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RNA POLYMERASE I INHIBITION: MECHANISM AND EXPLOITATION IN CANCER TREATMENT

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RNA POLYMERASE I INHIBITION: MECHANISM AND EXPLOITATION IN CANCER TREATMENT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Asimina Zisi

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"Life is an unfoldment, and the further we travel, the more truth we can comprehend."

-Hypatia

-

To the memory of my Late Grandfather, Thomas

ABSTRACT

Cancer is an umbrella term for diseases characterized by uncontrollably proliferating abnormal cells that often have also gained the ability to spread and invade other tissues. It is one of the leading causes of death worldwide and the second-leading cause of death in Sweden. Chemotherapy is a commonly used treatment approach, where the drugs preferentially target cellular processes needed for cancer cell proliferation, leading to cancer cell growth arrest or death. Albeit a potent tool in managing cancer, the overall success rate remains low for certain cancer types, highlighting the need to identify new chemotherapeutic targets and strategies.

Ribosome biogenesis (RiBi), a fundamental process that supplies cells with ribosomes, represents an emerging target, with several cancer types relying on high RiBi rates to maintain high proliferation rates. Small-molecule-mediated RiBi inhibition induces nucleolar stress, a cellular response resulting in cell cycle arrest, and apoptosis, often dependent on p53. Pre-clinical studies have shown promising results in a variety of cancer types; however, the compounds available are limited, and their mechanistic details are yet to be explored. Thus, the characterization of cancer-specific biological effects of RiBi inhibition, together with the identification of new RiBi targets and inhibitors, may expand the therapeutic promise of this strategy, accelerate the clinical development of drug candidates and potentially facilitate the selection of patients who might benefit from the clinical use of RiBi inhibitors in the future.

The primary aim of the Thesis was to study

1. the pharmacological inhibition of RiBi focusing on RNA polymerase I (Pol I), and repurposing of clinically approved drugs with underappreciated RiBi-inhibitory effects for cancer treatment
2. the effects of Pol I inhibition in high-grade gliomas (HGG) and identify synergistic treatment strategies to prevent potential resistance development
3. alternative druggable RiBi-associated protein targets

In **Paper I**, we identified an FDA-approved antimalarial drug, amodiaquine, with previously unknown Pol I inhibitory effects. We designed and synthesized a chemical analog with comparable efficacy to limit potential toxicity and demonstrated the effectiveness of the analog series in a panel of colorectal cancer cell lines.

In **Paper II**, we reported the relevance and effectiveness of RiBi as a target in HGG, uncovered a novel cellular response to nucleolar stress, mediated by the Fibroblast Growth Factor 2 (FGF2)- Fibroblast Growth factor receptor 1 (FGFR1) signaling axis, and proposed a highly synergistic combination with FGFR inhibitors to limit glioma cell growth.

In **Paper III**, we further characterized the functional role of the DEAD-Box Helicase and Exon Junction Complex protein, eIF4A3, and suggested its relevance as a target for drug discovery, showing its involvement in RiBi and highlighting its association with tumor aggressiveness.

LIST OF SCIENTIFIC PAPERS

I. Jaime A. Espinoza, **Asimina Zisi**, Dimitris C. Kanellis, Jordi Carreras-Puigvert, Martin Henriksson, Daniela Hühn, Kenji Watanabe, Thomas Helleday, Mikael S. Lindström, Jiri Bartek. *The antimalarial drug amodiaquine stabilizes p53 through ribosome biogenesis stress, independently of its autophagy-inhibitory activity.* Cell Death & Differentiation. 2020; 27(2):773-789.

II. **Asimina Zisi**. Dimitris C. Kanellis, Simon Moussaud, Ida Karlsson, Helena Carén, Lars Bräutigam, Jiri Bartek, and Mikael S. Lindström. *Small Molecule-mediated Disruption of Ribosome Biogenesis Synergizes With FGFR Inhibitors to Suppress Glioma Cell Growth* (Manuscript)

III. Dimitris C. Kanellis, Jaime A. Espinoza, **Asimina Zisi**, Elpidoforos Sakkas, Jirina Bartkova, Anna-Maria Katsori, Johan Boström, Lars Dyrskjøt, Helle Broholm, Mikael Altun, Simon J. Elsässer,Mikael S. Lindström, Jiri Bartek. *The exon-junction complex helicase eIF4A3 controls cell fate via coordinated regulation of ribosome biogenesis and translational output.* Science Advances. 2021; 7(32):eabf7561.

Scientific papers not included in the Thesis

Asimina Zisi, Jiri Bartek, Mikael S. Lindström. *Targeting Ribosome Biogenesis in Cancer: Lessons Learned and Way Forward*. Cancers. 2022; 14(9):2126.

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
ActD	Actinomycin D
AKT	Protein Kinase B
ALK	Anaplastic lymphoma kinase
AMPK	AMP-activated protein kinase
APC	Adenomatous polyposis coli
ATM	Ataxia-Telangiectasia Mutated
ATR	Ataxia Telangiectasia And Rad3 Related
AUC	Area Under the Curve
AQ	Amodiaquine
BRCA1/2	Breast Cancer Type 1 and 2 Early Onset Gene
CCGA	Chinese Glioma Genome Atlas
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A
CDKN2A	Cyclin-dependent kinase inhibitor 2A/B
CHK1/2	Checkpoint Kinase 1 And 2
CIN	Chromosomal Instability
CQ	Chloroquine
CRC	Colorectal Cancers
DDR	DNA Damage Response
DHODH	Dihydroorotate Dehydrogenase
DNA-PK	DNA-Dependent Protein Kinase
DSBs	Double Strand Breaks
EGFR	Epidermal Growth Factor Receptor
EIF4A3	Eukaryotic Translation Initiation Factor 4A3
EJC	Exon Junction Complex
ERK	Extracellular Signal-Regulated Kinases
FBL	Fibrillarin
FGFR	Fibroblast Growth Factor Receptor
GBM	Glioblastoma multiforme

GSCs	Glioblastoma Stem Cells
G4	G-Quadruplex
HGG	High-grade Glioma
HIF-1	Hypoxia-Inducible Factor
HMG	High-Mobility Group Domain Proteins
HTS	High Throughput Screening
GI_{50}	Half Maximal Growth Inhibitory Concentration
IC_{50}	Half Maximal Inhibitory Concentration
IDH1	Isocitrate Dehydrogenase 1
ITS1 and 2	Internal transcribed spacer 1 and 2
IMPDH2	IMP Dehydrogenase-2
INK4	Inhibitor of Cyclin-Dependent Kinase 4
IRBC	Impaired Ribosome Biogenesis Checkpoint
MAPK	Mitogen-Activated Protein Kinases
MDM2	Mouse Double Minute 2
mTOR	Mechanistic Target Of Rapamycin
NCL	Nucleolin
NMD	Nonsense Mediated Decay
NORs	Nucleolar Organizer Regions
NPM1	Nucleophosmin
NSC	Neural Stem Cells
PDGFR	Platelet-Derived Growth Factor Receptor
PI3K	Phosphoinositide 3-Kinase
PIC	Pre-Initiation Complex
Pol I	RNA Polymerase I
Pol II	RNA Polymerase II
Pol III	RNA Polymerase III
PTEN	Phosphatase And Tensin Homolog
Rb	Retinoblastoma Protein
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA

RiBi	Ribosome Biogenesis
ROS	Reactive Oxygen Species
RPs	Ribosomal Proteins
rRNA	Ribosomal RNA
SL1	Selective Factor 1
SOX2	SRY-Box Transcription Factor 2
ssDNA	Single-Strand DNA
SSU	Small subunit
TCGA	The Cancer Genome Atlas
TIF-I	Transcription Initiation Factor I
TFIIIB	Transcription factor IIIB
TFIIIC	Transcription factor IIIC
UBF	Upstream Binding Factor
zPDX	Zebrafish Patient-derived Xenografts
γH2AX	Phosphorylated Histone 2X

1 INTRODUCTION

1.1 Cancer

A tumor, an abnormal cell mass, can develop in any tissue and is classified as benign or malignant based on, among other parameters, its growth pattern. Benign tumors grow slowly, show distinct borders, and do not spread in nearby tissues or other organs, whereas malignant tumors, known as cancers, display more aggressive features, infiltrate adjacent tissues and metastasize (Patel 2020). Failure to control tumor growth and/or metastasis can affect vital organs, followed by severe, often deadly consequences, while the complications of systemic therapies or surgery may also have fatal outcomes in advanced stages of cancer. Regardless of the tremendous ongoing basic and clinical research efforts, cancer is a leading cause of death, highlighting the urgency for developing successful anti-cancer strategies. This becomes more of a challenge since cancer is a heterogeneous group of diseases.

1.1.1 Hallmarks Of Cancer

Cancer cells fail to obey rules governing intra- and multicellular homeostasis and instead acquire functional capabilities that allow them to adapt, proliferate, migrate to surrounding tissues and hide from the immune system. Six acquired traits that collectively allow malignant progression were proposed as Hallmarks of Cancer; these are "*self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis."* (Hanahan et al. 2000). A decade later, two emerging alterations defined as *"cellular energetics"* and *"avoiding immune destruction",* were introduced to the core set of Hallmarks (Hanahan et al. 2011). The same study introduced the concept of *"enabling characteristics"* to approach the intricacy of cancer pathogenesis and provide the processes required to enable the acquisitions of the hallmark traits, that is, the genomic instability and the tumor-promoting inflammation. In 2022, the Hallmarks scheme was further refined, and four more emerging hallmarks and enabling characteristics were introduced: *"unlocking phenotypic plasticity," "non-mutational epigenetic reprogramming," "polymorphic microbiomes," and "senescent cells"* (Fig. 1) (Hanahan 2022). Overall, the Hallmarks of Cancer conceptualization has been integral to addressing the increasing complexity of cancer pathogenesis, understanding the molecular characteristics of malignancy, and strategically designing tailored cancer treatments.

