most DSBs are rapidly repaired by NHEJ in the fast phase. The remaining DSBs were repaired by resection-dependent HR in the slow phase of DSB repair. NHEJ-deficient cells fail to efficiently resolve DSBs due to the absence of the primary DSB repair pathway. Illustration adapted from Shibata & Jeggo, 2020 (Created with BioRender.com).

Chromatin remodeling is crucial for facilitating the repair of DSBs, particularly in heterochromatin. Various chromatin-modifying enzymes, such as HATs (histone acetyltransferases) and HMTs (histone methyltransferases), modify histones to relax chromatin, making a more accessible space for repair factors. Proteins like KAP1(KRAB-associated protein 1) and HP1 (Heterochromatin Protein 1) are phosphorylated to relax chromatin, allowing HR repair. Additionally, ATM, phosphorylates histone H2AX (γH2AX), to recruit MDC1, 53BP1, and BRCA1, which coordinate the DNA repair. These chromatin modifications is necessary for repair kinases effective operation and prevention of DNA lesion accumulation (84,85).

1.3.7. Cell Cycle and DSB

Mitotic cell cycle in human cells has two phases: interphase (duplication of cellular content) and M phase (mitosis) (segregation of cellular content) for ensuring the timely and correctly duplication of cells and its genomic material.

During S phase (synthesis phase), cells initiate DNA replication. There are to gap phases or G phases before (G1) and after S phase (G2). During G1 phase, cell undergoes growth and preparation for DNA replication. In the G2 phase, DNA replication, which is initiated in the S phase is completed, and the cell prepares for mitosis by checking for DNA damage, repairing errors, and producing proteins necessary for chromosome segregation. M phase has four parts: prophase (chromosome condensation, spindle formation, and nuclear envelope breakdown), metaphase (chromosome alignment), anaphase (chromatid separation), and cytokinesis (cytoplasmic separation).

In interphase, cell cycle progression is controlled before and after S phase. The main regulator of cell cycle progress is activity of CDK (Cyclin-Dependent Kinase) (86,87). CDK1, regulated M phase, CDK2 regulates G1 to S phase progression, and CDK4/6 regulates early G1phase and G1 to S phase progression. CDK needs cyclin protein to become active. Cyclins (A, B, C, D, and E) are proteins that bind to CDKs to control the transition between different cell cycle phases. For example, in G1, Cyclin D binds to CDK4/6 to help cell to pass through G1 checkpoint. Cyclin E binds to CDK2 to prepare cell for DNA replication in late G1 phase (passing G1 checkpoint) and in the beginning of S phase. Cyclin A, regulates the DNA replication and transition to M phase. Cyclin B drives the cell into M phase for proper cell division. Blocking CDK activity can push the cell from the G1 phase into the G0 phase, a resting state (88).

Cell cycle mainly control DNA replication and subsequent segregation of DNA between daughter cells. In cancer cells, cell cycle checkpoints are not defective and only specific parts of cell cycle control is disruptive. The recent studies showed that cancer cells are mainly disable to exit from cell cycle and as a result they don't stop dividing. Therefore, most of cell cycle machinery must be functional for cancer cells to survive. This point, suggests the importance of difference between DNA damage checkpoint (preventing DNA damage accumulation) and DNA replication stress checkpoints (prevent replication stress-induced DNA damage). Cancer cells often have impaired DNA damage checkpoint, allowing continuous proliferation of cells with accumulation of DNA lesions. But, replication stress checkpoint are rarely defected in cancer cells due to dependency of cancer cells on them to tolerate intense replication stress. Also, cancer cells are dependent to mitotic checkpoint for prevention of chromosome mis-segregation (89).

Cell cycle checkpoints are activated in response to DNA damage during interphase, replication stress in S phase, and spindle assembly failure in M phase. CHK2 (Checkpoint Kinase 2) is crucial for halting cell progression from G1 to S and from G2 to M phase. DSBs can activate CHK2 in interphase via ATM. Then, ATM phosphorylates and activates p53, a tumor suppressor protein that regulates apoptosis and cell cycle arrest. Phosphorylated p53 blocks CDK2 activity and halts the cell cycle. DNA damage in the form of

SSBs activates ATR. ATR then recruits CHK1 to block the activation of CDK1/2, which leads to the inhibition of mitotic entry (90,91)

Cell cycle checkpoints play a crucial role in performing accurate DSB repair and preventing genomic instability. These checkpoints act as surveillance mechanisms that stop cell cycle progression in response to DSB, giving time for repair processes to correct the lesions before the cell proceeds to mitosis. The G2/M checkpoint is particularly important for preventing the segregation of damaged chromosomes to enter mitosis with unresolved DSBs. The checkpoints also coordinate to the replication forks stabilization and regulate the mitotic DNA damage response, to ensure lesion processing after escape from checkpoint surveillance (92). This coordination is done by preventing of excessive origin firing and inhibiting replication fork collapse to avoid accumulation of SSB (91)

During mitosis, main DNA repair pathways are inactivated, but checkpoints stop transition of DNA lesion into mitosis (93). NHEJ is active in G1/S/G2 phase, while HR perform only in S/G2 phase after DNA replication. (94,95) (Fig. 5).

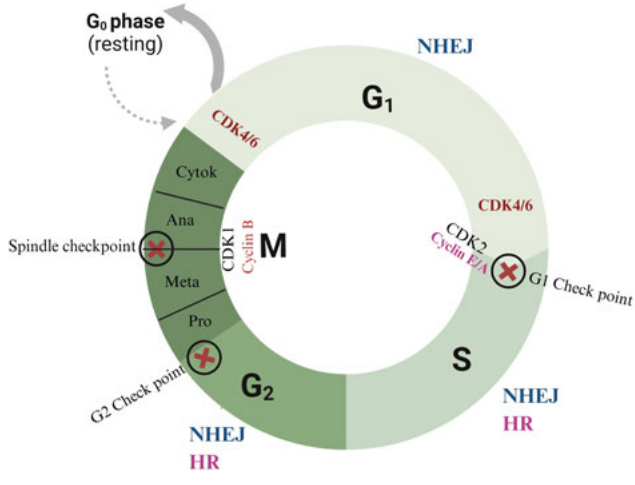

Figure 5. Cell cycle regulation phases and main DSB repair pathways (Created with BioRender.com).

1.4. DNA damage and apoptosis

The apoptotic response is the most sophisticated form of programmed cell death that associates with chromatin condensation, nucleus fragmentation, DNA cleavage, and cell volume reduction (96).

Apoptosis can be activated in both ways of DNA damage dependent and independent. Here DNA damage-dependent apoptosis is briefly introduced.

DSB is one of the main activators of DNA damage-dependent apoptosis. Damaged DNA can trigger apoptosis directly through death receptor FASR (through JNK), mitochondria (JNK), and caspase3/7 (CAD) activation. It can also promote apoptosis indirectly through ATM, ATR, and MYC. Direct activation of FASR, leads to caspase 8/10 phosphorylation which activates caspase 3/7 apoptotic kinase or initiates mitochondrial apoptosis through BID. After phosphorylation of ATR by DNA damage, it activates caspase 2 or p53. Here caspase 2 can initiate mitochondrial apoptosis or activates caspase 3/7 via the mediation of ICAD. Activated p53 elevates the activation of the death receptor FASR and promotes mitochondrial apoptosis through BAX, BCL-2, BCL-XL, and BAK. Phosphorylated ATM, activates the CHK2 checkpoint, then p53 becomes activated and promotes mitochondrial apoptosis or the FASR pathway. The involvement of MYC in DNA damage-dependent apoptosis is still not fully revealed. It is shown that activating MYC after DNA damage, phosphorylates NOXA, which leads to mitochondrial apoptosis (97).

1.5. DNA damage and necrosis/necroptosis

While apoptosis is well defined after DNA damage, emerging evidence suggests that DDR can also trigger necrosis/necroptosis under certain conditions (98). Necrosis considered an unregulated, accidental form of cell death caused by severe stress like overwhelming DNA damage. However, recent data show that specific DDR failures can lead to necrotic cell death. Complex DSBs from radiation or genotoxic agents can overwhelm NHEJ and HR repair machinery leading to ATP (Adenosine Triphosphate) depletion, mitochondrial dysfunction, and plasma membrane rupture leading to necrosis (99). Necroptosis is a programmed form of necrosis, mediated by receptor-interacting protein kinases RIPK1 (Receptor-Interacting Protein Kinase 1) and RIPK3, along with the pseudokinase MLKL. DDR pathways related to activation of PARP and ATM, have been associated to necroptosis (100). When PARP is overactivated under extensive DNA damage, NAD+ (Nicotinamide Adenine Dinucleotide) and ATP will be depleted leading to necroptosis activation. Some studies showed that PARP1 inhibition downregulates necroptosis in favor of necrosis after DNA damage due to preventing loss of energy through NAD+ and ATP depletion. Other studies showed opposite effect due to shifting cell death from caspase-dependent apoptosis to caspase-independent necroptosis (101). DNA damage typically induces apoptosis via p53-dependent pathways. However, when apoptosis is inhibited (e.g., by caspase inhibitors), DDR can activate necroptosis through RIPK3 and MLKL, especially in apoptosis-resistant cancer cells (102,103). DDR can exploit to manipulate necroptosis for cancer

treatment. For example, treating apoptosis-resistance cancer cells with PARP or ATM inhibitors could enhance treatment effect by inducing necroptosis (104).

1.6. DNA damage repair and cancer

1.6.1. Genomic Instability and Mutation Accumulation

DNA damage is critical in the development and progression of cancer. The mechanisms of DNA damage involve interactions between genetic and environmental factors, resulting to genomic instability, mutations, and uncontrolled cell proliferation. Genomic instability, is one of hallmarks of cancer, originates from defective DNA repair mechanisms. If this instability leads to mutations in critical genes, such as tumor suppressors and oncogenes, over time, these mutations could drive tumor formation, cancer progression, and metastasis (105,106). There are three main contributors to genomic instability: replication stress which drives from oncogene activation and rapid cell proliferation leading to replication errors (107). Chromosomal instability from defects in DNA repair pathways which leads to chromosomal abnormalities and rearrangements (108). Epigenetic alterations such as DNA methylation and histone modifications can impair DNA repair machinery gene expression (109).

1.6.2. Defects in DNA Repair Pathways

DNA repair pathways are vital for genomic integrity maintenance. However, defects in these pathways can lead to DNA damage accumulation and cancer development. Defects in these pathways, mainly HR and MMR, are significantly associated with cancer development (110). Many cancer types are associated with different types of genetic instability related to DNA damage repair genes such as deficiency in mitotic spindle checkpoint with chromosomal instability in breast cancer, BRCA1 and BRCA2 mutations in hereditary ovarian and breast cancer (impairing HR), and mismatch repair deficiency in hereditary non-polyposis colorectal cancer (38,111). DNA-PK has lower expression associated with genomic instability in the head and neck, and breast cancer (112) whereas overexpression of DNA-PKcs in several cancer types has been linked to poor prognosis. Defective ATM protein leads to ATR upregulation in oral squamous cell carcinomas and higher stages of breast cancer tumors are associated with elevated ATR and pCHEK1n (113).

1.6.3. DNA Damage in Tumor Progression

Once cancer is initiated, DNA repair impairment fuels tumor progression in three ways. Mutation in cell cycle checkpoint regulatory genes such as *TP53*, allow cancer cells to escape from proliferation arrest and apoptosis (114). Genomic instability, can accelerate acquisition of mutations that gives cancer cells the ability of invasion and metastasis (115). DNA repair pathway defects can render resistance trait to cancer cells against chemotherapy and radiation (116).

1.6.4. Targeting DNA Repair Pathways

Defects in DNA repair pathways provide a window of opportunities for targeted therapies. For example, PARP inhibitors can yield synthetic lethality (explained later) in cancer cells with HR deficiency (BRCA-mutated tumors) (117). ATR and CHK1 Inhibitors target replication stress in cancer with HR impairment or high replication stress. DDR inhibitors such as DNA-PK and ATM inhibitors have the potential of tumor radiosensitization (118).

1.6.5. Combination therapy

Combining DDR inhibitors with other cancer therapy modalities has shown promising results. The data showed that DDR inhibitors can enhance DNA-damaging agent's effect by hindering cancer cells from DSB repair, when combined with irradiation. For example, in ovarian cancer, the DDR is frequently defective, which is linked to the cancer's development and resistance to chemotherapy. These alterations are significant in high-grade serous carcinoma (HGSC), where about half of the cases show defects in HR pathway which can be targeted by inducing DSB via radiation and then inhibiting the DSB repair by HR inhibitors (119). Several studies suggests that DDR defects can elevates cancer cell's immunogenicity, making combination with immune checkpoint inhibitors an effective strategy. DDR inhibitors like PARP induce DSBs and genomic instability, leading to micronucleus (MN) formation and cytosolic DNA (DNA presence in cytoplasm instead on nucleus) accumulation in cancer cells. The innate immune system detects MN and cytosolic DNA as pathogenic, similar to viral nucleic acids, triggering interferon (IFN) signaling (cGAS-STING-IFN pathway) and subsequent immune activation (120). For example pamiparib (PARP inhibitor) treatment elevates the PD-L1 expression in pancreatic cancer cells, which in combination with PD-L1 mAb, enhances therapy effectiveness over PARP monotherapy (121).