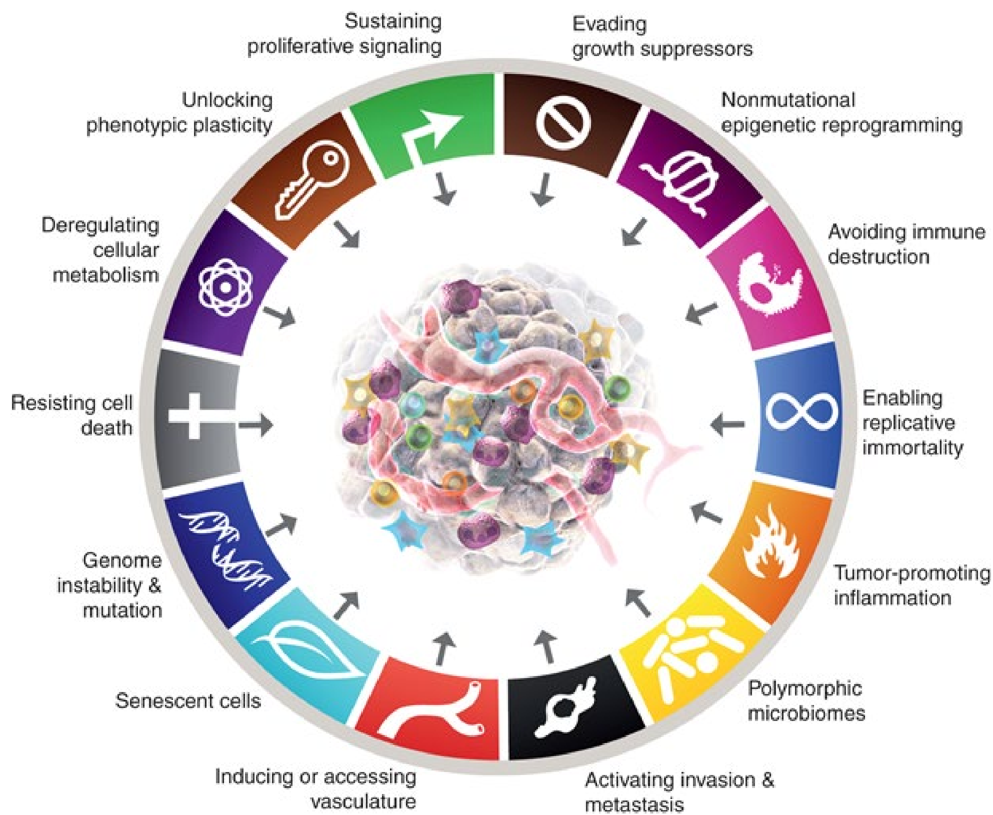

Figure 1: *The Hallmarks of Cancer: New Dimensions. (Hanahan 2022) Copyright © 2022 American Association for Cancer Research. Reprinted with permission.*

1.1.2 Cancer Development

The transformation of a normal cell into a cancer cell, a process called carcinogenesis pr tumorigenesis, results from a series of genetic and cellular alterations that affect molecular control mechanisms, disrupt cell homeostasis and allow aberrant cell proliferation (Weinberg 1996). It is believed that carcinogenesis emerges upon genetic alterations triggered by pathogenic stimuli such as genotoxic chemicals, ultraviolet irradiation, or infectious agents, while an ample proportion of cancer types are considered to be of sporadic etiology (Takeshima et al. 2019). Most commonly, genetic alterations include oncogenic activation via gain-of-function mutations in proto-oncogenes that induce cell proliferation and enhance cell survival and via loss-of-function of critical negative regulators of uncontrolled cell proliferation, the tumor suppressor genes (Fig. 2) (Lee et al. 2010). The imbalance between the fundamental processes of regulating cell proliferation and programmed cell death results in an abnormal cell mass formation that can eventually grow to perturb the body's physiological conditions.

Oncogene activation

Oncogene activation most commonly arises from mutations, chromosomal translocations, or genomic amplifications (Sadikovic et al. 2008). **Mutations** often alter the protein structure, enhancing the transformation to its active state, as seen in the frequently mutated RAS oncogene group, resulting in continuous cell growth signal transduction (Bos 1989). **Chromosomal translocations** result in fusion protein-coding genes with oncogenic activity,

such as the fusion gene of nucleophosmin (NPM1) and ALK. NPM-ALK is a constitutively activated tyrosine kinase and shows potent oncogenic activity (Morris et al. 1994, Drexler et al. 2000). Alternatively, translocations can lead to transcriptional oncogene activation by moving a proto-oncogene gene close to a T-cell receptor or an immunoglobulin gene, which will be then subjected to transcriptional control by the regulatory elements of the latter. **Genomic amplifications** emerge via redundant DNA replication and are defined as the multiplication of 0.5-10 Mb-long intra-chromosomal regions, while DNA copy number increase is characterized by larger chromosomal areas that occur via translocations or aneuploidy and is defined as gain (Myllykangas et al. 2006). Some of the oncogenes reported to be activated by this mechanism are *CCND*, *EGFR*, *FOS*, and *c-MYC*.

Tumor Suppressor Inactivation

Tumor suppressors are inactivated by intergenic mutations, deletions, or allelic loss of genes (Sadikovic et al. 2008). **Mutations** are frequently observed in genes regulating the cell cycle and programmed cell death. One of the most common examples across a wide range of tumors is the mutational inactivation of p53, a transcription factor that induces the expression of target genes controlling cell fate by triggering cell cycle arrest or apoptosis (Hollstein et al. 1991, Soussi et al. 2007). Other examples include the *BRCA1* and *BRCA2* genes and the *APC*, *CDKN1A*, and phosphatase and tensin homolog (*PTEN*) (Negrini et al. 2010). **Deletions** of larger chromosomal regions or entire chromosomes is another common mechanism of inactivation observed in important regulators of the cell cycle, such as the retinoblastoma gene (*RB*) and the *CDKN2A/INK4A* locus coding for $p14^{ARF}$ and $p16^{INK4A}$, involved in the p53 pathway and the G1 to S cell cycle transition regulation (Sadikovic et al. 2008). Regions encoding PTEN, a modulator of the phosphatidylinositol-3-kinase (PI3K) pathway and Protein Kinase B (AKT) activation, is also among the most frequently deleted loci in several cancer types (Li et al. 1997). Collectively, these alterations lead to dysfunctional cell cycle checkpoints and amplified growth signaling.

Oncogene-induced Genomic Instability

Oncogene activation often leads to deregulated DNA replication and cell proliferation, followed by replication stress and genomic instability, which are crucial during the early tumor evolution (Bartkova et al. 2006, Di Micco et al. 2006). Oncogene-induced replication stress cause DNA replication fork stalling and collapse, triggering the formation of DNA double-strand breaks (DSBs) and activating DNA Damage Response (DDR) pathway and p53 to halt cancer progression (Halazonetis et al. 2008). Impairment of DDR or p53 mutations overcomes this barrier, fueling cancer development. Replication stress is also associated with severe

chromosomal alterations, collectively termed chromosomal instability (CIN), a driver of cancer heterogeneity and evolution (Burrell et al. 2013, Bakhoum et al. 2017).

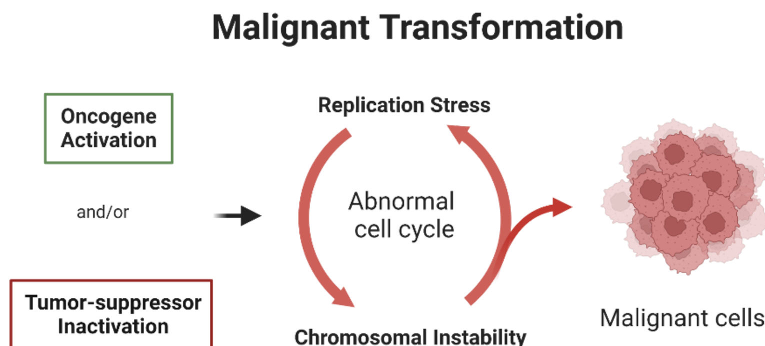

Figure 2. *Malignant Transformation as a result of Oncogene Activation and/or Tumor Suppressor Inactivation, Created with Biorender*

The DNA Damage Response

DNA lesions, under normal conditions, engage the DDR pathway that activates cell cycle arrest to allow for DNA repair. Failure to repair these mechanisms may lead to cellular senescence or cell death. (Jackson et al. 2009, O'Connor 2015). Depending on the DNA lesion type, DDR employs four types of repair mechanisms, summarized in Fig. 3.

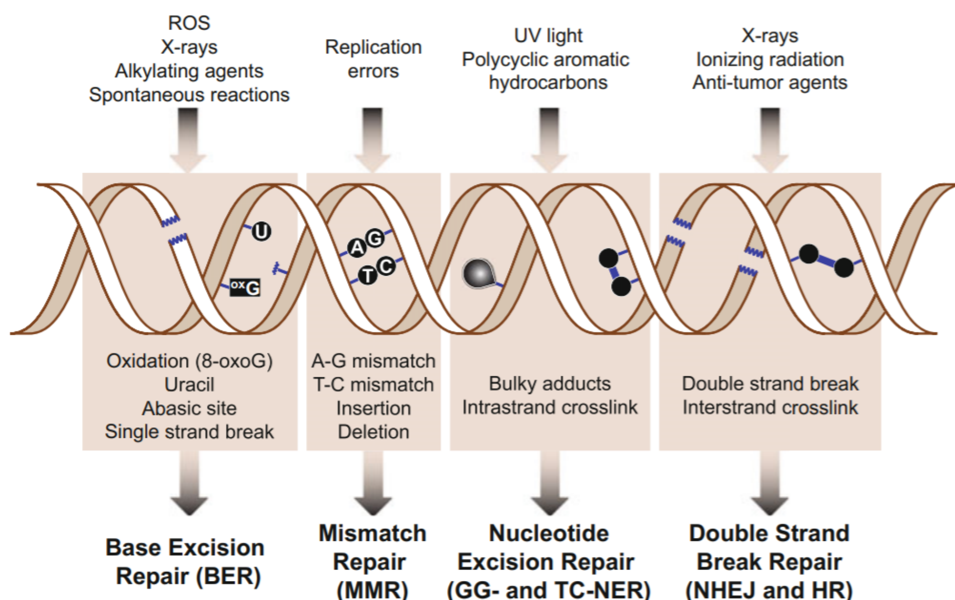

Figure 3. *DNA Damage type, response pathways and repair. (Dexheimer 2013) Reprinted with permission from Copyright © 2013, Springer Science Business Media Dordrecht*

DNA damage activates the central transducers DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM), and rad-3 related (ATR), checkpoint kinases, which with the effector kinases checkpoint kinase 1 (CHK1), checkpoint kinase 2 (CHK2), mediate the phosphorylation of downstream targets, leading to cell cycle arrest and orchestrating the downstream DNA Damage response (Fig. 4). For instance, p53 phosphorylation and

downstream p21 upregulation that activates cell cycle checkpoints, while phosphorylation of histone H2AX close to DNA lesions attracts DNA repair proteins to the site.

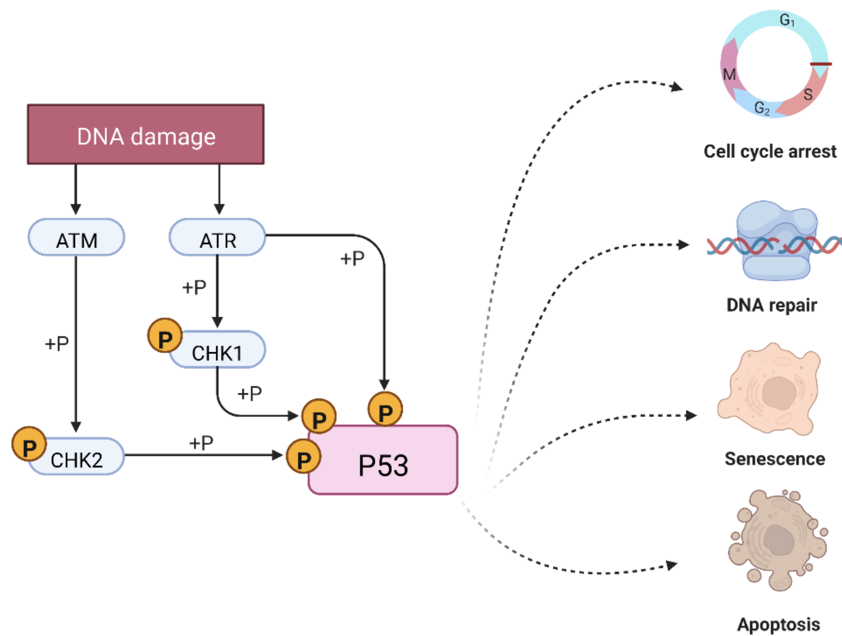

Figure 4. *DNA Damage Response-mediated p53 activation, Created with Biorender*

1.1.3 Cancer Therapy

The ultimate goal of cancer therapy is to selectively target and remove or kill cancer cells while leaving normal cells unaffected. Cancer therapy options have traditionally included chemotherapy, radiotherapy, and surgery. Radiotherapy and surgery were the first approaches to be employed as early as the late 19th Century (Arruebo et al. 2011). Chemotherapy was established as a term later in the 1930s by Paul Ehrlich and was applied to cancer treatment a decade later. The observation that alkylating agents, which soldiers were exposed to during the two World Wars (mustard gas), reduced leukocyte counts (Gilman 1946, Arruebo et al. 2011), led to the first use of alkylating agents in the treatment of lymphomas. The development of chemotherapeutic agents continued with the synthesis of more alkylating agents such as cyclophosphamide, antimetabolites (e.g., methotrexate and 5-fluorouracil), the anti-mitotic vinca alkaloids, the platinum-based drugs (e.g., cisplatin) and the Topoisomerase poisons (e.g., Doxorubicin), still in use today against several cancer types. The targets of these chemotherapeutics summarized in Fig. 5 are fundamental cellular components and processes, such as DNA replication and cell proliferation, while the rationale behind chemotherapy is that rapidly proliferating cancer cells will exhibit increased sensitivity to these agents compared to the normal ones (Avendaño et al. 2015).

Tumor resistance and recurrence were evident from the early years of chemotherapy, motivating the introduction of combination therapy in the 1960s to achieve superior therapeutic effects (Frei et al. 1958, Keating et al. 2013). This strategy was further established with the increasing understanding of cancer heterogeneity and the individual molecular characteristics, which led to the development of targeted therapy, that is, targeting the cellular components or pathways required for tumor growth in a given cancer subtype (Gerber 2008, Sherr et al. 2017).

Regardless of the constantly increasing implementation of targeted cancer therapies, a significant proportion of the clinically used drugs are still conventional chemotherapeutics targeting DNA by alkylation, cross-linking, intercalation, or by causing other DNA lesions and, in turn, activate the DDR pathway and the p53, leading to cell-cycle arrest, DNA damage repair, senescence or apoptosis (Fig. 4) (Jackson et al. 2009, Avendaño et al. 2015, O'Connor 2015).

However, these genotoxic agents may cause severe side effects and are associated with increased risks of tumor recurrence and the emergence of secondary malignancies (Housman et al. 2014). Thus, developing alternative, non-genotoxic activators of p53 has been a strategy of particular interest, especially in treating childhood cancers that show a higher risk for secondary cancers (Choi et al. 2014).

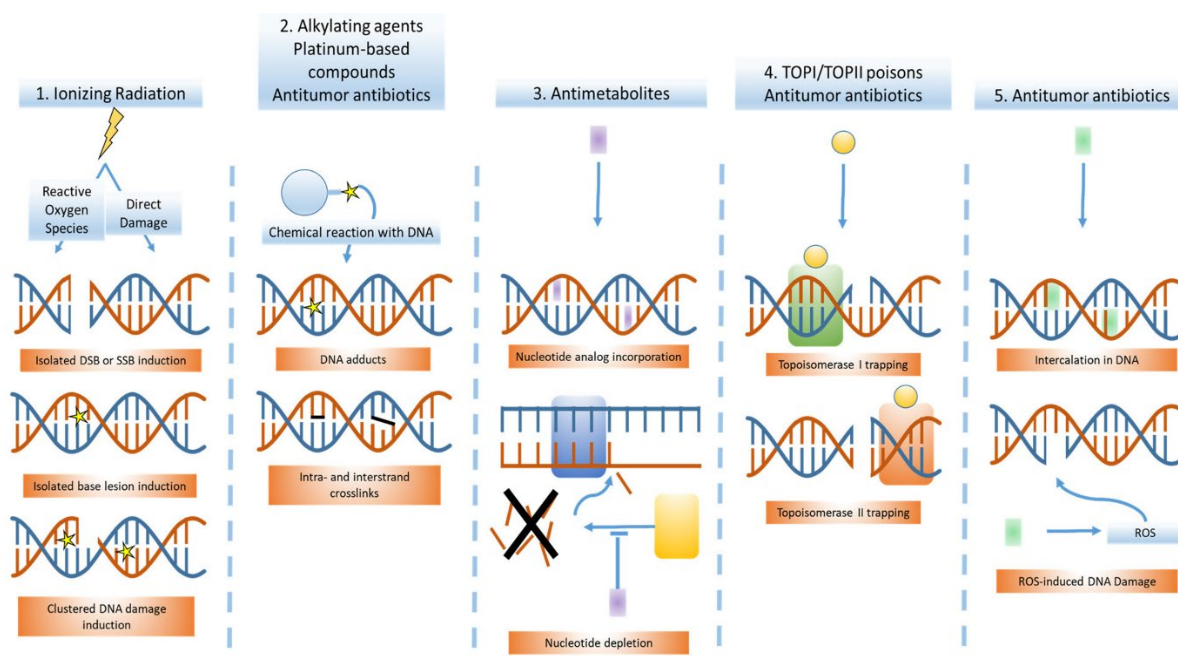

Figure 5. *Summary of the Radio- and Chemotherapy-induced DNA damage, adapted from (Reuvers et al. 2020), © 2020 by the authors. Licensee MDPI, Basel, Switzerland*

1.1.4 Chemotherapy-Induced Cellular Responses and Death

The end goal of chemotherapy is to cause tumor cell death. **Apoptosis** is one of the major mechanisms of chemotherapy-induced death (Elmore 2007, Strasser et al. 2020). It is executed by the intrinsic (triggered by intracellular stress, e.g., DNA damage, hypoxia, oncogenic activation, ROS) or the extrinsic pathway (triggered by external death signals). The intrinsic pathway is activated following p53 stabilization and transcription activation of pro-apoptotic genes of the Bcl2 family. External stimuli activate the extrinsic, receptor-mediated apoptotic pathways, following ligand-receptor binding of death receptor complexes. The activator protein families are members of the tumor necrosis factor (TNF) and their receptors (TNFR). Both pathways converge to activating caspases, proteolytic enzymes that dismantle the cellular structures (Ricci et al. 2006).