1.6.6. Synthetic lethality

Although these kinds of mutations make cancer cells hard to eradicate but might open a therapeutic opportunity, based on DSB repair biology called synthetic lethality. To overcome the treatment resistance problem, we need to aim at the common characteristics of a wide range of tumors. Induction of DSB by oncogenes is a common condition of all cancer cells. Oncogenes induce aberrant proliferation of cancer cells which elevates DNA replication stress and DSB rate with the possibility of telomere erosion and caretaker genes mutations. Induced DSB in cancer cells trigger the DDR and put the cell in the repetitive cycle of DSB and DDR. Cancer cells' DDR pathways (partially defective but working and active) normally rely on the S/G2 checkpoint for continuous proliferation which makes it an interesting target to stop the cell from dividing. It means we can use the cancer cell`s survival strategy against itself based on the synthetic lethality concept (122). Synthetic lethality refers to a condition that one defective cellular pathway enforces the cell to shift to another pathway as a backup to survive which is not to be used under the cell`s normal status (6). Simultaneous perturbation of both pathways will end in cell death. But this strategy is applicable just for the portion of cancer types or subtypes that has defective DNA repair pathways. For capturing the therapeutic window in all cancer types we need to induce conditional synthetic lethality by manipulating of cell`s metabolic condition and environmental stress (123).

For example, it was shown that genotoxic stress induction by radiation and HSP90 inhibitor onalespib led to overcoming cisplatin resistance in ovarian and head and neck cancer (124). Many DNA repair proteins like ATM and DNA-PKcs are HSP90 protein clients. Another example of targeting the DNA repair system is the use of PARP inhibitors. HR pathway deficiency has been detected in pancreatic, ovarian, breast, and prostate cancers which increases tumor cells' genomic instability. HR becomes defective when BRCA 1 and BRCA 2 genes are not functional, then the 5′ DSB end can’t be resected and as a result 3′ end overhang can’t be loaded by RAD51. The BRCA-defective cells can normally repair this lesion by using BER instead, but the use of PARP-inhibitors will block BER and lead to synthetic lethal therapy of BRCA-deficient tumor cells. PARP inhibitors can kill cancer cells from multiple pathways. One way is by blocking BER which leads to the conversion of SSB to DSB and death of the cell for being not able to repair these DSBs during the S phase by HR. The other mechanism is the trapping of the PARP1 enzyme inside chromatin (125–128).

2. Aim

The overall aim of this thesis is, first, to understand how to exploit DSB formation and NHEJ repair pathways in the context of combination therapy to sensitize cancer cells to chemotherapy and irradiation. Second, to reveal the nature and involvement of DSB and NHEJ repair pathways in different types of DNA damage with varying complexities in cancer cells.

The specific aims of the thesis are presented below in the form of the studies performed:

- The aim of **paper I** was to investigate the DSB-inducing potential of the new generation of HSP90 inhibitors, onalespib, to overcome cisplatin resistance in ovarian cancer

- In **paper II** the goal was to explore the DSB-inducing potential of HSP90 inhibitors, onalespib, to overcome radiotherapy resistance in patient-derived glioblastoma

- The aim of **paper III** was to explore the nature and involvement of DSBs induced by Ra-223 in different prostate cancer cells

- The goal of **Paper IV** was to explore the effect of ascorbate-induced oxidative stress on DSB generation and NHEJ pathway in colorectal cancer cells

- The aim of **paper V** was to investigate the effect of different levels of DSB complexity on NHEJ pathway

3. Results

3.1. Paper I

Overcoming Limitations of Cisplatin Therapy by Additional Treatment with the HSP90 Inhibitor Onalespib

3.1.1. Introduction and Aim

Cisplatin has been in use for over 40 years and is a cost-effective first-line treatment for several solid cancers, including ovarian, head and neck, and testicular (129). It works by forming DNA inter- or intra-strand crosslinks through binding to reactive metal-binding sites of N7 atoms of guanine and adenine bases in the DNA major group. The drug also interacts with many nucleophilic non-DNA targets in the cytoplasm due to its highly electrophilic properties leading to additional cytotoxic effects against cancer cells (130). The effectiveness of this compound varies with different cancers, with ovarian and head and neck cancers providing the greatest challenge due, in part, to the development of resistance (131,132). Acquisition or innate resistance of cisplatin has been linked to multiple mechanisms, primarily involving reduced uptake and accumulation of the drug in cancer cells due to the downregulation of copper 1 and 2 transporters (CTR1 and CTR2) as cisplatin intracellular active transporters and increasing cisplatin efflux from through activity of copper efflux proteins (ATP 7A and ATP7B) (133–136). Additionally, altered nucleotide excision repair (NER) pathways by upregulating ERCC1 which acts in DNA excision, and activation of general stress response pathways via heat shock proteins have been linked to increased cisplatin resistance (133,137).

HSP90 is a highly conserved molecular chaperone that plays an important role in the formation and maturation of proteins involved in a variety of cellular pathways, making it a promising target in cancer therapy (138,139). Over 300 HSP90 client proteins have been discovered such as epidermal growth factor receptor family (EGFR), signal transduction proteins (AKT and ERK), and DNA damage response proteins (ATM and DNA-PK) (140). Many of these proteins are related and overexpressed in cancer which makes malignancy addicted to them. This cancer cell dependency gives the opportunity of cell killing using HSP90 inhibitors by blocking multiple oncogenic pathways simultaneously. HSP90 inhibitors can block the client protein chaperoning

function by targeting N-terminal ATPase. Newer generations of HSP90 inhibitors like onalespib (AT13387) with less hepatotoxicity, and better biological function are in phase II of clinical trials in treatment of solid tumors (141). Due to the similar effect of ionizing radiation and cisplatin we hypothesize that maybe onalespib can potentiate the cisplatin effect via elevated DSB levels. This combined treatment is of great interest as it provides more accessible and cheaper cancer treatment.

In this study, we investigated the ability of the new HSP90 inhibitor onalespib to increase the effectiveness of cisplatin and reverse cisplatin resistance *in vitro* in cancer cell lines. We tested the effects of the drugs on the head and neck squamous cell carcinoma cell line H314 and on ovarian cancer cell lines SKOV3, A2780, and its resistance clone A2780CIS, and also studied the molecular mechanisms of the combination treatment.

3.1.2. Methods

Cell lines: the human ovarian cancer cell line SKOV3, human head and neck squamous cell carcinoma cell line H314, human ovarian cancer cell lines A2780, and the cisplatin resistant clone A2780CIS.

Assays: XTT viability assay, clonogenic survival assay, wound healing assay, *Trans*-well migration assay, western blot, flow cytometry analysis of apoptosis, cell-cycle distribution, γH2AX and 53BP1 expression analysis via Immunofluorescence Staining (Confocal Microscopy).

3.1.3. Results

The optimal concentration of cisplatin and onalespib were defined by XTT viability assay and clonogenic assay after 24 h and 72 h of incubation with drugs.

XTT assay: The cisplatin monotherapy decreased all cell lines' viability measured by XTT assay in a concentration-dependent manner. The A2780 cell line was the most sensitive cell to 500 nM cisplatin mono treatment (72 h) by losing >50% of viability. The viability of SKOV3 and H314 cells decreased by 57.5 and 53 % when exposed to 10 μM cisplatin and by 70 and 71 % when exposed to 25 μM. Similarly, 10 μM and 25 μM cisplatin caused a decrease in viability in the A2780CIS cells of 58 and 98 %, respectively. The XTT test showed that as the concentration of onalespib increased, its effect of decreasing cellular viability intensified. At 100 nM there was an 11% and 45% drop in SKOV3 and H314 cells, respectively, while 1 μM caused a decrease of 60% and 90% in the same cells. The strong effects were seen in A2780 and A2780CIS cells which were much more sensitive to the monotherapy of onalespib. With 100 nM, a decrease of 80% and 70% were recorded, and nearly complete elimination of viability was seen when the concentration was increased to 1 μM. At 72 h, a significant decrease in viability was seen in all

combination treatments with 10 μM cisplatin compared to monotherapy, effectively reducing survival to near zero. The effects of the combination were greater at higher concentrations of cisplatin (500 nM) and onalespib (≤100 nM).

Clonogenic assay: In the clonogenic assay, combination therapy of cisplatin and onalespib decreased survival fractions (SF) across all cell lines compared to untreated controls and onalespib mono treatment, with A2780 and H314 cells being the most sensitive. The 500 nM cisplatin and 100 nM onalespib combination significantly decreased cell colony-forming ability compared with onalespib monotherapy in all cell lines ($p \leq 0.05$). Clonogenicity of H314 and A2780 cells decreased significantly ($p \leq 0.0001$) in all combinations. Combinational exposure led to smaller, and more irregularly shaped colonies (Fig. 6).

Cell cycle: Cell cycle analysis of SKOV3 cells with PI staining after combinational exposure (500 nM cisplatin and 100 nM onalespib) for 96 h showed an increased signal of G2/M arrest (32.5%) compare to cisplatin (12.1%) and onalespib (26.6%) mono treatment.

Foci assay: Cisplatin and onalespib dual therapy significantly elevate the DSB biomarker's (γH2AX and 53BP1) response level compared to monotherapy in SKOV3 and A2780CIS cells (Fig. 7).

Apoptosis assay: The dual treatment significantly increases apoptotic response in SKOV3 and A2780CIS cancer cells compared to mono treatment groups. Flow cytometry analysis with Annexin V and caspase 3/7 showed that the combination therapy of 500 nM cisplatin and 100 nM onalespib yielded 45% Annexin V and 72% caspase 3/7 activity in SKOV3 cells and 20% Annexin V and 27% caspase 3/7 activity in A2780CIS cells which significantly more than either mono treatments (Fig. 8).

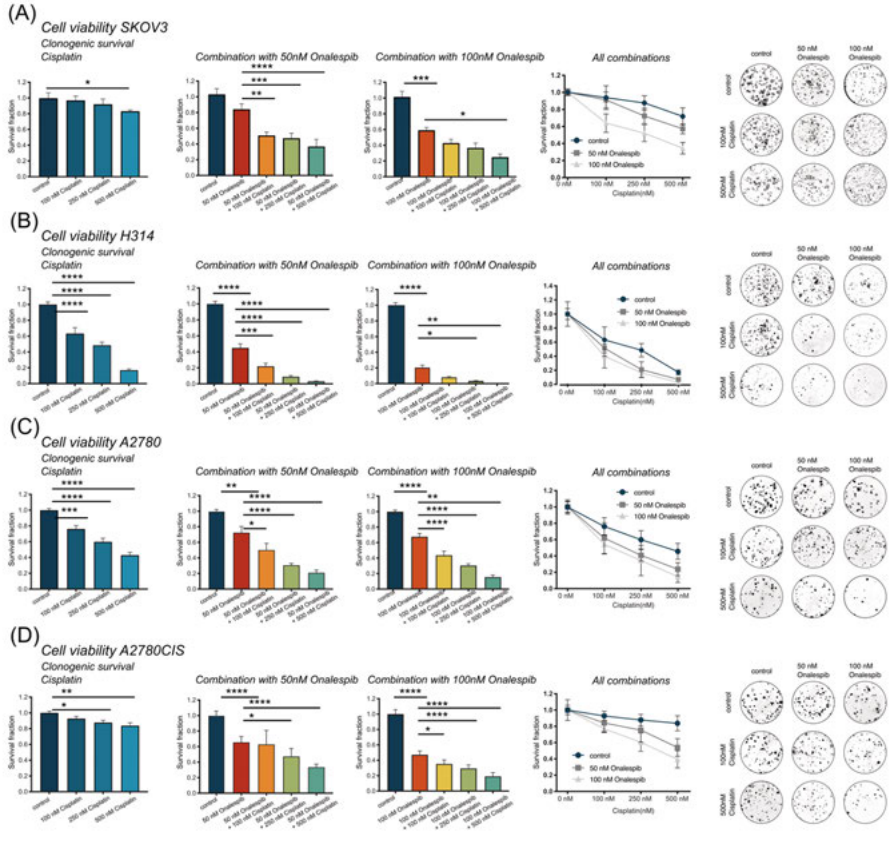

Figure 6. Clonogenic assay results of SKOV3, H314, A2780, and A2780CIS cells after exposure to 0, 100, 250, and 500 nM cisplatin or in combination with 50 or 100 nM onalespib for 24 h. N = 3, error bars = SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

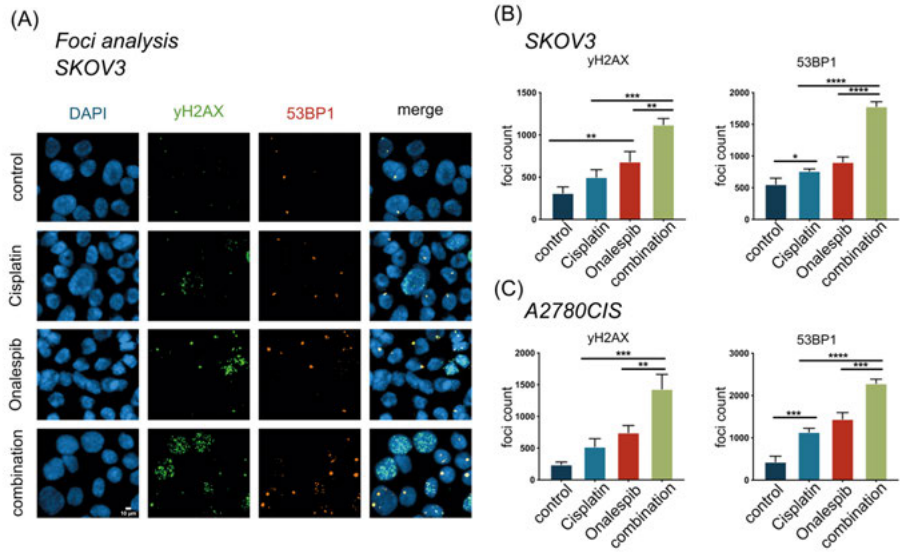

Figure 7. The DSB biomarkers of γH2AX and 53BP1 in SKOV3 cells were significantly more expressed in combinational treatment group (500nM cisplatin + 100nM onalespib, 96 h exposure) than monotreatment. error bars = SD. **p < ***p < 0.001, ****p < 0.0001.*

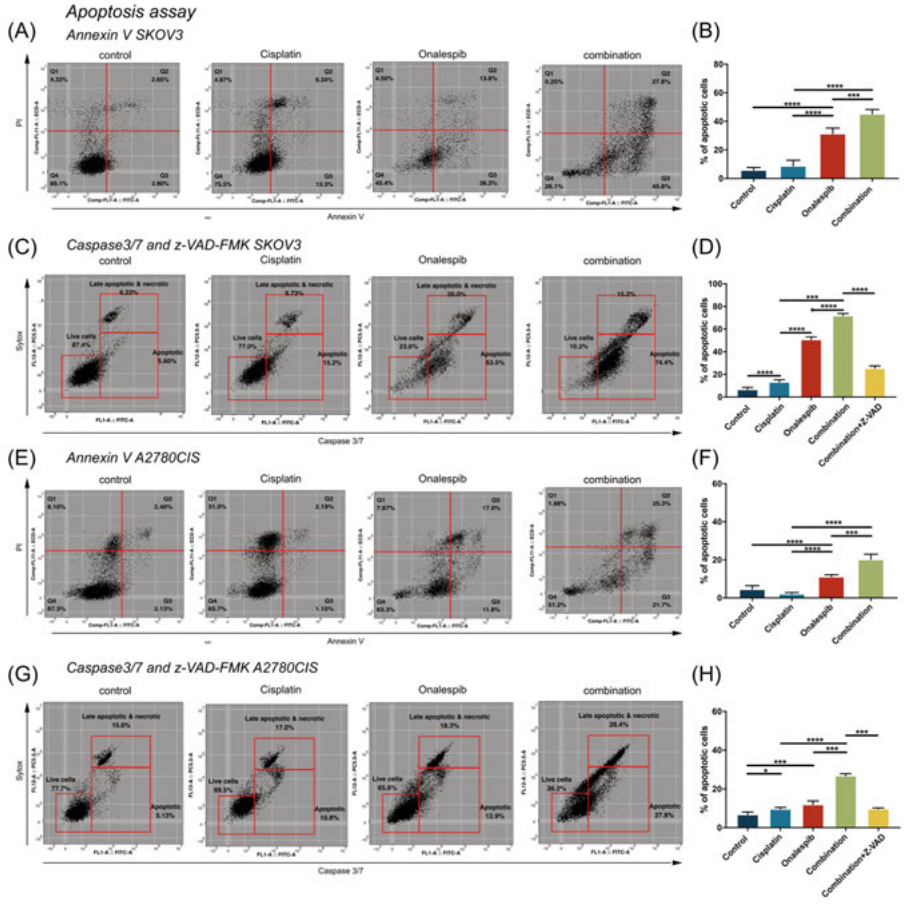

Figure 8. Apoptotic biomarkers (Caspase3/7 and Annexin V) were significantly increased after combinational exposure (500 nM cisplatin and 100 nM onalespib exposure for 96 h) in comparison with mono treatment at SKOV3 and cisplatin-resistant cell line A2780CIS. error bars = SD. **p < ***p < 0.001, ****p < 0.0001.*

3.1.4. Discussion and conclusion

Cisplatin, one of the most widely used cancer drugs, despite of high initial response, has limitations such as rapid resistance development and adverse side effects, leading to the exploration of combination therapies (131). Multiple studies have shown that using HSP90 inhibitors like onalespib in combination therapy, is effective in reversing drug resistance in several cancers, due to blocking a wide range of vital proteins for the survival of cancer cells such as DNA damage repair proteins. However, there is still limited knowledge of the mechanisms behind this. In this study, four cell lines (SKOV3, H314, A2780CIS, and A2780) were selected because of their cisplatin-resistant development. The A2780 is cisplatin sensitive and the parent of the A2780CIS cisplatin-resistant cell line. Results showed that onalespib monotherapy was

effective against all cell lines, with A2780 being particularly more sensitive than others. However combinational exposure was significantly more effective than either mono treatment with consistent results at both XTT survival and clonogenic assay in accordance with previous studies (142). The potent effect of combined treatment is likely due to the DNA repair inhibition by onalespib after DNA damage induction of cisplatin. Even though cisplatin monotherapy did not affect the proliferation and migration of cells in wound healing assays, combination therapy reduced it in a dose-dependent manner. The combination therapy reduced the proliferation of H314 without affecting its migration but it reduced both traits at SKOV3. Dual treatment significantly increased SKOV3 cell apoptotic levels consistent with previous studies (143).

Onlaspib monotherapy significantly downregulated EGFR and AKT expression at both 50 and 100 nM but cisplatin treatment did not affect their expression significantly which made it difficult to interpret the combination treatment effect using Western blot.

The main DNA damage effect of cisplatin is through intra-strand crosslinks which activate the NER repair pathway. The ERCC1 is the main player in the NER repair pathway which is upregulated in cisplatin resistance. However, nucleotide excision of cisplatin-generated DNA adducts via NER can produce DSBs. As a result, these DSBs could not repair effectively due to the general inhibition of ATM, ATR, and DNA-PKcs by onalespib. This inhibition can lead to DSB accumulation and shift the dominant repair pathway from NER to the NHEJ pathway. Significantly increased γH2AX and 53BP1 DSB biomarkers in the combination group supported this fact.

In conclusion, our results showed the effective participation of onalespib in the elevation of cisplatin potency and toxicity in cisplatin-resistant cells leading to higher DSB induction and apoptotic rate.

3.2 Paper II

Enhancing Glioblastoma Therapy: Unveiling Synergistic Anticancer Effects of Onalespib-Radiotherapy Combination Therapy

3.2.1. Introduction and Aim

Glioblastoma (GBM) is the most prevalent brain tumor in adults, with a median survival time of less than 15 months despite treatment (144). Its occurrence in children constituting 3–15% of primary central nervous system (CNS) tumors. Despite the rarity, pediatric GBM is resistant to therapy and has a high mortality rates, with a 5-year survival of less than 20% (145,146). The upregulation of DNA damage response (DDR) have been described as important contributors to GBM drug resistance (147). The mechanisms behind GBM

resistance to treatment are poorly understood, emphasizing the urgent need for new treatment modalities.

Recent data showed the molecular chaperone heat shock protein 90 (HSP90) as a promising target for radiation treatment improvement in GBM (139,148–152). HSP90 inhibitors have higher affinity for the intertumoral HSP90 compared to the HSP90 in normal cells, due to the increased ATPase activity of HSP90 in tumor cells (153). We used Onalespib, a second-generation HSP90 inhibitor with tolerable toxicity profile (154) and the ability of penetrating the blood-brain barrier (155). Onalespib already has undergone phase I clinical trials (156). Furthermore, efficacy of Onalespib against gliomas as a single agent or as a combination therapy with TMZ have been demonstrated *in vitro* and *in vivo* (155). This onalespib effectiveness can be associated to ability of targeting several DDR pathways (151).

We aim here to investigates the effectiveness and molecular mechanisms of combining the HSP90 inhibitor Onalespib with external beam radiotherapy in GBM cell lines in vitro, providing a robust model to explore GBM's genetic diversity. The widely used U343 MG and U87 MG cell lines, characterized by wild-type p53, are valuable for studying radiation resistance and tumor invasiveness. However, their long-term culturing may have diminished their molecular complexity. In contrast, the patient-derived lines U3013MG and U3024MG retain genetic heterogeneity (157), exhibit sensitivity to specific therapies, and display distinct DNA repair defects. This combination approach bridges personalized GBM therapy development with the continuity of existing research.

3.2.2. Methods

Cell lines: glioblastoma cell lines U87 MG (HTB-14), U343, the human patient-derived GBM cells U3013MG and U3024MG.

Assays: XTT viability assay, clonogenic assay, Migration/Proliferation assay, Proteomic analysis Proximity Extension Assay, Proximity Extension analysis, cell cycle distribution, Immunofluorescent Biomarker of DSB repair, western blot.

3.2.3. Results

XTT assay: Four cell lines U343 MG, U87 MG, U3013MG, and U3024MG were treated with varying Onalespib doses, followed by radiation after 24 hours, with viability measured 72 hours post-treatment. Results showed dose-dependent reductions in cell viability for established cell lines U343 MG and U87 MG under both monotherapies, with Onalespib (100 nM) reducing viability by 47% and 44.5%, respectively. U87 MG was more radiation-sensitive, with 46.4% survival at 4 Gy compared to 68% for U343 MG. Combined treatment further decreased viability, demonstrating synergistic effects at higher

drug doses (>10 nM) and 6 Gy radiation. Additive effects were seen at lower radiation doses unless Onalespib concentrations exceeded 50 nM for U343 MG. In patient-derived lines, U3013MG and U3024MG were less responsive to low Onalespib concentrations, with significant reductions only at 100 nM. However, both lines were highly radiation-sensitive, with viability reduced by 70.1% and 82% at 2 Gy, respectively. Synergy was observed at specific drug-radiation combinations: U3013MG showed synergy at 10–50 nM with 6 Gy, while U3024MG exhibited synergy at 100 nM combined with 4 or 6 Gy. Overall, combined treatment was most effective across all cell lines, particularly at higher drug and radiation doses.

Clonogenic and spheroid growth assay: Clonogenic and 3D tumor spheroid assays confirmed the potent efficacy of Onalespib combined with radiation in glioblastoma models. Clonogenic assays revealed that both treatments independently reduced survival in a dose-dependent manner, with complete colony loss at 50 nM Onalespib, regardless of radiation. Combination therapy showed enhanced efficacy, significantly reducing clonogenicity even at lower radiation doses. For instance, 2 Gy with 25 nM Onalespib decreased colony formation by 78.2% (U343 MG) and 83.5% (U87 MG). In 3D tumor spheroids, combined treatment also outperformed individual therapies, though spheroids exhibited lower sensitivity than 2D models. Untreated spheroids showed rapid growth, with volumetric increases of 1386% (U343 MG) and 3720% (U87 MG) after 14 days. In contrast, 250 nM Onalespib and 6 Gy slowed growth to 14% and 41%, increasing doubling times to 67.31 and 26.23 days, respectively. Synergistic effects were confirmed with Loewe synergy scores >10 for several drug-radiation combinations. Live-dead cell analysis revealed higher doses increased dead cell populations, despite similar spheroid sizes three days post-treatment. Limiting dilution assays demonstrated a dose-dependent decline in spheroid-forming efficiency for U87 MG, U3013MG, and U3024MG cells. Untreated controls formed spheroids efficiently, while combination therapies substantially inhibited this capacity (Fig. 9).

DSB repair Foci assay: We assessed DDR (53BP1 and γH2AX foci) in U343 MG and U87 MG cells treated with Onalespib, radiation, or their combination. Onalespib and radiation monotherapies significantly increased foci, with Onalespib causing a stronger effect than radiation. Combining Onalespib with radiation further amplified DSB accumulation. At 6 Gy, the combination led to dramatic increases in 53BP1 and γH2AX foci, indicating overwhelming DNA damage and impaired repair. U87 MG cells showed a smaller increase in foci compared to U343 MG, but the combination still resulted in poor DSB repair. Foci co-localization of 53BP1 and γH2AX was observed, with some proteins localized in distinct nuclear regions. Western blot analysis confirmed increased γH2AX expression in all treatment groups, especially with radiation. Overall, Onalespib combined with radiation effectively decreased DSB

repair capacity by inducing complex DSBs, significantly impairing repair in both cell lines (Fig. 10).

Cell cycle distribution: We used flow cytometry to analyze the cell cycle distribution in U343 MG and U87 MG cells after 48-hour exposure to Onalespib (100 nM), radiation (2 and 4 Gy), and their combinations. Radiation (4 Gy) reduced the G0/G1 phase population and increased G2/M phase cells, with a stronger G2/M arrest observed when combined with Onalespib. In U343 MG, 21% were arrested in G2/M, and in U87 MG, 31% were arrested. Onalespib also reduced S-phase cells, particularly in U87 MG. These changes were statistically significant. Western blotting revealed Onalespib suppressed p21 expression, while radiation alone or with Onalespib increased p21, indicating cell cycle arrest and activation of cell death pathways.