Another form of caspase-independent programmed cell death shown to have important role in cancer cell response to therapy is necroptosis. The name reflects the morphological similarities with **necrosis**, an additional type of cell death considered to be passively executed following extended cellular insults (Gong et al. 2019, Strasser et al. 2020). Necroptosis is activated by the receptor-interacting protein (RIP) kinases RIPK1 and RIPK3 kinases, which engage the membrane pore-forming mixed lineage kinase domain-like pseudokinase (MLKL), and is believed to be induced upon failure of caspase-dependent apoptosis (Gong et al. 2019, Strasser et al. 2020). Caspase-independent programmed death occurs via ferroptosis, which is also connected to the efficacy of anticancer therapies (Strasser et al. 2020, Zhang et al. 2022). It is induced by excessive ROS generation and depends on the presence of iron ions, and is morphologically similar to necrosis (Galluzzi et al. 2018).

Another common cellular response to chemical stress is **Autophagy** (or macroautophagy)**,** a catabolic process, which is considered another form of programmed cell death, even though it is not always synonymous with death (Ricci et al. 2006, Sui et al. 2013, Galluzzi et al. 2018, Strasser et al. 2020). It is engaged upon metabolic stress and executes the degradation of internal organelles and proteins by lysosomal hydrolases, following encapsulation of cytoplasmic content in autophagosomes and fusion with lysosomes. Autophagy is negatively regulated by the mammalian target of rapamycin (mTOR) pathway and positively by the AMP-activated protein kinase (AMPK) signaling pathway, which suppresses mTOR signaling (Sui et al. 2013). Tumor suppressors (e.g., p53) or metabolic stress (e.g., ER stress) have been shown to stimulate autophagy. While autophagy is routinely executed at basal levels in normal tissues to enable nutrient turnover, excessive autophagic activity upon stress may lead to death. However, increasing evidence suggests that autophagy activation following chemotherapy can also have a pro-survival role. Moreover, concomitant inhibition of autophagy enhances the cytotoxic effect and is thus considered a promising anticancer strategy, which is under investigation in preclinical and clinical settings (Liu et al. 2020).

Non-lethal processes are also observed following chemotherapy. M**itotic catastrophe** is induced by several chemotherapeutics and occurs during mitosis due to extensive DNA damage and loss of cell cycle checkpoints. Mitotic catastrophe may lead to intrinsic apoptosis, however, it does not always lead to death (Galluzzi et al. 2018). Finally, **cellular senescence**, that is, permanent growth arrest, is triggered by telomere shortening-induced DNA damage (Ricci et al. 2006). Other types of cellular stresses, including oncogene activation and chemotherapy, can induce senescence, predominantly via tumor suppressors such as p53 and Rb. However, senescent cells have been associated with tumor resistance, as senescence reversibility has been proposed (Lee et al. 2019). In addition, senescent cells exhibit a secretory phenotype and have been reported to induce microenvironmental changes, promoting invasion, stemness, and metastasis (Laberge et al. 2012, Milanovic et al. 2018, Zeng et al. 2018).

1.2 Ribosome Biogenesis

Ribosome Biogenesis (RiBi) is a fundamental, multistep cellular process through which ribosomes, the protein factories of the cells, are formed (Grummt 2010, Goodfellow et al. 2013)

(Fig. 6). The process employs three DNA-dependent RNA polymerases, approximately 80 ribosomal proteins (RPs), together with ~200 transiently incorporating non-ribosomal factors.

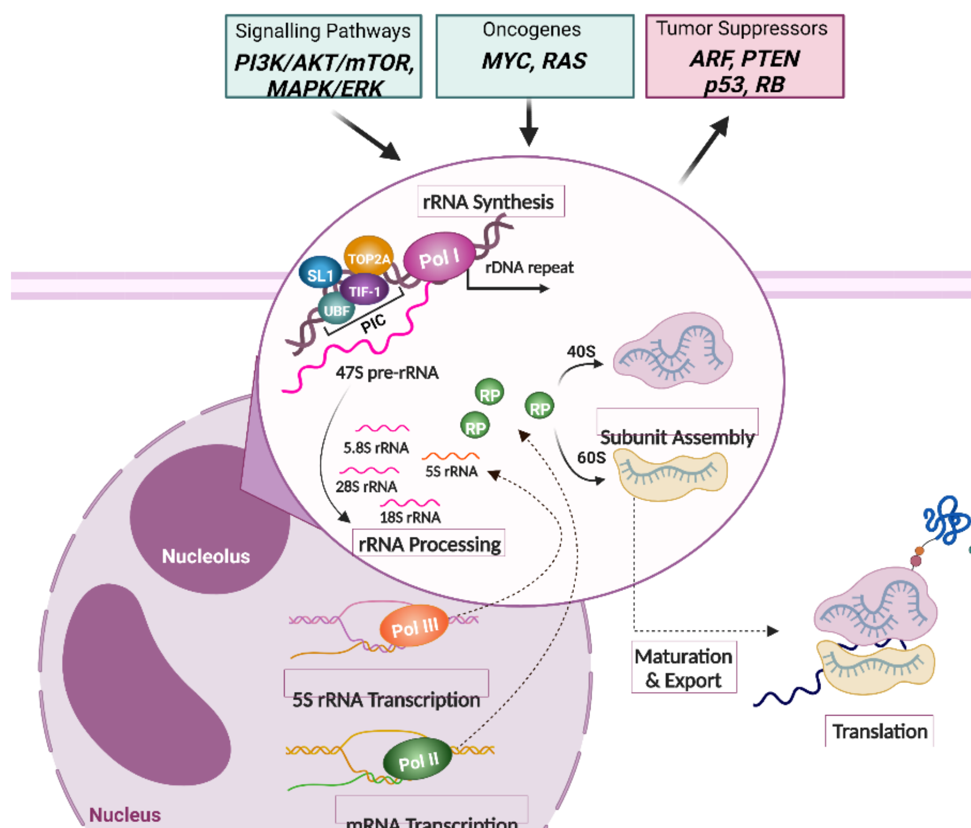

Figure 6. *The steps of ribosome biogenesis, along with the positive and negative regulators of the process. Adapted from (Zisi et al. 2022), © 2022 by the authors. Licensee MDPI, Basel, Switzerland.*

Upon the assembly of the pre-initiation complex (PIC) at the rDNA promoter, the rate-limiting step of the ribosomal DNA (rDNA) transcription into ribosomal RNA (rRNA) is executed by RNA polymerase I (Pol I) (Drygin et al. 2010, Bywater et al. 2013, Goodfellow et al. 2013). PIC includes several components, including the transcription initiation factor I (TIF-I), the promoter selectivity factor, SL1, and the upstream binding factor (UBF). rDNA binding of UBF forms a nucleosome-like structure enabling the recruitment of Pol I and Pol I-associated factors (Drygin et al. 2010, Bywater et al. 2013, Panov et al. 2021). rRNA synthesis generates the 47S rRNA precursor (47S pre-rRNA), which following cleavage, modification, and further processing, forms the mature 18S, 5.8S, and 28S rRNAs. RNA Polymerase II (Pol II) and III (Pol III) transcribe the RP genes required and the 5S rRNA, respectively (Thomson et al. 2013). Their final products translocate from the cytoplasm to the nucleolus, the site of RiBi, and are assembled into the large and small ribosomal subunits together with the mature rRNAs. Mature ribosomes are formed following export to the cytoplasm and further maturation steps.

1.2.1 Ribosome Biogenesis and Connection with Cancer

Nucleoli are membrane-less, sub-nuclear organelles formed at active transcription sites of rRNA tandem arrayed copies or nucleolar organizer regions (NORs) (Pederson 2011, Hein et al. 2013). The shape and size of the nucleoli often correlate positively with rRNA transcription

rates and, as has been suggested by several studies, to the degree of tumor malignancy (Fig. 7) (Derenzini et al. 1998). Consequently, pathologists used nucleolar morphology as a diagnostic marker for malignancy over a century ago when a common feature shared by cancer cells was observed: hypertrophied and abnormal nucleoli, considered to be a hallmark of malignancy (Montanaro et al. 2008).

Pol I machinery and the nucleolus represent a critical sensory hub of intra- end extracellular stimuli (Boisvert et al. 2007, Pederson 2011, Lindstrom et al. 2018, Correll et al. 2019, Weeks et al. 2019, Lafontaine et al. 2021). Multiple growth signaling and stress pathways converge on the nucleolus, assigning it important functional roles such as its regulatory activity of cell cycle progression, cell growth, and cellular stress response (Grummt 2010, Quin et al. 2014). Fast-dividing cancer cells require increased rates of ribosome production to meet the high demand for protein synthesis and sustain the elevated metabolism and proliferation rates (Pelletier et al. 2018). Numerous oncogenic signaling pathways, tumor suppressors, and other nucleolar proteins, commonly affected in human cancers, have been identified to regulate Pol I activity by directly controlling the transcription and activity of the genes involved in rRNA synthesis (Bywater et al. 2013).