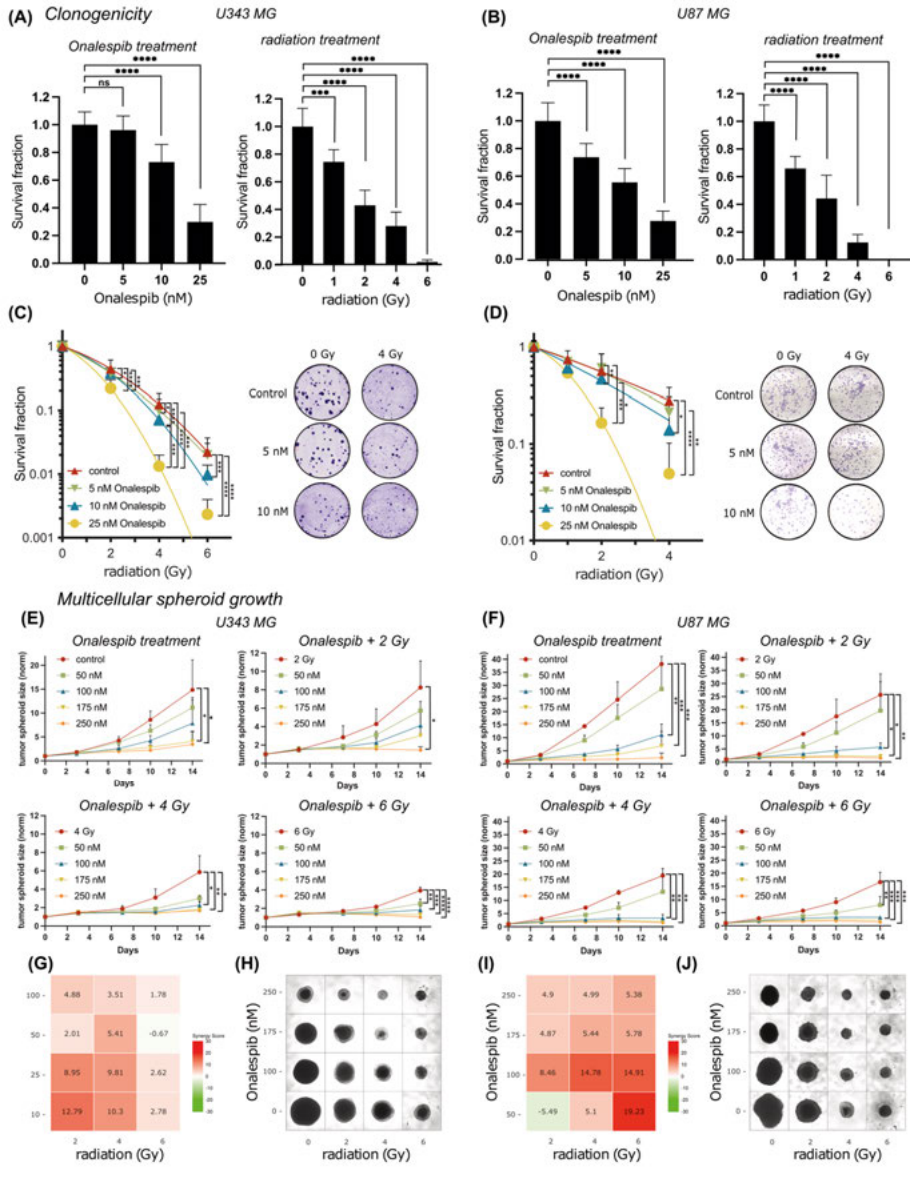

Figure 9. Colonogenic survival and spheroid growth of U343 MG and U87 MG cells. SF of Onalespib and X-ray of U343 MG A) and U87 MG cells B) SF of Onalespib and X-ray combination of U343 MG C) and U87 MG D). Colony images of the mono and combined exposures of Onalespib and X-rays. Onalespib mono and combine therapy with X-ray in spheroid of U434 MG E) and U87 MG F). Normalized spheroid volume (mm3) over time, (means ± SD, n ≥ 3). Synergy scores for U34mg G) and U87 MG I). Representative spheroid images of the U343 MG and U87 MG at the endpoint (H) and (J). Data plotted as means ± SD, n ≥ 3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

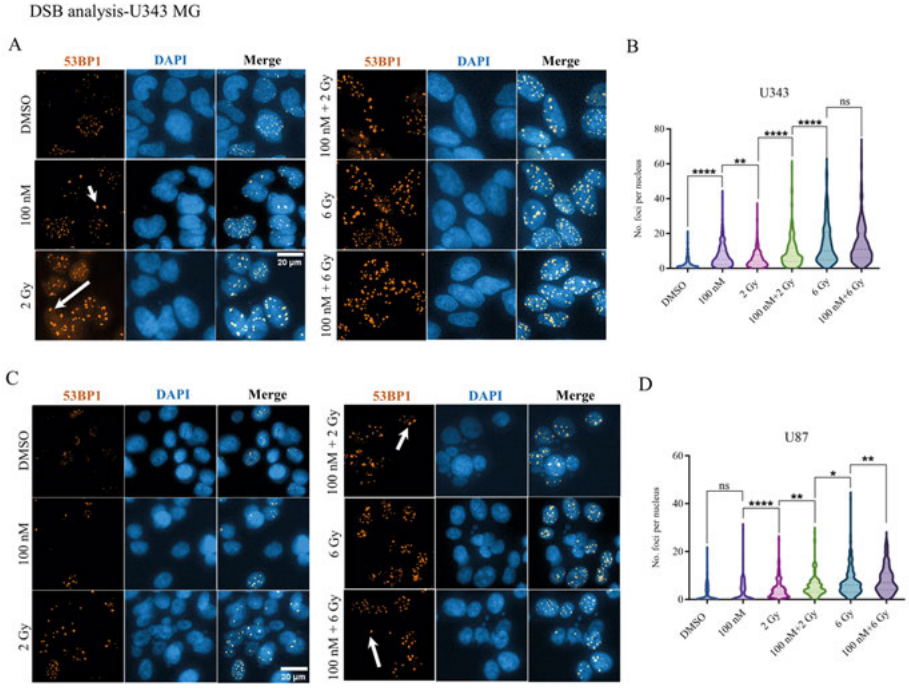

Figure 10. 53BP1 foci quantification of U343 MG and U87 MG cells. A) Confocal mages of U343 MG cells exposed to 100 nM Onalespib and 2-6 Gy X-rays. Arrows show counted 53PP1 foci. B) Plots of U343 MG, 53BP1 foci per cell. C) Microscopy images of U87 MG cells exposed to 100 nM Onalespib and 2-6 Gy X-rays. Arrows show 53BP1 foci. D) Plots of U87 MG, 53BP1 foci per cells. Data presented as means ± SD, n = 2. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Proteomics: Proteomic analysis of U343 MG cells treated with Onalespib, radiation, and their combination showed substantial protein expression changes impacting cancer pathways. Onalespib predominantly downregulated tested proteins, radiation upregulated them, and combination therapy amplified necrosis and apoptosis markers like c-Flip and caspases. Radiation-induced DNA damage increased p21, reversing Onalespib's suppression and promoting cell cycle arrest. Onalespib reduced FOLR3 and FOLR1, counteracting radiation's folate demands and possibly reducing tumor aggressiveness. VEGFA expression dropped significantly with combination therapy, suggesting impaired tumor vascularization. TRAIL, linked to apoptosis and necroptosis, surged with combination therapy, intensifying tumor cell death. Functional pathway analysis highlighted reduced growth signaling, enhanced immune modulation, p53-driven DNA damage responses, and increased apoptosis, emphasizing the combination treatment's potential to synergistically disrupt tumor progression (Fig. 11).

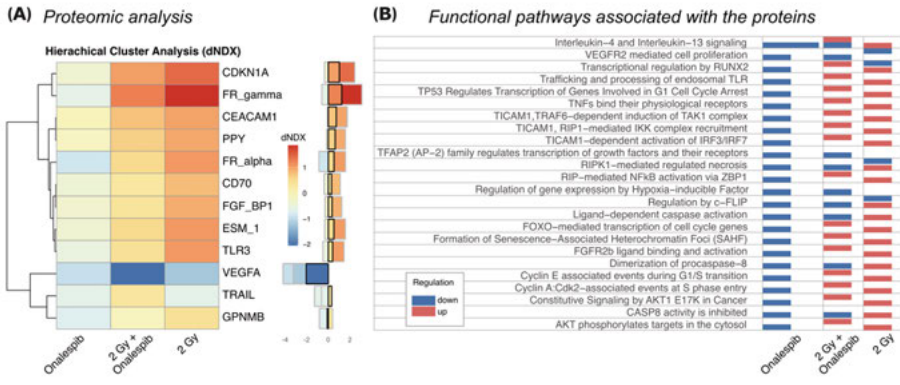

Figure 11. A) Panel of differential proteins expressed between treatments (SD>0.5). Hierarchical clustering analysis shows the most notable changes in protein expression degrees observed in U343 MG cells treated with X-ray, Onalespib, and their combination, relative to control. The value highlighted in red, indicates higher expression compared to control, and negative values are in blue, showing lower expression. B) Functional analysis indicating the biological pathways where the differential protein expression is depicted.

3.2.4. Discussion and conclusion

GBM's poor prognosis stems from HSP90 overexpression and aggressive growth. HSP90 is 2- to 10-fold higher in cancer cells, aiding survival during tumorigenesis (155,158). Targeting HSP90 with agents like Onalespib, which passes the blood-brain barrier, may improve treatment efficacy (159,160). Combination therapy, including Onalespib and radiotherapy, enhances anti-tumor effects by decreasing cell viability and clonogenicity in glioma cell lines.

In tumor spheroid models, mimicking hypoxic conditions, Onalespib reduced proliferation and spheroid formation, with combination therapy showing the strongest effects. This aligns with other studies showing radiosensitizing effects of HSP90 inhibitors in GBM spheroids (161,162). Furthermore, Onalespib reduced wound healing, impacting migration and invasion, though the specific interaction with EPHA2 was not observed in our analysis (163,164).

Onalespib disrupts DNA repair, increasing DSBs, as evidenced by γH2AX and 53BP1 foci, suggesting inhibition of repair pathways, including homologous recombination and non-homologous end joining. This enhanced radiosensitivity was seen in combination treatment, particularly in U343 MG, though U87 MG showed resistance, likely due to its stronger DNA repair capacity (151). Studies also indicate Onalespib's synergistic effects with TMZ in glioma models (155). Moreover, it increases radiosensitivity in hypoxic regions, often resistant to radiotherapy (147,161).

Proteomic analysis confirmed Onalespib's impact on protein expression in several functional pathways, particularly p21, which was upregulated in the combination treatment, highlighting its role in cell cycle arrest and DNA repair (165–168). VEGFA, linked to angiogenesis, was reduced by both treatments, with the combination showing stronger inhibition. TRAIL, involved in apoptosis and necroptosis, was notably elevated in the combination group, suggesting enhanced cell death pathways (169–171).

Despite promising results, this study has limitations, such as the use of the U87 MG cell line, which may not fully represent the original model (172). Further studies with glioma organoid models and traditional validation methods like Western blotting are needed. While Onalespib showed no adverse effects in other tumor models (149), its potential neurotoxicity in brain tissue requires further investigation. Preclinical studies should assess its impact on brain cell survival and neurogenesis.

Overall, our findings support combining Onalespib with radiotherapy as a promising strategy for GBM treatment, especially for resistant cases. However, more research is needed to optimize dosing and evaluate toxicity in clinical settings.

3.3 Paper III

Ra-223 Induces Clustered DNA Damage and Inhibits Cell Survival in Several Prostate Cancer Cell Lines

3.3.1. Introduction and Aim

Radium-223 dichloride, an analog of calcium that can bind to bone hydroxyapatite in newly formed bone structures, is one of the alternatives for the treatment of bone metastatic prostate cancer. In this type of targeted alpha therapy, the patient's survival increases to 3.6 months with less pain (173). However, the molecular mechanism of Ra-223 action is not fully understood especially in the DSB generation and repair context. In this study, we produce a bone-like structure to mimic the bone metastatic condition to study Ra-223 interaction with bone and prostate cancer cells. First, we explored the bone surface interaction and cell internalization of Ra-223. Then the survival and growth of cells were examined in 2D and 3D spheroid models. The DSB repair and generation were studied using PFGE and immunofluorescent staining of DSB biomarkers. Finally, we examined cell fate using an apoptosis assay. The study results showed extremely stable bonding of Ra-223 with the hydroxyapatite (HAp) without cancer cell internalization. The conditioned Ra-223 on the HAp-coated surface reduced cell clonogenicity and 3D spheroid growth higher than free Ra-223. Also, drug treatment activated NHEJ consistent with high-LET radiation outcomes

leading to high levels of apoptosis. The toxicity of Ra-223 is likely related to α-particles decay and its interaction with the nucleus.

3.3.2. Methods

Cells: Prostate cancer PC3 and brain metastatic prostate cancer DU145 cells, and Prostate cancer 22RV1 cells.

Assays: Clonogenic assay, Spheroid assays, apoptosis, PFGE, DSB repair foci assay, flowcytometry, western blot, real-time affinity measurements with LigandTracer.

3.3.3. Results

LigandTracer: Real-time affinity quantification of Ra-223 with HAp surface and with DU145 cells using LigandTracer showed extremely strong binding of Ra-233 and HAp within 2 h with >99% continuous affinity signal until 24h without disassociation (Fig. 12A). Interestingly cells didn't uptake Ra-223 within 2 h (Fig. 12B).

Clonogenic assay: The clonogenicity of all three cell lines (PC3, DU145, and 22RV1) significantly decreased after exposure to media containing different concentrations of Ra-223 in a similar pattern. Interestingly when PC3 and 22RV1 cells were exposed to HAp-coated surface conditioned with 500 Bq/ml Ra-223, the colony-forming ability was extremely reduced compared to free Ra-223 treatment.

Spheroid assay: After six days of being exposed to 250 Bq/ml and 500 Bq/ml of free Ra-223, the growth of 22RV1 spheroids was significantly suppressed ($p = 0.0248$ and $p = 0.0019$, respectively). Treatment with 100 Bq/ml of Ra-223 caused a noticeable reduction in growth after eight days ($p = 0.0182$;). At the end of the experiment (18 days), the 250 Bq/ml and 500 Bq/ml groups showed complete growth inhibition, while the lowest activity (100 Bq/ml) reduced the growth by half compared to the control sample.

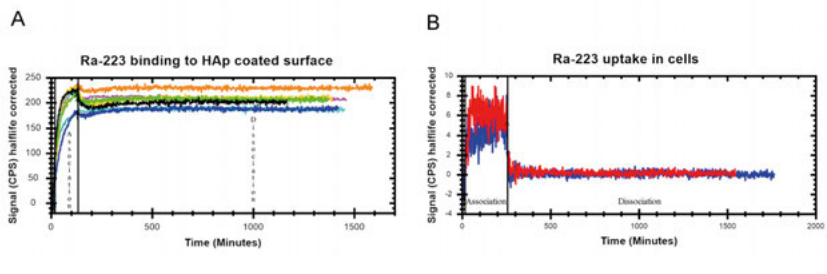

Figure 12. The Ra-223 binds strongly to HAp without any cell uptake. (A) The Ra-223 binding kinetics to the HAp (B) The Ra-223 is not taken up in cells. The solid vertical lines show the border of association and dissociation.

Spheroid outgrowth assay: We tested how spheroid outgrowth was impacted when grown on a HAp surface that was pre-treated with or without Ra223 to simulate *in vivo* conditions. On day 4, 22RV1 spheroid outgrowth was significantly reduced in the 0.5 kBq/ml and 1 kBq/ml groups and this effect persisted until day 10. The treatment did not affect DU145 spheroid outgrowth. However, the treatment did reduce cell density in the central and peripheral regions, making it difficult to score cellular outgrowth at later stages.

PFGE: The Ra-223 DNA fragmentation induction was measured in DU145 cells by PFGE and compared with high-LET X-rays radiation. The result showed random DSB distribution for X-rays and non-random for Ra-223 with the elevated generation of small DNA fragments (<1 Mbp sizes) as a hallmark of high-LET radiation (Fig. 13A).

Foci assay: We evaluated classic immunofluorescence DSB biomarkers of γH2AX and 53BP1. The confocal imaging results showed a tracklike traverse of the 53BP1 DSB repair signal as a signature of α-particles travel through the nucleus (Fig. 13B)

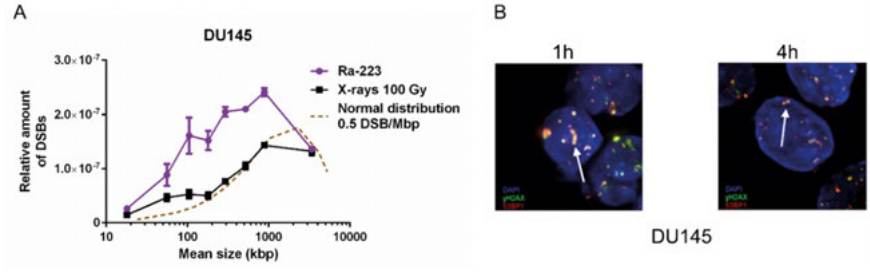

Figure 13. (A) Comparison of DSB fragment distribution from Ra-223 and high-LET X-rays (100 Gy) in DU145 cells using PFGE. (B) Immunostaining of DSB biomarkers (53BP1 and γH2AX) after Ra-223 (100 kBq/ml) treatment showed track lines (white arrows) of α-particles traveling through DU145 prostate cancer cells nucleus after 1 h and 4 h repair time.

Flow cytometry: Flow cytometric analysis of DSB repair key protein DNA-PKcs (T2609 phosphorylation) of PC3 and DU145 cells showed fast and efficient repair of DSBs 4 h after irradiation with 6-11% residual DSB remaining. 22RV1 cells were slower and less efficient in DSB repair.

Apoptosis assay: The Ra-223 apoptotic effect was evaluated by flow cytometric analysis of caspase 3/7-sytox and Annexin V/PI double staining. All three cell lines' apoptotic response for both biomarkers was significantly higher than the control showing strong apoptosis stimulation of Ra-223.

3.3.4. Discussion and conclusion

Ionizing radiation-generated DSB is the most harmful damage to the cell repair system. If these DSBs are not removed or properly repaired, it can result in genetic instability and tumorigenesis (174). Therefore, quantification of

DSB induction and repair is necessary. For DSB induction measurement, and DNA fragment distribution we used PFGE. DNA fragmentation distribution analysis showed clustered DSBs with <1 Mbp after Ra-223 exposure in prostate cancer cells. This finding is in line with the result of high-LET ion irradiation results (175,176). To measure DSB repair, we tried using immunostaining of DSB biomarkers. However, during this study, we realized that foci counting is not an accurate way of DSB quantification of alpha particles since it may underestimate the DSB number. In this case, one detected focus may have several aggregated DSBs close together (177). At the same time, these foci images gave valuable information about the physical shape of induced clustered damage. The image acquisition of fluorescently stained γH2AX and 53BP1 foci demonstrated track-like distributions of DNA clustered lesions inside the nucleus. These clusters are associated with a track line of alpha particle travel through the folded chromatin (178).

To overcome the quantification of aggregated DSBs we used the relative fluorescence intensity of phosphorylated (T2609) DNA-PKcs, as a marker of the DSBs repair kinetics and NHEJ activation (179). The results showed that NHEJ repair activation was the result of clustered DSB induction by Ra-223. The activated NHEJ repair pathway and the early repair kinetics measured by flow cytometry were in line with previous PFGE analysis measuring rejoining of DNA after ion irradiations (180) suggesting that the high toxicity of the DNA damage caused by the high-LET irradiation is due to the clustering of highly complex DSBs. As a result of toxic DNA clustered, Ra-223 reduced cell survival regardless of the cell type, indicating the potential use of Ra-223 treatment in ADT resistance bone metastases prostate cancer.

Conditioned HAp, reduced cell survival more than free Ra-223 in 2D. Free Ra-223 in the growth media was able to decrease the cell survival in monolayers and 3D spheroids and induce high levels of apoptosis (181). However, this does not reflect the actual *in vivo* condition, as only a small number of the α-particles will be able to hit the cells when Ra-223 is evenly distributed in the media. To address this issue, we quantified Ra-223 binding to the HAp surface by using LiganTracer. The results demonstrated strong binding of Ra-223 to the HAp surface without cell internalization, showing that all damages are induced by decay events outside of the cell. The results were consistent with previous observations which showed Ra-223 redistributed to the bones within 2 h of being injected into the bloodstream (182).

Cells (22RV1 and PC3) and spheroids (22RV1) seeded on top of the HAp surface, conditioned with Ra-223, showed greater cell growth inhibition compared to free Ra-223 exposure in media. This is likely due to the α-particle source being located at a closer distance to cells in the HAp-conditioned setup. However, DU145 spheroids outgrowth was not affected by placing them on top of the conditioned HAp layer, even though their survival was compromised. This result suggests that Ra-223 cannot inhibit the migration of all

prostate cancer cell types. The possible explanation could be cells entering apoptosis or locating far from the reach of α-particles.

In conclusion, the stable localization of Ra-223 inside the HAp structure without entering the cell shows how this radionuclide acts *in vivo* by migrating to the bone remodeling site and irradiating invading cancer cells to the metastatic node. This finding validates previous biodistribution studies (182,183). The elevated apoptotic response of all three studied cell lines suggested that the severity of DNA clustered damage forced cells toward programmed cell death due to the unrepairable nature of initial DNA damage. These data are in line with previous investigations (184). The role of prostate cancer Arv7 in Ra-223-induced DNA damage needs more investigation in future studies.

3.4 Paper IV

Ascorbate Induces G2/M Arrest and Necrosis Without Generation of Direct DNA double-strand breaks

3.4.1. Introduction and Aim

Intravenous high-dose infusion of pharmacological ascorbate (Asc; vitamin C) is used as an adjuvant agent in cancer treatment (185,186). Ascorbate's (Asc) cytotoxicity in cancer cells is attributed to DNA damage caused by the generation of reactive oxygen species (ROS) and H_2O_2. While many studies have reported Asc-induced single-strand DNA breaks, growing evidence points to its ability to induce DSBs, as indicated by H2AX phosphorylation and other biomarkers (187,188). However, the mechanisms involving chromatin modification, the DSBs origin, and the activation of NHEJ remain unclear.

This study aimed to explore the role of DSBs in Asc-induced cell death. Using two NHEJ-deficient cell lines (XRCC4 KO and DNA-PKcs KO), we demonstrated that Asc does not directly cause DSBs but alters chromatin structure by increasing 53BP1 expression and inducing pan-nuclear γH2AX patterns 24 hours post-exposure, indicative of nuclear DNA degradation. Additionally, Asc triggered necrosis without activating apoptosis in all tested cell lines. XRCC4 KO cells showed a tendency for greater sensitivity to Asc, potentially linked to their inherently greater accumulation in G2/M phase. These findings provide new insights into DSB formation and their involvement in Asc-induced cytotoxicity.

3.4.2. Methods

Cell lines: Human colorectal cancer cell line HCT116, HCT116 DNA-PKcs KO, HCT116 XRCC4 KO, Prostate cancer PC3, Human skin fibroblast GM5757.

Assays: western blot, colonogenic assay, CellTiter-Glo® 2.0 Luminescent viability assay, spheroid assay, pulsed-field gel electrophoresis (PFGE), γH2AX and 53BP1 foci assay, flow cytometric analysis of apoptosis and necrosis, cell cycle distribution.

3.4.3. Results

We used parental HCT116 with intact NHEJ and two NHEJ deficient derivates of it (DNA-PKcs KO and XRCC4 KO) besides human prostate cancer cell PC3 and normal human skin fibroblast GM5757 cell line with functional NHEJ pathway.

Clonogenic assay: Asc reduced clonogenicity in all cell lines in a concentration-dependent manner, with similar sensitivity at lower concentrations. XRCC4 KO cells were the most sensitive, losing clonogenic potential beyond 150 μM, followed by DNA-PKcs KO and HCT116 cells. Colony sizes decreased more in HCT116 cells compared to KO lines. The DNA-PKcs inhibitor NU7441 confirmed the KO phenotype.

Spheroid size and viability assay: Asc caused significant, concentration-dependent reductions in spheroid size and viability across all lines. XRCC4 KO spheroids showed higher sensitivity at higher concentrations, with the greatest size reductions observed between 1000 and 2500 μM. At 5000 μM, spheroids lost structural integrity, becoming irregular and disorganized, leading to their exclusion from analysis (Fig. 14).

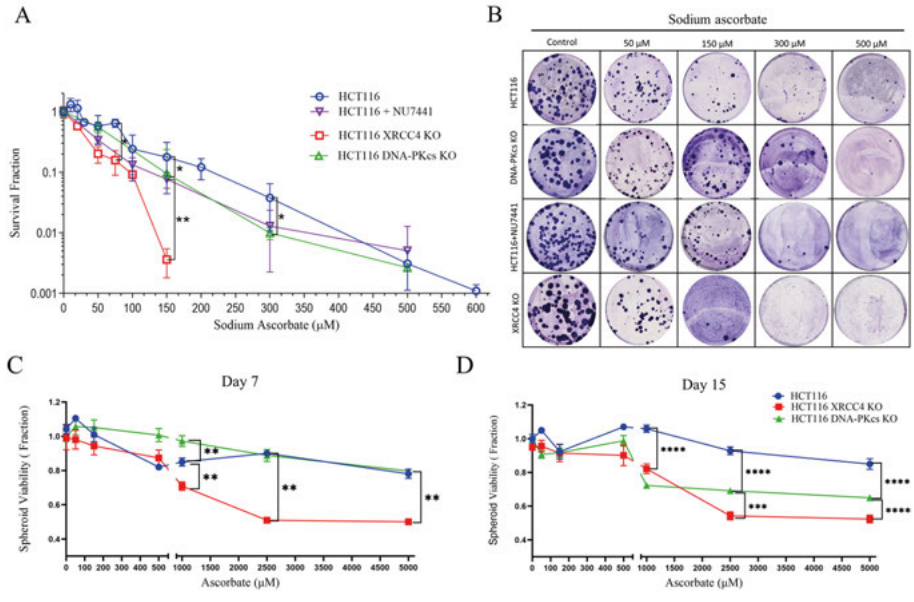

Figure 14. Clonogenic assay and spheroid viability results after treatment with Asc. (A) clonogenicity SF plot of 0-600 µM Asc exposure (2h of HCT116 (±NU7441), XRCC4 KO, and DNA-PKcs KO cells. Colonies with >50 cells counted for SF. (B) Colony images (6-well plate). Spheroid viability after 0-5000 µM Asc treatment on HCT116, XRCC4 KO, and DNA-PKcs KO cell's (C) day 7 and (D) day 15. Error are ± SD, N=3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

PFGE: To examine DSB formation following Asc treatment, PFGE analysis was performed. In HCT116 cells, no dsDNA fragments was observed, and the FAR, representing migrated DNA, remained comparable to the control across all Asc concentrations. In contrast, positive controls (40 and 100 Gy X-rays) induced significant DNA fragmentation (Fig. 15A,B). Chromatin alteration during Asc exposure was assessed by quantifying 53BP1 foci in three cell lines after 2 hours of Asc treatment, followed by a 1-hour recovery in fresh medium. The distribution of 53BP1 foci per cell showed no dependency on Asc dose or correlation with the NHEJ status of the cells (Fig. 15C,D,E).