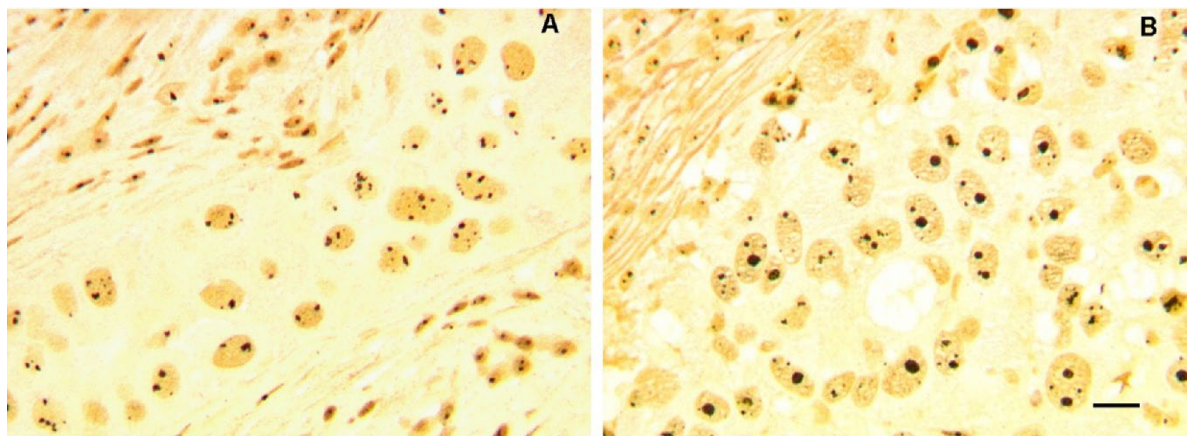

Figure 7. *AgNOR histological staining of the nucleoli shown as brown dots in two breast cancer samples with (A) wt p53 and smaller nucleoli and with (B) mutated p53 and deleted RB, showing enlarged nucleolar area (Derenzini et al. 2017) © 2017 Elsevier GmbH. All rights reserved. Reprinted with permission*

Positive regulators, such as growth factors, activate **oncogenic pathways** and induce rDNA transcription (Stefanovsky et al. 2001). Growth signal-induced activation of the PI3K/AKT, for instance, converges on the mTOR pathway via the c-MYC oncogene. c-MYC promotes Pol I by mediating SL1 recruitment to rDNA loci, while it also induces Pol II and Pol III transcriptional activity to upregulate RPs and 5S rRNA levels (Boon et al. 2001, Gomez-Roman et al. 2003, Arabi et al. 2005, Grandori et al. 2005, Ruggero 2009, van Riggelen et al. 2010). mTOR also activates Pol I via TIF-IA phosphorylation and Pol III via TFIIIB and TFIIIC interaction with 5S rRNA (Mayer et al. 2006, Iadevaia et al. 2012). Upregulation is also mediated by the activation of RAS mitogen-activated protein kinases (RAS-MAPK) that boost rDNA transcription by phosphorylating UBF, SL1, and TIF-IA (Mayer et al. 2006, Drygin et al. 2010, Gaviraghi et al. 2019).

Negative regulators of Pol I activity are **tumor suppressor** genes commonly mutated or deleted in tumor cells (Bursac et al. 2014, Bursać et al. 2021). Normally, tumor suppressors dampen Pol I activity by interacting with PIC components to prevent its formation, such as in the case of p53 interacting with SL1 (Drygin et al. 2010). The Rb protein, when in its active conformation, also binds to UBF and TFIIIB, inhibiting Pol I and Pol III activity, respectively (Voit et al. 1997, Hannan et al. 2000). The tumor suppressor p14^{ARF} has been shown to localize in the nucleolus, interact with the rDNA promoter and associate with UBF, also affecting the formation of PIC and reducing rRNA transcription (Lindström et al. 2000, Ayrault et al. 2004, Ayrault et al. 2006). Furthermore, p14^{ARF} binds to the multifunctional nucleolar phosphoprotein NPM1 and promotes its degradation (Itahana et al. 2003). NPM1 is involved in key cellular processes related to RiBi, such as rRNA processing, ribosome protein nuclear import, ribosome assembly, and ribosome subunit nuclear export. Another vital tumor suppressor, PTEN, interferes with the SL1 and the PIC complex and affects Pol I activity (Zhang et al. 2005). Together, oncogenic activation and tumor suppressor inactivation in cancer cells disrupt the regulatory mechanisms of RiBi, resulting in its upregulation and enabling aberrant ribosome production to support uncontrolled cell growth and division (Hein et al. 2013, Quin et al. 2014).

1.2.2 The Nucleolar Stress Response

Along with the indirect, DNA damage-dependent regulatory effect of p53 stabilization on RiBi, there is a direct connection between RiBi and p53 (Beckerman et al. 2010, Holmberg Olausson et al. 2012, Golomb et al. 2014, Weeks et al. 2019). Impairment of any step in RiBi triggers the nucleolar stress response, a key surveillance

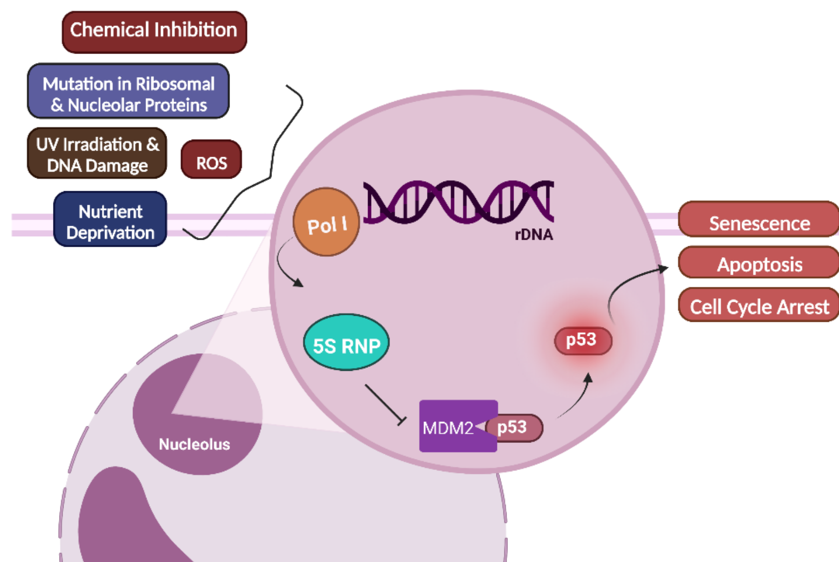

Figure 8. *Insults leading to the activation of the IRBC, leading to p53 stabilization. Created with Biorender.*

pathway, leading to p53 activation and induction of apoptosis, cell cycle arrest, and senescence (Fig. 8) (Fumagalli et al. 2009, Fumagalli et al. 2011, Nicolas et al. 2016, Turi et al. 2018). In detail,

following nucleolar stress, the RPs RPL5 and RPL11 form the 5S RNP complex together with the 5S rRNA, which sequesters the Mouse double minute 2 homolog protein (MDM2) (Zhang et al. 2003, Lindstrom et al. 2007, Macias et al. 2010, Donati et al. 2013, Sloan et al. 2013). MDM2 regulates p53 protein levels by two mechanisms; it binds the N-terminus of p53 and hampers its interaction with the Pol II transcription machinery, resulting in impaired transcriptional activity, while it is also able to modify the stability of p53, acting as an E3 ubiquitin ligase. Following 5S RNP-mediated sequestration, MDM2 is unable to ubiquitinate p53 and drive its 26S proteasomal degradation, resulting in p53 stabilization. The formation of the 5S RNP and its interaction with MDM2 following the RiBi-disrupting event is frequently described as impaired ribosome biogenesis checkpoint (IRBC). Interestingly, p53-independent mechanisms sensing nucleolar stress have also been reviewed, as RiBi impairment affects cell growth in p53 mutant or null cells (Holmberg Olausson et al. 2012, James et al. 2014).

1.2.3 Ribosomopathies

Mutations and defects in factors involved in rRNA synthesis and ribosome assembly may trigger nucleolar stress, p53 activation, and a reduced level of canonical ribosomes, causing cellular malfunction and disease (Nicolas et al. 2016, Aspesi et al. 2019). The consequences of ribosome-related genetic defects are illustrated in a number of congenital syndromes, collectively known as ribosomopathies. Ribosomopathies are associated with loss-of-function mutations or haploinsufficiency in ribosomal proteins or ribosome biogenesis factors. The term was established during the early 2000s, when defects in RiBi factors were discovered in with the inherited bone marrow failure syndromes Dyskeratosis Congenita and Diamond–Blackfan anemia (DBA) (Heiss et al. 1998, Draptchinskaia et al. 1999). Albeit the essentiality of ribosomes in all cellular types, they present with tissue-specific defects associated with upregulated p53 signaling and growth arrest, such as bone marrow failure, anemia, or developmental defects (Yelick et al. 2015, Bowen et al. 2019). The genes affected may code for any of the numerous factors involved in RiBi; however, the association of the clinical phenotypes to the defective genes remains elusive (Aspesi et al. 2019). Thus, diseases are classified into those where (i) ribosomal factors are the target of disease-causing mutations, such as DBA, Shwachman-Diamond syndrome (SDS) or Treacher Collins syndrome (TCS); (ii) defective genes coding factors that, apart from RiBi, are involved in additional cellular functions, and thus, are considered disease modifiers, influencing the severity of the disease phenotypes, such as the X-linked dyskeratosis congenita (XL-DC) and cartilage-hair hypoplasia-anauxetic dysplasia (CHH–AD), and, (iii) the causality of the detected mutations in ribosomal factors to the disease phenotypes are uncertain (Aspesi et al. 2019).

The nucleolar stress response and the 5SRNP-mediated p53 activation are considered one of the primary causes of the pathogenesis in ribosomopathies, with several studies confirming the critical role of p53 in disease models of DBA, SDS, TCS, and XL-DC, among others (Danilova et al. 2008, Jones et al. 2008, Dutt et al. 2011, Fok et al. 2017, Warren 2018, Oyarbide et al. 2020). Moreover, genetic ablation or pharmacologic inhibition of p53 has been shown to rescue or improve the disease-associated phenotypes (Danilova et al. 2008, Jaako et al. 2015, Lau et

al. 2016, Noack Watt et al. 2016, Fok et al. 2017). Another underlying cause of disease pathophysiology is the altered translational capacity following RiBi defects, which results in decreased levels of functional ribosomes and potentially increased ribosome heterogeneity at the RPs or rRNA content (Simsek et al. 2017, Kampen et al. 2019). Limited ribosomes may induce a differential translational landscape, following the competition of specific mRNA populations, influencing cell fate decisions (Aspesi et al. 2019). Additionally, heterogeneous ribosomes with variable composition may exhibit specialization towards distinct mRNA types (Shi et al. 2017), and together with changes affecting speed and accuracy of the translation, could potentially explain the pathogenesis and tissue specificity of ribosomopathies (Kampen et al. 2019, Kang et al. 2021).

Ribosomopathies patients are predisposed to cancer, a paradox termed Dameshek's riddle, where the early hypo-proliferative phenotypes associated with the disease transition to hyper-proliferative cancer phenotypes later in life (Dameshek 1967). While the intermediate events leading this transition and promoting oncogenesis remain to be deciphered, two main oncogenic molecular mechanisms have been proposed. The first and most apparent one is the key role of p53 in the hypo-proliferative syndromes, which could lead to selective pressure for loss-of-function p53 mutations, to promote cell survival, alleviate hematological symptoms, but ultimately lead to an unstable cellular environment and increase the risk for secondary mutations and cancer (Aspesi et al. 2019, Kampen et al. 2019). On the other hand, the altered translational output described above could disrupt the proteomic and cell metabolism balance, while the emergence of non-canonical, specialized ribosomes could promote the translation of oncogenic factors to overcome growth impediments, leading to malignant transformation. Another connection of defective RiBi with cancer has been highlighted by the increasing evidence of oncogenic somatic RP mutations in several cancer types with sporadic etiology. RPL5 loss-of-function mutations are among the most frequent in sporadic cancers of different types and have been suggested to be a haploinsufficient tumor suppressor (Fancello et al. 2017). Overall, these observations support a clear connection between ribosomal defects to cancer and motivate further research to illuminate the molecular mechanisms of oncogenesis.

1.2.4 Targeting The Nucleolus

Diverse triggers induce nucleolar stress, such as nutrient deprivation, changes in REDOX balance, DNA damage, mutations of nucleolar and RPs involved in ribosomal subunits biogenesis, and, most importantly, perturbation of rRNA synthesis and processing (Holmberg Olausson et al. 2012, Chen et al. 2019). The diversity of the stressors highlights the central role of the nucleolus and the importance of protecting the cell against uncontrolled cell growth.

A number of traditional chemotherapeutic drugs commonly used in the clinic have been reported to elicit their anticancer effects partly by disrupting the process of RiBi (Burger et al. 2010, Bruno et al. 2017). Most of these drugs cause DNA damage and activate the DDR pathway. DDR stabilizes p53 by engaging checkpoint kinases, including ATR and ATM, which lead to indirect Pol I inhibition (Kruhlak et al. 2007, Jackson et al. 2009). Albeit the contribution of RiBi impairment to the therapeutic responses shown in the clinic is challenging

to define, these observations intuitively highlight RiBi as an important, often underappreciated target of cancer chemotherapy and lay the foundations for nucleolus-targeted drug development.

1.2.5 RNA Polymerase I inhibition

One of the most eminent strategies to target RiBi is the inhibition of Pol I and the synthesis of rRNA; such an inhibitor could constrain aberrant rDNA transcription in cancer cells with functional p53, promote the subsequent activation of the IRBC and induce the safeguarding functions of p53 (Drygin et al. 2010), or exert antiproliferative effects via p53-independent mechanisms in cancer cells with defective/null p53. Extensive work has been undertaken in the field toward this direction, leading to the emergence of several small molecule inhibitors that preferentially target rRNA transcription by inhibiting Pol I activity, as well as the repurposing of clinically approved compounds.

Most compounds that interfere with Pol I function are planar aromatic molecules capable of DNA intercalation into GC-rich sequences, which are abundant in rDNA (Zisi et al. 2022). Interaction with DNA leads to indirect inhibition of the enzyme by hampering the PIC formation, Pol I holocomplex assembly, or transcription elongation. The most prominent example of this category is **Actinomycin D** (ActD), a licensed chemotherapeutic and the most widely used probe for studying rRNA synthesis. ActD preferentially inhibits Pol I in low concentrations (~5-30 nM), without being genotoxic (Perry 1970). However, increasing concentrations inhibit Pol II and Pol III, and generate double-strand brakes.

The recently discovered BMH-21 demonstrated an improved mechanism of Pol I inhibition. The acridine-like quinazolinone derivative was discovered in a drug screening campaign, searching for compounds that activate p53 in the absence of DNA damage (Peltonen et al. 2010). It was shown to intercalate into rDNA, impair rDNA transcription elongation and induce the proteasomal degradation of Pol I catalytic subunit, RPA194, a feature that makes it one of the most specific rDNA inhibitors to date (Peltonen et al. 2014, Wei et al. 2018, Jacobs et al. 2022). While the molecular events underlying the RPA194 degradation remain to be deciphered, this mechanism has received increased attention as a desirable trait of Pol I inhibition. BMH-21 robustly induces nucleolar stress in low nanomolar concentrations in the absence of γH2AX formation and has shown promising anti-proliferative activity *in vitro* and *in vivo* in a p53-independent manner (Peltonen et al. 2014). While BMH-21 is the most characterized compound among this family, similar activity has been reported by its analogs **BMH-9**, **BMH-22** (also known as **CID-765471**), and **BMH-23** (Colis et al. 2014, Morgado-Palacin et al. 2014, Peltonen et al. 2014).

The **acridine** family also exhibits structural features that can be associated with rDNA transcription inhibition. Namely, acridine analogs **aminacrine** and **ethacridine** were reported to intercalate into rDNA, stabilize p53 in a DNA damage-independent fashion, and dose-dependently induce the degradation of RPA194 (Morgado-Palacin et al. 2014), while in the case of aminacrine, rRNA processing alterations were also observed (Pestov et al. 2008).

Within the same family, **quinacrine**, an authorized drug used against malaria, was shown to exhibit anticancer effects, downregulate the expression of POLR1A and trigger nucleolar stress (Eriksson et al. 2015, Oien et al. 2021). Another group with antimalarial drugs that share rDNA transcription inhibitory effects is that of **4-aminoquinolines**. In detail, our group showed that **Amodiaquine (AQ)** and three of its analogs share a common mechanism with BMH-21 by inducing RPA194 degradation and stabilizing p53 via the IRBC in a DNA-damage independent mechanism (Espinoza et al. 2020).

Despite the promising preclinical activity of the recently discovered RPA194-degrading compounds, none has progressed into clinical testing to date. A possible explanation could be the structural limitations of the molecules that are associated with potential toxicity. In addition, these molecules are often pleiotropic and display ambiguous mechanisms of action (MoA), increasing the probability for off-target effects and highlighting the need for "cleaner" inhibitors. Indeed, a series of optimized BMH-21 analogs have been designed to reduce off-target activity and prevent toxicity and are hopefully expected to progress into the clinical phase in the near future (Dorado et al. 2022). Moreover, alternative drugs targeting Pol I and RiBi via diverse mechanisms are currently investigated in clinical trials and will hopefully provide essential information about the clinical potential of this therapeutic strategy. These compounds are extensively discussed in the review recently published by our group (Zisi et al. 2022).

1.3 Glioma

Classification

Gliomas are malignant primary brain tumors classified into four histological grades, with high-grade gliomas (HGG), being the most frequent and aggressive intracranial tumors in adults (Wen et al. 2020). The WHO grading system has been based on histopathological features such as necrosis, mitotic index, infiltrative growth, and microvascular proliferation, and has been revised to include molecular parameters, new subtypes, and updated diagnostic technologies (Louis et al. 2016, Louis et al. 2021) Grade IV HGG, known as glioblastoma multiforme (GBM), account for ~50% of all gliomas and is associated with the worst survival rates (Grochans et al. 2022). The standard of care for GBM is maximal-safe surgical resection followed by radiotherapy and chemotherapy with an alkylating agent, Temozolomide (TMZ). However, the overall survival lies within 12 to 18 months, with less than 5% of newly diagnosed patients surviving longer than five years.