DSB biomarkers assay: We measured 53BP1 and γH2AX foci per cell 24 hours after Asc exposure to assess chromosomal stress signals. At 500 µM, 53BP1 foci increased in all cell lines, with HCT116 cells showing a sustained elevation at 1000 µM. However, higher Asc concentrations led to a decline in 53BP1 foci, suggesting impaired DNA damage response activation. Validation with 2 Gy X-rays confirmed 53BP1 as a reliable biomarker. For γH2AX, the response was less pronounced, with no significant differences across cell lines or concentrations. At 500 µM, γH2AX foci decreased, and nearly disappeared at 5000 µM, indicating potential loss of DSB signaling. Pan-nuclear

γH2AX signals displayed distinct dose-dependent profiles, especially in XRCC4 KO cells, where the signal increased from 50% at 500 µM to nearly 90% at 5000 µM. These findings suggest chromatin change and DNA degradation processes leading to DSB formation over time (Fig. 16A,B,C,D).

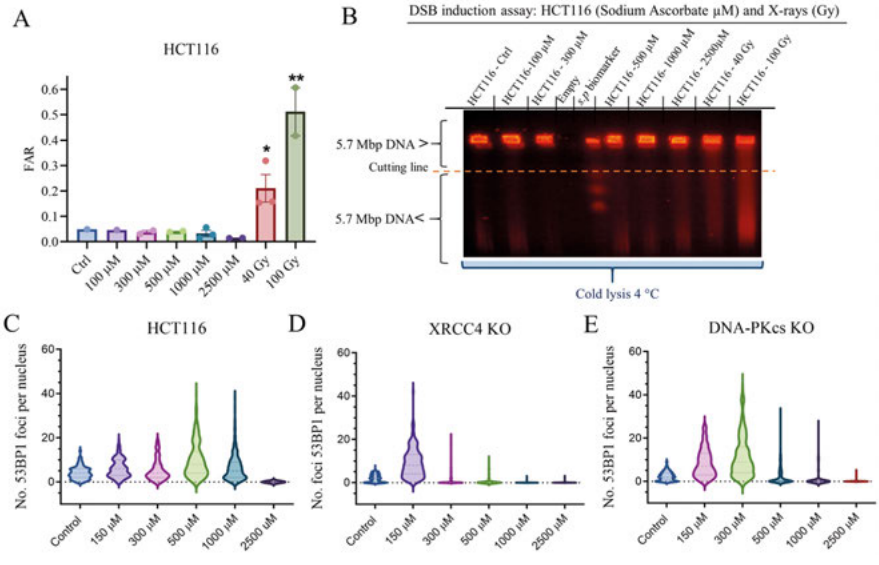

Figure 15. Prompt DSB (>5.7 Mbp) and 53BP1 foci after Asc and X-rays treatment. (A) Prompt DSB measured with PFGE after 2 h Asc exposure (0-2500 µM of Asc), and with 40 and 100 Gy of X-rays. Error bars are SD, N=3. *$p < 0.05$, **$p < 0.01$. (B) Sample gel image showing DNA migration in HCT116 cells. 53BP1 foci quantification after 2 h exposure followed by 1 h recovery in (C) HCT116, (D) XRCC4 KO, and (E) DNA-PKcs KO cells (0-2500 µM Asc treatment).

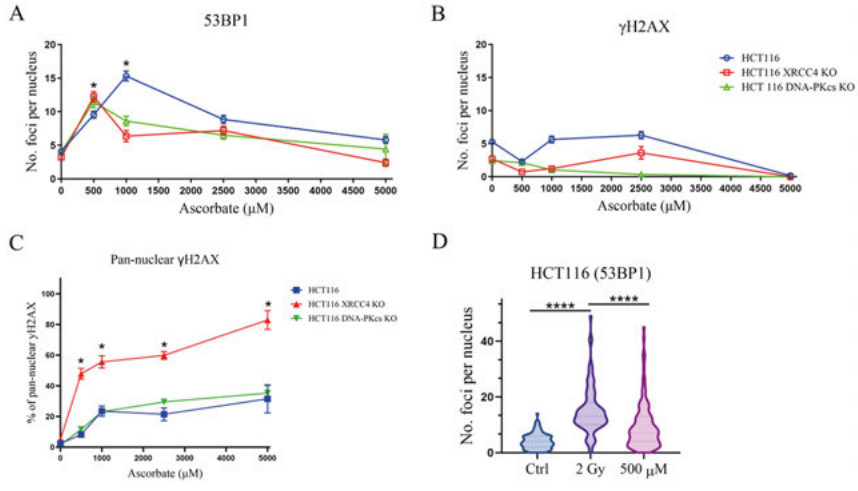

Figure 16. Chromosomal DSB biomarkers expression (γH2AX, 53BP1), and pan-nuclear γH2AX formation in cells after Asc treatment. (A)53BP1, (B) γH2AX foci, and (C) pan-nuclear γH2AX after exposure of cells to 0-5000 μM of Asc for 2 h, followed by 24 h incubation with medium for repair. (D) 53BP1 foci comparison with X-rays and Asc (1 h). Error bars present ±SEM from around 300 cells per condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

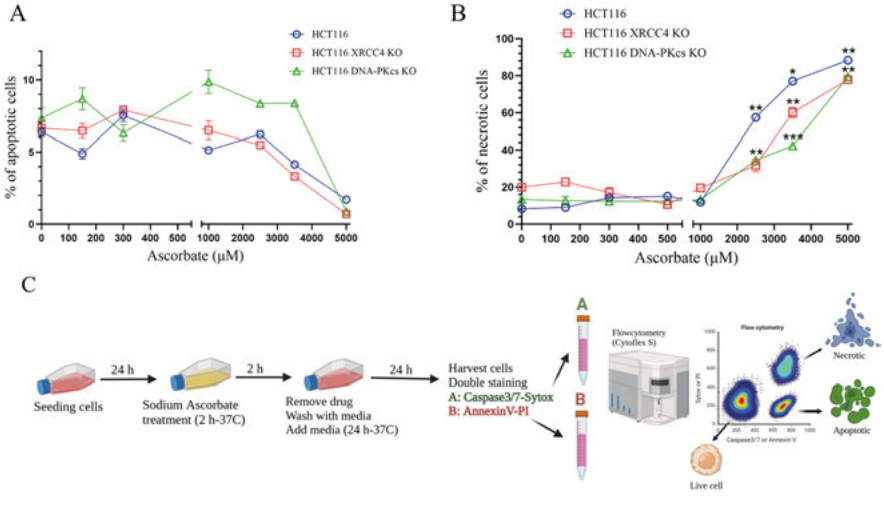

Figure 17. pharmacological Asc generates strong necrotic response in HCT116 cells without apoptosis. (A) Asc treatment (2h) (0-5000 μM) followed by a 24h incubation in medium before measuring caspase-3/7 signal in HCT116, XRCC4 KO, and DNA-PKcs KO cells by flow cytometry. (B) Necrotic response of 0-5000 μM Asc (2h), followed by a 24 h incubation in medium using Sytox signal intensity via flow cytometry. (C) Schematic view of cell preparation and assay performance for apoptosis/necrosis measurement. Error bars are ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Apoptosis and necrosis analysis: To explore the involvement of apoptosis and necrosis pathways, HCT116 and KO cells were treated with various Asc concentrations for 2 hours, followed by 24 hours of incubation with fresh media (48 hours for parental HCT116). Flow cytometry with Caspase 3/7-sytox and annexin V-PI double staining was used to assess apoptotic and necrotic responses. No significant effects on apoptosis or necrosis were observed at 100-1000 µM Asc concentrations after 24 hours. However, concentrations of 2500, 3500, and 5000 µM led to dose-dependent necrosis without activation of apoptosis (Fig. 17).

Cell cycle analysis: The results indicate that the cell cycle distribution of HCT116 cells remained unchanged until 2500 and 5000 µM Asc, which caused G2/M arrest. XRCC4 KO cells exhibited a greater accumulation of cells in G2/M phase under untreated conditions, making them more sensitive to Asc. These cells showed increased sensitivity to moderate Asc concentrations, with a dose-dependent rise in G2/M arrest at 150, 300, and 500 µM. At higher concentrations, XRCC4 KO cells accumulated significantly in the G2/M phase and displayed a dose-dependent increase in sub-G1. DNA-PKcs KO cells also exhibited increased G2/M arrest at 300 and 500 µM, followed by a substantial rise in both G2/M and sub-G1 phases at 1000 and 2500 µM. Interestingly, at the highest concentration, there was no further increase in G2/M arrest, but the sub-G1 phase elevated. Overall, high Asc concentrations caused G2/M arrest in all cell lines, with a stronger effect in XRCC4 KO cells, and increased sub-G1 cells in both KO cell lines (Fig. 18).

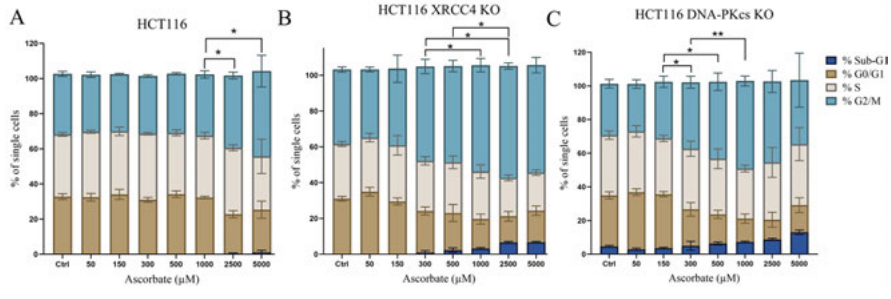

Figure 18. G2/M arrest after Asc treatment. Cell cycle analysis of (A) HCT116, (B) XRCC4 KO, (C) DNA-PKcs KO cells after Asc treatment. Cells were treated with Asc and incubated in medium for 24 h. Error bars represent ± SD, N=4. *$p < 0.05$, **$p < 0.01$.

3.4.4. Discussion and conclusion

The study investigated the effects of Asc on DNA damage, focusing on DSB formation and NHEJ using various cell lines, including XRCC4 and DNA-PKcs deficient colorectal cancer cells, and NHEJ-proficient cells (PC3 and GM5757). Results showed that Asc decreased cell survival and viability in

both 2D and 3D models in a dose-dependent manner, with XRCC4 KO cells being the most sensitive, possibly due to having naturally greater number of cells in G2/M phase. Although Asc did not induce direct DSBs (as observed with X-rays), it increased 53BP1-positive cells after 24 hours, suggesting that DSBs may result from SSBs converting into DSBs during replication or due to apoptosis and necrosis (124,188,189). γH2AX foci expression was weak in response to Asc but showed a strong, dose-dependent pan-nuclear γH2AX response, particularly in XRCC4 KO cells, indicating oxidative stress-driven overactivation of ATM (190).

Asc did not activate apoptosis but induced necrosis, with ATP depletion playing a key role in promoting necrosis, independent of caspase activation and NHEJ status. GM5757 cells were less sensitive to necrosis than HCT116 cells, consistent with the higher oxidative stress tolerance in normal cells (191). Our study suggested that Asc-induced oxidative stress may shift cell death mechanisms from apoptosis to necrosis, and highlighted that Asc can induce G2/M arrest in all cell lines, with XRCC4 KO cells showing a more pronounced effect (192–194).

Overall, Asc does not directly induce DSBs but contributes to their formation through cellular degradation processes. The XRCC4 KO cells' sensitivity is likely due to having greater number of cells in G2/M phase. These findings may help develop new cancer therapies combining Asc with radiation, cytotoxic drugs, or DNA repair inhibitors (195–197).

3.5 Paper V

The Role of Non-homologous End Joining in the Repair of Different Types of DNA Double-Strand Breaks

3.5.1. Introduction and Aim

Cancer therapies involving irradition and cytotoxic drugs kill cancer cells by inducing DSBs, the most harmful form of DNA damage (17). DSBs, however, vary in complexity, ranging from relatively clean, enzymatically induced breaks to more complex ones, such as those associated with additional DSBs, SSBs, or base lesions (18).

The complexity of DSBs can be classified based on lesion type, number, and proximity. For example, calicheamicin (CLM), a potent DSBs inducer, cleaves DNA at pyrimidine-rich sites (19). Phleomycin (PHL), generates DNA adducts that disrupt DNA polymerase, yielding numerous SSBs and a few DSBs (20). X-rays induce clustered DNA damage (27,29,198). Etoposide (ETP), causes DNA fragments to become permanent DSBs at ETP high concentrations (21–23). Temozolomide (TMZ), generates base pair mismatches and SSBs with no DSB (24,25). Additionally, the chromatin's heterogeneous

packaging likely affects the localization of cytotoxic drugs, and DNA-binding sites (34).