Molecular Characteristics

GBM displays high inter- and intra-tumoral heterogeneity, hindering the development of effective treatments, and regardless of the significant progress that has been made in the field concerning the biological aspects of gliomas and the improved therapeutic strategies, the survival rate has remained almost unchanged over time. Profiling of the extensive genetic alterations observed in GBM introduced an additional classification system into four subtypes based on the genetic signature of the tumor: the classical, characterized by high EGFR

expression, wild-type TP53, PTEN, and CDKN2A deletions, and Rb inactivation; the proneural, showing TP53 alterations and mutations of the IDH1, PI3K and PDGF genes; the neural, characterized by the normal cell-like gene expression signatures; the mesenchymal characterized by NF1 alterations (Sasmita et al. 2018, Zhang et al. 2020).

Glioblastoma Stem Cells

Moreover, GBM is characterized by angiogenesis, invasiveness, and inherent resistance to radiation and chemotherapy, making recurrence inescapable, a property considered to be fuelled by the existence of glioblastoma stem cells (GSCs). GSCs are believed to exhibit characteristics of self-renewal and pluripotency and were initially described as a subpopulation of cells expressing stemness-related markers capable of tumor initiation and recapitulation of tumor heterogeneity upon orthotopic injection into mice (Singh et al. 2003, Yuan et al. 2004, Gimple et al. 2019). GSCs display distinguished transcriptional, epigenetic, and metabolic features compared to the differentiated tumor progeny, highlighting the challenge of effective targeting of GBM heterogeneous populations and their importance to the rational development of multimodal regimens for the effective management of the disease.

2 RESEARCH AIMS

The overall focus of this thesis was to study a non-conventional chemotherapy target and expand the repertoire of available chemotherapy strategies against cancer. RiBi, especially Pol I, are emerging targets for anticancer therapeutic interventions; however, mechanistic details and biological effects are not fully explored. Also, available drugs and probes, albeit very effective in most cases, often display ambiguous mechanisms of action, limiting the understanding of drug-protein interactions, structure-activity relationships, and the signaling events involved. In this context, our goal was to identify new compounds with Pol I inhibitory activity and characterize their mechanism of action and anticancer effect while comparing them with other inhibitors available. We also sought to understand RiBi-related cancer dependencies, evaluate the effect of RiBi on cancer malignancy, and explore further candidate enzymes for drug development to perturb RiBi.

The specific aims were to:

Paper I: discover new compounds among FDA-approved drugs that target rDNA transcription via Pol I inhibition, and characterize the mechanism of action, structure-activity relationships, and efficacy against cancer cells

Paper II: evaluate RiBi as a cancer therapy target in high-grade gliomas, study the efficacy of pol I inhibition, and identify synergistic treatment strategies

Paper III: explore and characterize alternative druggable enzymatic targets to perturb RiBi

3 METHODOLOGY

This chapter discusses the key methodology applied in this thesis work

3.1 Phenotypic Drug Profiling

An extended toolkit of standard phenotypic and cell-based methods has been employed to characterize the cellular responses to drug treatments, such as antibody-based techniques, brightfield and fluorescence microscopy, and assays quantifying cell viability and death. Detailed protocols can be found in the attached research papers.

The methodology has been divided into three main parts to approach the mechanistic aspects of nucleolar function and translation following chemical RiBi inhibition:

(I) Nucleolar Morphology

Nucleolar size and numbers have been addressed with microscopy-based methods. For the initial qualitative assessment of nucleolar alterations during drug treatments, a conventional brightfield microscope was used to screen for compounds suspected to affect the nucleolus in a non-invasive, time and cost-efficient manner. Standard immunofluorescence (IF) following endpoint treatments, fixation of the cells, and staining with antibodies against protein markers of nucleolar integrity allowed for the thorough analysis of nucleolar effects induced by the chemicals of interest. In detail, stainings for FBL (fibrillarin), UBF, NPM1, and NCL (nucleolin), among others, were used to evaluate nucleolar size, shape, and number upon normal and nucleolar stress conditions. Imaging with a high-resolution microscope (Nikon Ti2, Nikon) allowed for high-quality visualization of the nucleolar morphology. High content imaging with the automated fluorescent microscope (IN Cell Analyzer 2000, General Electric), followed by tailored image analysis using Cell Profiler (Stirling et al. 2021) (v. 4.2.1, https://cellprofiler.org) was also implemented, which allows for the study and quantitation of diverse morphological features, such as nucleolar number, circularity, diameter, signal intensity and nucleolar protein localization in a single cell level that enhanced statistical robustness of our analysis.

Further qualitative information for selected compounds was obtained by silver (AgNOR) staining and Transmission Electron Microscopy (TEM). AgNOR is useful for the analysis of the Nucleolar Organizer Regions (NORs). NORs are defined as chromosomal segments containing proteins with acidic regions that are selectively stained by silver nitrate at low pH (Valdez et al. 1995). Under light microscopy, NORs are visualized as dark dots considered to represent fibrillar centers. AgNOR staining together with TEM provide additional information at the ultrastructural level (Trerè 2000).

(II) rRNA synthesis and processing

Methods quantifying rRNA synthesis and processing were highly important for the conduct of the work presented in this Thesis. Several assays are available, including northern blotting and

metabolic pulse labeling using radiolabelled probes and tracers. However, we had to focus on nonradioactive protocols due to increased safety concerns. To quantify the output of Pol I activity, that is, the levels of the precursor 47S pre-rRNA, as well as those of the mature rRNA species, we have instead used qRT-PCR with primers designed against the External Transcribed Spacer (5'ETS, 3'ETS) and Internal Transcribed Spacer (ITS1 and 2) regions of the 47S precursor, as described in detail in paper I. This approach has been used to measure rRNA synthesis and is considered to sufficiently reflect changes in the levels of 5' ETS of 47S rRNA, which shows a short half-life (Stefanovsky et al. 2016). It is, however, believed that as rDNA is highly abundant in mammalian cells, neither Northern blot nor qRT-PCR analysis can detect rapid changes in synthesis rates or accurately discriminate between nascent RNA from steady-state levels, leading to the development of nonradioactive, metabolic labeling protocols (Burger et al. 2016). Thus, we have also employed the Click-It Chemistry by pulse-labeling cells with 5-Ethynyl-uridine (5-EU), a uridine analog incorporated into nascent RNA and used to measure the *de novo* RNA synthesis in replicating cells (Jao et al. 2008). Following pulse labeling, cells are fixed and fluorescently labeled via the Cu(I)-catalyzed click chemistry reaction. High-content imaging followed by image analysis enables the quantification of global RNA synthesis signal intensity, not only in the nucleus but also in the nucleolus, where the signal is usually stronger and can be more robustly segmented by co-staining with nucleolar proteins such as FBL, given that rRNA synthesis accounts for about 30% of the total gene transcription in proliferating cells (Moss et al. 2007, van Sluis et al. 2017, Bryant et al. 2022).

An additional method we used to quantify Pol I activity involved a luciferase reporter assay. In detail, we transiently transfected the pHrD-IRES luciferase reporter plasmid containing human rRNA promoter spanning −410 to +314 bp (pHrD-IRES-Luc), together with a Renilla luciferase reporter plasmid driven by HSV-tk promoter (pRLTK), as an internal control (Ghoshal et al. 2004). Thus, the transcriptional activity of the rRNA promoter is assessed by measuring the Firefly signal, while the transfection activity is normalized by the Renilla signal, providing a Firefly:Renilla ratio that indicates the activity rate.

rDNA promoter occupancy following drug treatments has also been assessed by the chromatin immunoprecipitation assay, focusing on the association of members of the PIC complex with rDNA (Paper I). Moreover, an RNA immunoprecipitation protocol was utilized to reveal protein interactors involved in the early rRNA processing steps (Paper III).

(III) Translation Monitoring

Inhibition of ribosome biogenesis may cause downstream perturbations in the translation machinery. Therefore, we have applied a diverse methodology to monitor changes in protein translation, an important aspect of paper III. To address the global translation rate, we used **puromycin-based protocols**. Puromycin is a natural aminonucleoside antibiotic and a structural analog of aminoacyl tRNAs. It incorporates into the nascent polypeptide chain, preventing translation elongation and causing premature termination (Nathans 1964); when used in low concentrations, it reflects the rate of ongoing mRNA translation in the cells. (Schmidt et al. 2009). We initially applied a standard immunochemical method, pulse-chasing

cells with puromycin, lysing them, and detecting the puromycin-labeled protein levels by immunoblot using an anti-puromycin antibody. To study translation on the single cell level, we pulsed cells with an alkyne analog of puromycin, **O-propargyl-puromycin** (OP-puro or **OPP**) that forms covalent conjugates with nascent polypeptide chains, which can be visualized by Click-It chemistry (Liu et al. 2012). Fluorescent high-content microscopy and image analysis allow for robust OPP signal quantification following drug treatments, making the assay one of the easiest and most reliable methods to monitor protein synthesis. As an alternative method to validate our findings, we used a nonradioactive approach similar to the traditional ^{35}S-methionine-based metabolic labeling method (Bonifacino 1999). The assay employs an alkyne-containing methionine (Met) analog called **L-homopropargylglycine (Hpg)**. Hpg competes with Met and effectively incorporates into the polypeptide chain, as mammalian cells do not synthesize Met (van Hest et al. 2000). Following that, newly synthesized proteins are labeled with Click-It chemistry and quantified by microscopy as described before (Beatty et al. 2006). While this method is useful for monitoring protein translation levels, it should be noted that it generates full-length labeled proteins and not nascent peptide chains. Met-free culture media must also be used to enable sufficient incorporation and labeling.