NHEJ is the main DSB repair pathway, repairing majority of all DSBs throughout the cell cycle by directly ligating DSB ends (199). First, the Ku 70/80 heterodimer recognize the DNA break ends. This high-affinity binding supports the DNA ends from nuclease's reach (68). Ku, bridge the DSB gap by recruiting DNA-PKcs. Next, DNA-end processing starts by DNA-PKcs recruiting Artemis (69). The KU and DNA-PKcs complex are responsible for DNA-end tethering and recruitment of XRCC4, XLF, DNA ligase IV, PAXX, and Artemis. XRCC4-ligase IV form a complex with XLF, for DNA end-ligation. The main factors for this step are the Ku heterodimer, XRCC4, ligase IV, and XLF (68). The complexity of DNA damage influences the efficiency and accuracy of NHEJ.

Despite extensive research, the mechanisms of repairing complex DNA lesions are not fully understood (200), particularly in terms of how lesion complexity affects the efficiency and specificity of NHEJ components in recognizing and processing DNA damage. Moreover, the relationship between DSB complexity and cell fate remains unclear. This study aims to investigate the roles of two core NHEJ kinases, DNA-PKcs and XRCC4, in recognizing and repairing DSBs of varying types or complexity. We used five DNA damaging agents and compared repair kinetics: TMZ, ETP, PHL, X-ray, and CLM.

3.5.2. Methods

Cell lines: Human colorectal cancer cell line HCT116, HCT116 DNA-PKcs KO, HCT116 XRCC4 KO.

Assays: western blot, colonogenic assay, pulsed-field gel electrophoresis (PFGE).

3.5.3. Results

Clonogenic assay: The long-term effects of DNA-damaging agents on HCT116 cells and their XRCC4 and DNA-PKcs knockout (KO) variants were evaluated using clonogenic assay. CLM: HCT116 cells displayed high resistance to CLM, with only minor reductions in survival fraction (SF) at higher concentrations. In contrast, both KO cell lines exhibited a dose-dependent decrease in clonogenicity. XRCC4 KO cells were significantly more sensitive than DNA-PKcs KO cells, particularly at 3, 5, and 8 pM CLM. X-rays: Radiation reduced clonogenicity across all cell lines in a dose-dependent manner. HCT116 cells were the most resistant, followed by XRCC4 KO and DNA-PKcs KO cells. While both KO cell lines were more sensitive than HCT116 cells, DNA-PKcs KO cells demonstrated slightly greater sensitivity than XRCC4 KO cells. ETP: HCT116 cells showed notable resistance compared to KO cells. Both XRCC4 KO and DNA-PKcs KO cells had similar

clonogenic loss, with only minor differences. PHL exposure led to dose-dependent reductions in clonogenicity across all cell lines. HCT116 cells were significantly more resistant than the KO lines, which displayed similar levels of sensitivity. TMZ: All cell lines exhibited a dose-dependent decrease in survival upon TMZ treatment. However, HCT116 and KO cells displayed similar reductions in clonogenicity (Fig. 19).

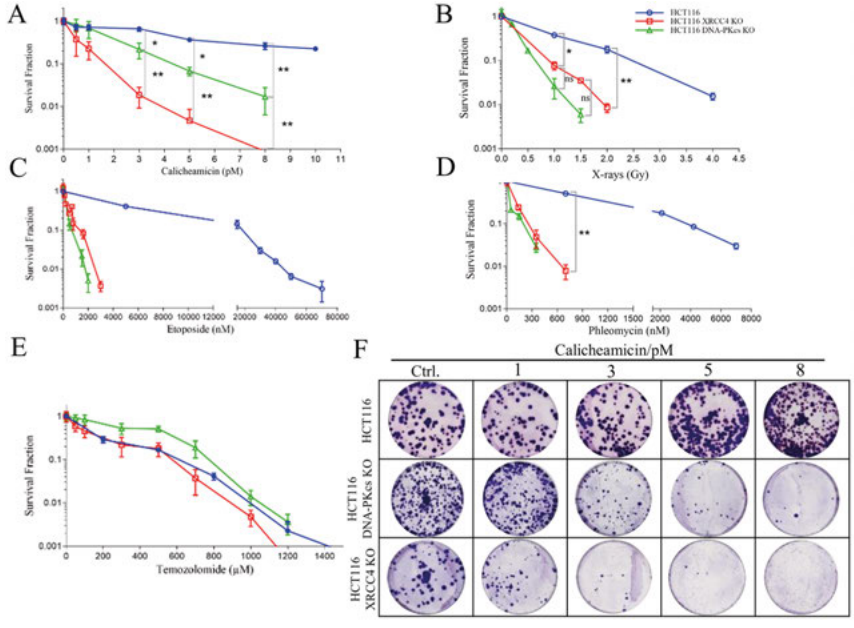

Figure 19. Clonogenic survival of HCT116, HCT116 XRCC4 KO, and HCT116 DNA-PKcs KO cells following treatment with DNA-damaging agents. (A) Clonogenic assay after CLM exposure in HCT116 cells (0-10 pM) and in XRCC4 KO and DNA-PKcs KO cells (0- 8 pM). (B) X-rays (C) ETP (D) PHL (E) TMZ. SF were quantified from colonies with more than 50 cells. Error bars represent ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (F) Representative colony image after CLM treatment.

Prompt DSB and heat-released non-DSB clusters: To evaluate the ability of DNA-damaging agents to induce prompt DSBs and heat-released non-DSB clusters in HCT116 cells, a DSB induction assay using PFGE was performed. CLM: Treatment with 0, 2, 5, 10, 25, and 50 nM CLM resulted in a dose-dependent increase in both prompt DSBs and heat-released DSBs, with only a small difference between the two (10-15% higher after heat treatment). X-rays: Increasing X-ray doses elevated DSB levels, and heat-release of non-DSB clusters increased DSB with 100%. ETP: DSB yields increased after treatment with 0-300 μM ETP. No heat-released DSB were observed below 50 μM but at higher concentrations, heat-treatment increased DSB with approximately 25%. PHL: Exposure to 0-70 μM PHL showed a dose-dependent

increase in DSBs and heat-treatment increased DSB with approximately 30%. TMZ: No DSBs were detected at concentrations up to 2000 µM. However, a slight increase in FAR was observed at 2500 µM, likely due to its high toxicity. Higher TMZ concentrations led to very high increase of heat-released non-DSB clusters (Fig. 20).

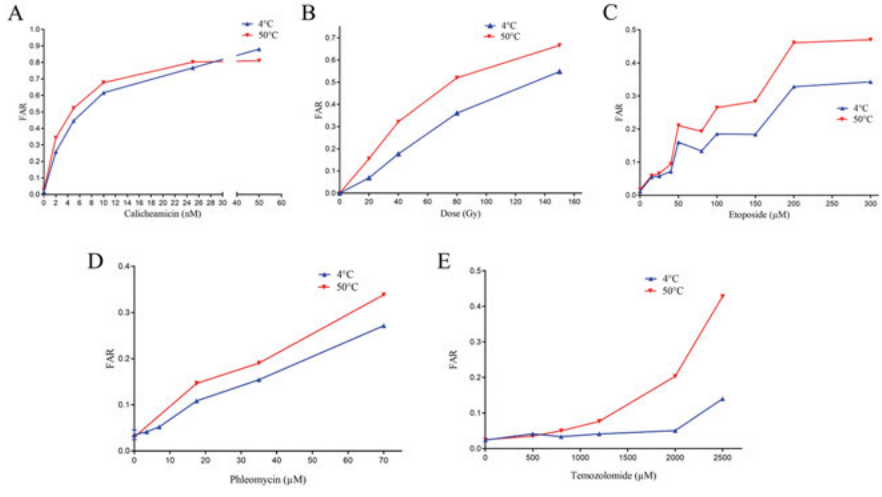

Figure 20. Prompt DSBs and heat-released non-DSB clusters generated by DNA-damaging agents measured by PFGE after treatment with (A) CLM, (B) X-rays, (C) ETP, (D) PHL, and (E) TMZ.

Prompt DSB Repair Kinetics: To evaluate the limitations of DSB repair capacity, DSB repair kinetics assay was performed using PFGE at various time points (0, 15, 30, 60, 120, 240, and 1500 minutes). Repairs occurring within the first 2 hours were classified as fast repair kinetics, while those between 2 and 24 hours were categorized as slow repair kinetics. CLM (2 nM, 30 min): HCT116 cells rejoined DSBs at all time points, achieving complete repair within 24 hours. DNA-PKcs KO cells began removing DSBs during the fast-repair phase but exhibited persistent DSBs later. XRCC4 KO cells showed minimal repair, resolving only ~40% of DSBs by 120 minutes, with an increase in unrepaired DSBs at later times. This highlights the significant sensitivity of both KO cell lines to CLM. X-rays (40 Gy): HCT116 cells rejoined nearly all induced DSBs, while both KO lines repaired only ~20%, underscoring the critical role of NHEJ in resolving radiation-induced complex DSBs. ETP (60 µM, 30 min): All three cell lines repaired ETP-induced DSBs effectively, showing minimal impact of NHEJ deficiency on this process. PHL (52.53 µM, 30 min): HCT116 cells resolved ~80% of DSBs during the fast-repair phase, with residual repair occurring later. KO lines rejoined ~40% of DSBs within 15 minutes, but persistent damage was observed at later time points. DNA-PKcs KO cells displayed greater sensitivity than XRCC4 KO cells. Overall, CLM and X-rays posed the greatest challenge to DNA repair in

the absence of NHEJ, with KO cells repairing only ~20% of DSBs. PHL allowed ~40% repair, while ETP-induced DSBs were effectively repaired (~80%) by both KO and HCT116 cells, suggesting alternative repair pathways, including HR, can partially compensate for NHEJ loss (Fig. 21).

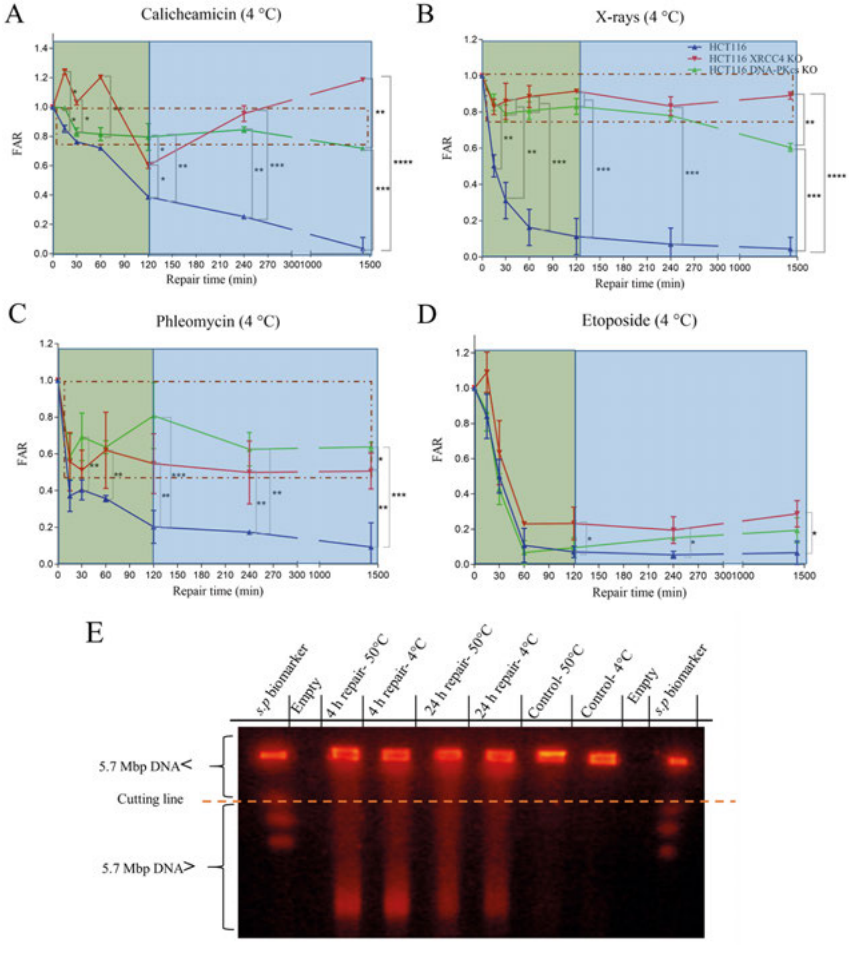

Figure 21. Prompt DSB repair kinetics of HCT116 cells after exposure to DNA damaging agents, measured by PFGE. (A)CLM (30 min), (B) 40 Gy X-rays, (C) 52.53 µM PHL (30 min), and (D) 60 µM ETP (30 min). Error bars ± SD, N=3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Green squares, represents the DSB fast repair kinetics (15-120 min) (euchromatin). Blue squares, shows the DSB repair slow kinetics (120-1500 min), (heterochromatin). (E) Sample of PFGE gel image showing DSB repair kinetics in HCT116 XRCC4 KO cells treated with PHL. (F)

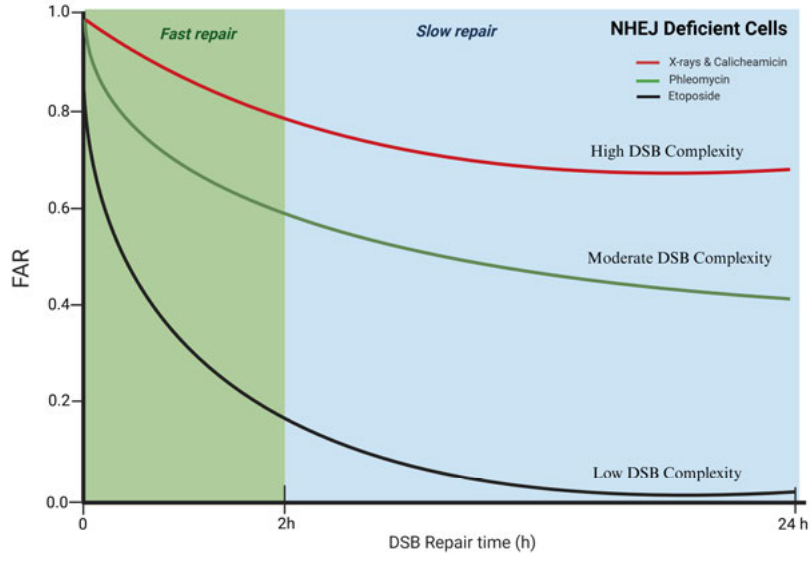

Figure 22. A model of DSB complexity in NHEJ-deficient cells. The illustration is adopted from Shibate and Jeggo, 2020. (Created with BioRender.com)

3.5.4. Discussion and conclusion

Cancer patients receive radio chemotherapeutics during the course of treatment. Cytotoxic agents kill cancer cells by inducing diverse types of DSBs with various levels of DSB complexity. NHEJ is responsible for repairing most of these DSBs. The structure of DNA ends is critical element of DSB complexity and repair. Conventional DSB repair studies typically use DSB ends with 5′-phosphate and 3′-hydroxyl termini, generated either through in vitro constructs or site-specific nucleases. However, this method fails to account for the DNA end structures created by actual DSB inducers in natural or clinical contexts (201).

Our findings revealed that DSB repair kinetics are closely related to the type and/or complexity of the DSBs. In the absence of NHEJ, there was essentially only a fast repair phase (<2h), whereafter there was no repair the following 1-24 hours. Interestingly, KO cells exhibited similar limitations in resolving DSBs induced by X-rays and extremely complex DSBs caused by CLM, suggesting that HR or Alt-NHEJ may be ineffective for repairing these DSBs.

Approximately 50% of DSBs induced by PHL were repaired in the NHEJ KO cells, whereas those caused by ETP (low complexity) were nearly fully resolved (<20% left unrejoined), emphasizing the influence of DSB type and potentially the localization within euchromatin or heterochromatin. In cells

with functional NHEJ, the repair pattern declined with increasing chemically induced DSB complexity, except for X-ray-induced clustered DNA damage. Clonogenic assays showed that NHEJ deficiency sensitized cancer cells to all DSB-inducing agents. However, when DNA damage did not involve DSBs, as seen with TMZ, NHEJ inactivation had no effect on clonogenic survival.

X-rays and CLM, displayed distinct clonogenic responses between XRCC4 and DNA-PKcs knockout cells. Notably, formation non-DSB clusters was observed following exposure to X-rays, PHL, and TMZ but less for CLM and ETP, suggesting that SSBs and heat-labile sites contribute to secondary DSB formation during heat treatment. The rapid resolution of heat-released DSB within 15 minutes was independent of NHEJ, highlighting distinct repair dynamics for this process.

The complexity of DSBs significantly influences the efficiency and accuracy of repair processes. Complex DSBs pose particular challenges due to inhibitory effects on NHEJ caused by end-group structures and lesions like nearby SSBs (202). These breaks often engage multiple repair pathways, potentially leading to competition or interference among them, reducing repair efficiency, slowing repair kinetics, and increasing the likelihood of errors. Despite these challenges, cells have evolved efficient mechanisms for addressing DSBs, often repairing them faster than other types of DNA damage (203). The choice between NHEJ and HR depends on the complexity of the break, with HR being more likely to resolve highly complex DSBs when available (204,205). This observation aligns with Okayasu et al. (2006), who showed that increasing DSB complexity from X-rays to carbon and iron ions, leads to reduced survival rates and repair efficiency in both NHEJ-proficient and deficient human and rodent cells (206). Similarly, our findings corroborate Pastwa et al. (2003), who demonstrated slower repair rates for complex, non-ligatable DSBs induced by bleomycin and γ-rays compared to simpler, ligatable blunt-ended DSBs produced by restriction enzymes (202). We observed nearly complete rejoining of DSBs during the rapid phase after low DSB complexity induction (ETP), both in the presence and absence of NHEJ. This suggests that intact NHEJ repaired almost all ETP-induced DSBs within 2 hours. Our previous findings indicated that, unlike rodent cells, human cells lack alternative NHEJ pathways (207). Therefore, in the absence of NHEJ, HR or Alt-NHEJ likely resolved all DSBs within 2 hours. Since most ETP-induced DSBs are located within euchromatin, our results indicates that the absence of NHEJ likely promotes the activation of HR or Alt-NHEJ in euchromatin regions.

In NHEJ KO cells, PHL-induced 40% of DSBs were resolved during the fast repair phase, most likely in euchromatin, and this may be due to the mixed nature of the damage affecting both euchromatin and heterochromatin (208–211). X-rays damage both chromatin types, but euchromatin, is likely damaged more immediately, making it easier for repair kinases to access. In contrast, heterochromatin is less accessible to repair proteins, requiring chromatin remodeling to make the DNA accessible, leading to slower repair (30). CLM

and X-rays, inducing breaks in both euchromatin and heterochromatin, result in slower and less efficient repair compared to ETP, emphasizing the importance of DSB localization within chromatin (30,212,212).

In conclusion, we explored the relationship between DSB type and complexity, DSB chromatin localization, repair pathway selection, and cell survival (Fig. 22). Our results emphasize the essential roles of DNA-PKcs and XRCC4 in resolving complex DSBs, while highlighting that HR or Alt-NHEJ pathways can partially or substantially compensate for NHEJ deficiencies in cases of moderate or less complex DSBs. Importantly, we show that HR or Alt-NHEJ is not limited to the slow phase of DSB repair but is also active during the fast phase for less complex DSBs, particularly those located in euchromatin. Additionally, the study highlights the significance of chromatin structure, SSB induction, and other cellular factors in interpreting the effects of DNA-damaging agents.

4. Concluding remarks

DSB induction and repair form the foundation of cancer therapy while also being a key factor in cancer resistance to radiation and chemotherapy. Nearly all cancer treatments, except surgery, aim to eliminate cancer cells by inducing DSBs while sparing healthy cells as much as possible. However, the identity, nature, and repair dynamics of DSBs in many cancer treatment modalities remain incompletely understood. A deeper understanding of how different types of DSBs are formed and repaired could lead to more precise and effective therapeutic strategies. Additionally, there is a growing trend in drug repurposing and combination therapy, aiming to enhance treatment efficacy and overcome resistance mechanisms. By integrating novel therapeutic agents with traditional DNA-damaging treatments, it may be possible to exploit DSB repair vulnerabilities and improve patient outcomes. Future research should focus on optimizing combination strategies, refining our knowledge of DSB repair pathways, and translating these insights into clinical applications to advance cancer treatment.

To achieve the goal of optimizing combination therapy and exploiting DSB repair pathway vulnerabilities in cancer cells, it is necessary to understand the DSB repair signatures of commonly used cancer therapeutics and how cancer cells respond to varying levels of DSB complexity arising from combination treatments. A deeper knowledge of these complexities is essential for strategically manipulating DSB repair mechanisms to maximize synthetic lethality in cancer cells while minimizing damage to healthy tissues.

This thesis addresses this challenge through a dual approach. First, we applied combination therapy in a practical setting to assess how DSB induction and repair capacity can be leveraged to sensitize cancer cells. Second, we adopted a fundamental radiochemo biology perspective to uncover the underlying mechanisms governing DSB repair responses to different levels of DSB complexity. By bridging these two approaches, this work contributes to a better understanding of DSB repair dynamics, with potential implications for improving cancer treatment strategies. The major findings of this thesis are summarized as follows:

In **Paper I**, we showed that HSP90 inhibition by onalespib can effectively overcome cisplatin resistance in ovarian cancer cells by increasing DSB lesions while reducing the DSB repair capability of cancer cells. This elevated DSB level was associated with increased apoptosis and G2/M arrest.

In **paper II**, we demonstrated that HSP90 inhibition by onalespib enhances GBM radiosensitivity by increasing DSB accumulation, triggering apoptosis, and promoting cell death in both 2D and 3D models. These findings highlight the potential of combining radiotherapy with HSP90 inhibition as a strategy to overcome GBM resistance.

In **paper III**, we showed that Ra-223 induces track-like clustered DSBs, activating NHEJ and leading to growth inhibition and apoptosis in prostate cancer cells. Notably, Ra-223 imposed its effects without cellular uptake.

In **paper IV**, we found that Asc induces delayed DSBs, marked by pan-nuclear γH2AX and G2/M arrest, leading to necrosis and reduced cell viability in both 2D and 3D models. This effect was more pronounced in XRCC4 KO cells but was driven by cell cycle regulation rather than NHEJ deficiency.

In **paper V**, we tested how NHEJ responds to different DSB types and complexities in colorectal cancer cells. Wild-type cells exhibited both fast and slow repair kinetics, while NHEJ-deficient cells showed only an initial fast repair phase, with no further repair over 24 hours. As the DSB:SSB ratio decreased across different inducers, non-DSB clusters increased but were rapidly removed in an NHEJ-independent manner.

Overall, our results highlight strategies for manipulating and optimizing DSB repair to overcome cisplatin resistance in ovarian cancer and enhance radiosensitivity in GBM cells. We also revealed the DSB repair response of prostate cancer cells to alpha-therapy with Ra-223. Additionally, we demonstrated the delayed DSB induction effect of Asc and examined the NHEJ response to different DSB complexities in colorectal cancer. These findings provide new insights into NHEJ response biology in cancer.

5. Future perspectives

DNA damage response is central to cancer initiation and progression. Understanding underlying mechanistic interaction of DDR and DSB complexity can improve targeted and combined cancer therapy. Monotherapy in cancer, dating back to the 1940s, revealed that single-agent treatments cannot sustain long-term remission. Since then, the pursuit of combination therapies targeting multiple pathways without overlapping mechanisms has intensified (213). The discovery of oncogenes in the 1980s fueled the rise of targeted therapies aimed at disrupting cancer's key pathways (214). However, the critical question remains: what combination of drugs can achieve a long term/lasting response? The driver of new ideas for combinational therapy can be from the origins of cancer resistant development, such as heterogeneity of tumor cells, mutations, non-genetic transcriptional pathway changes, epigenetic tumor changes, and cell state transition.

In **papers I** and **II**, we explored DSB optimization through HSP90 inhibition in combination therapy to enhance tumor sensitization. However, these findings require further *in vivo* validation to move closer to clinical application. A future approach building on **papers I** and **II** is *in vivo* loss-of-function genetic screening (215) to identify key targets for achieving synthetic lethality. This can be complemented by next-generation CRISPR–Cas technology for proof-of-concept validation and drug synergy testing. Data revealed that cancer cells with defects in certain DNA repair pathways showed increased immunogenicity, opening the door for combining DDR inhibitors with immune checkpoint inhibitors, such as PARP or DNA-PKcs inhibitors with PD-L1 mAb for enhanced therapeutic impact (121). Recent advancements in implantable devices for delivering multiple drugs at nanodoses to different tumor regions, combined with multiplex imaging, offer a promising strategy to enhance our study model (216). Moreover, the integration of computational power and AI with large-scale cancer datasets such as Pan-Cancer Analysis of Whole Genomes, The Cancer Genome Atlas, and DepMap (Cancer Dependency Map), alongside lab-generated data, can refine small-scale screening results within the broader cancer landscape, providing more precise combination therapy strategies (214).

The identity, nature, and repair dynamics of DSBs induced by different cancer treatments remain incompletely understood. A deeper insight into how DSBs form and resolve under various therapies is crucial for advancing cancer

treatment. In **papers III**, **IV**, and **V**, we tackled these challenges, yet a key unresolved question remains: What drives the fast repair (<30 min) in NHEJ-deficient cells? Is HR or alt-NHEJ compensating within this rapid phase? Addressing these questions requires not only mechanistic studies but also the development of advanced DSB detection techniques capable of capturing rapid repair dynamics, such as flow cytometry (179).

Although we examined chromosomal responses to DSBs through 53BP1 and γH2AX, key regulators of DNA damage recognition and repair, the structural and functional changes in chromatin architecture following DSB induction remain largely unexplored (35,217). Understanding the spatio-temporal dynamics of chromosome structure in response to different DSB types, particularly in the context of cancer therapy, is crucial. Advanced imaging techniques, chromatin conformation assays, and computational modeling could be instrumental in addressing this gap. Understanding these mechanisms may reveal chromosomal vulnerabilities, identify potential druggable chromatin targets, and pave the way for diagnostic strategies based on chromosomal DSB biomarkers.

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