Finally, to get an in-depth understanding of translational changes, we performed **polysome profiling**, a sucrose-gradient-based technique that separates polysome-associated and thus effectively translated mRNAs from the poorly translated ones associated with monosomes (Gandin et al. 2014). The term polysome refers to the number of ribosomes bound to a certain mRNA during translation elongation. The method includes immobilization of the ribosomes on the mRNA using chemical compounds that inhibit translation elongation and cell lysis and separation on sucrose gradients by ultracentrifugation. Lysates are subsequently fractionated based on the number of interacting ribosomes. The RNA-containing fractions can be further analyzed and provide information on the mRNA distribution changes following drug treatments or genetic manipulation, either by qRT-PCR or deep sequencing, as discussed in detail in Paper III. Alternatively, the method can be coupled with immunoblotting or proteomics to detect proteins present in the fractions that are either associated with the ribosomes or are part of initiation complexes (Chassé et al. 2016). The drawbacks of this assay are the technically challenging, multistep, and often non-standardized protocols leading to highly variable and low throughput data, as well as the high amounts of starting material (cell extracts) required as an input. Overall, this method provides information on the translation levels and differential translational programs that may be executed following external stimuli and stress.

3.2 Drug Synergy Studies And High Throughput Screening

To detect, **quantify and characterize drug synergies**, we have utilized a broad range of cell-based assays with orthogonal readouts, starting with measuring cell viability assays. The implementation of a viability assay that is robust, inexpensive, and scalable can significantly increase the data output and enable the parallel testing of as many drug pairs and ratios as possible. Therefore, we selected the **resazurin assay** to evaluate drug cytotoxicity and screen

for synergistic effects. The assay is based on mitochondrial respiratory chain-mediated reduction of the non-fluorescent, blue resazurin salt to the red fluorescent dye resorufin following 2-6 hours of incubation (Czekanska 2011). The fluorescent signal intensity is measured using a plate reader with an excitation/emission filter of 560/590nm and is proportional to the numbers of metabolically active cells, while it has increased sensitivity even in low cell numbers. However, the cell type, density, and incubation time can influence the performance in many ways, and thorough optimization is needed. Additionally, further methods are required to confirm the cytostatic or cytotoxic effects of the drug treatments in question.

We used a checkerboard matrix experimental layout to detect synergistic effects among two drug pairs, which allows for parallel testing and comparison of the single agents alone, the single agents combined in different ratios, and serial dilutions of those ratios. The concentrations range for each compound is determined based on the recorded IC50 values.

The **drug combination data** was analyzed for synergistic or antagonistic effects using a web-based application called **SynergyFinder** (https://synergyfinder.fimm.fi/), by which the overall synergy score is calculated as the deviation of phenotypic responses compared to the expected values, averaged over the full dose-response matrix(Ianevski et al. 2017). SynergyFinder provides four models for implementing synergism, including the most popular BLISS independence model (Bliss 1939). Several models for synergism analysis are described in the literature; however, they are highly dependent on the dose-response curves of the single agents to be combined and, therefore, are not widely applicable (Meyer et al. 2020). The suitability and robustness of the models are the subjects of intense discussion, with some of the studies suggesting that more than one approach should be used based on drug-, cell- and assay-specific characteristics since there is no appropriate reference methodology available (Foucquier et al. 2015). A recent study has challenged further the traditional approaches to address synergy, as insufficient to discriminate between different types of synergy termed synergistic potency (limit toxicity by minimizing doses) and synergistic efficacy (improve outcomes by an escalating effect) (Meyer et al. 2019). Ongoing research has promoted the development of new methods by using computational tools, artificial intelligence, and multi-omics data, which are expected to change the analysis framework currently available (van Sluis et al. 2017, Kumar et al. 2022, Rani et al. 2022).

Nevertheless, orthogonal methods to validate the synergistic or additive effects observed are highly recommended to confirm the initial predictions made by the applications and models mentioned above. Herein, the initial synergy scores obtained by SynergyFinder were confirmed by performing **complementary viability assays** (e.g., Cell Titer Glo) to quantify **IC50 shifts and AUC area**, cell death by measuring apoptosis induction, as well as by addressing the combinatorial effects on biological features including colony forming capacity and migratory potential using tailored assays, as described in detail in paper II.

To further explore synergistic patterns across diverse chemical compound libraries, we designed a **high throughput drug combination screening.** The purpose of the high

throughput screening (HTS) methodology is the rapid and accurate screening of large numbers of small-molecule compounds to detect candidates with desirable pharmacologic effects, termed “hits” (Malo et al. 2006). It has become an integral part of biomedical and pharmaceutical research over the last 20 years, providing tool compounds that have assisted basic research as well as lead compounds under pre-clinical and clinical development (Macarron et al. 2011). There is a long list of experimental pipelines, hit identification strategies, data processing, and validation routines available, selected based on the final goal of the screening. Factors that cause concern during the screening conduct are liquid handling errors, positional effects within the plates, plate batch effect, and thresholding of background noise, among others. To prevent or monitor such factors of variability, rigorous optimization is required before the run, while statistical tools available can assess the quality of the readout and detect with confidence the accuracy of the hits, minimizing the rates of false-positive and false-negatives.

In our case, we chose a **cell-based, phenotypic assay on a 384-well format, measuring cell viability** upon treatment with compounds from a library with 500 oncology compounds either used in the clinic or being under clinical investigation. The cell model was chosen to be A172, an adherent glioma cell line with a fast proliferation rate in standard growth conditions in serum-containing culture media. The selection of an easy-to-work-with cell line helps the liquid handling stage and can reduce well-to-well deviations due to issues such as uneven cell seeding or cell detachment. The resazurin assay was used as the readout, given its HTS amenability and cost-effectiveness. As a no-cell control, we used a biocide called benzalkonium chloride (0% viability), while DMSO or DMEM only were used as untreated controls (100% viability). Following assay miniaturization, extensive assay optimization was performed to define the best incubation window, cell density, and equipment used and evaluate the overall assay performance. The **statistical parameters used** were the signal-to-background ratio (S/B), the coefficient of variation of signal and background (CV%), and the Z’ factor.

The S/B ratio, [$\mathbf{S/B=Mean_{signal}/Mean_{background}}$], is calculated by dividing the signal intensity mean by the background intensity mean. It indicates how well positive and negative controls are separated and is useful in the initial step of assay development (Macarrón et al. 2011). However, as this parameter is not affected by variability, it needs to be complemented by additional indicators (Zhang et al. 1999). Variability provides information on the stability of the assay and the precision of the equipment used (Macarrón et al. 2011).

CV% [$\mathbf{CV=100\times SD/Mean}$ (%)] is the ratio of the standard deviation to the mean and represents a measure of relative variability, with higher CV% values indicating a broader data dispersion around the mean (Macarrón et al. 2011).

Finally, the Z’ factor [$\mathbf{Z'=1-3\times(SD_{signal}+SD_{background})/|Mean_{signal}-Mean_{background}|}$] combines the signal window and the variability, the most important factors to assess the quality of an assay, and has been widely used since its publication (Zhang et al. 1999). Z′ factor ranges from 1 to 0, where signal and background measurements start to overlap. Generally, an assay with Z’> 0.4-0.6 is considered of acceptable quality (Macarrón et al. 2011).

Following assay optimization, we designed the **experimental layout** to plot two dose-response curves per compound tested; each compound was tested in five doses, combined with a constant BMH-21 concentration or DMSO. **IC50s shifts and AUC** were calculated for each curve, while the compounds were ranked based on the AUC% difference between the curves of the compound combined with DMSO and the compound combined with BMH-21. As a positive synergy control, we used Temozolomide with BMH-21, based on the data obtained from the previously-conducted synergy analysis. A more detailed description can be found in Paper II, Figure 4. **Hit compounds** were selected and re-evaluated by performing with a 10-point dose response using the same parameters, together with compounds dispensed in a plate that displayed higher variability. The final lead compounds were independently validated in a low throughput format using additional viability assays and more cell lines, while synergy was further analyzed for the **lead compounds** using a checkerboard matrix and Synergyfinder. The initial findings were followed up by addressing biological features such as colony forming capacity and migratory potential using tailored assays, as well as cell death by measuring apoptosis induction as described in detail in paper II.

3.3 Experimental Models In The Study Of High-Grade Glioma

3.3.1 Cell-based Models

Over the past decades, extensive research efforts aimed to create representative experimental models for the study of gliomas. As in many cancer types, cell culture has played a fundamental role in understanding glioblastoma biology (Gómez-Oliva et al. 2021). The most commonly used glioblastoma cell lines that include most of the cell lines used in Paper II have been isolated from tumor specimens and grown adherently in serum-containing culture media (Giard et al. 1973, Westermark et al. 1973, Bigner et al. 1981). These cell lines, frequently called established glioma lines, are commercially available, cost-effective, and characterized by a rapid and unlimited supply of cells, while not posing any major ethical concerns. Thus, they represent a simplified, reference experimental model for the study of glioblastoma.

However, these cell lines cannot mirror the complex microenvironment in the brain. An additional consideration is the serum-based growth conditions applied for these lines; serum is known to induce differentiation of neural stem cells, which share similarities with cancer stem-like cells present in gliomas (Gage et al. 1995, McKay 1997). Also, serum-cultured glioma cells have been observed to be unresponsive to differentiation stimuli (Ledur et al. 2016, Ledur et al. 2017). At the same time, they show genomic alterations that are not present in the original tumors and have altered gene expression patterns. On the contrary, maintenance of glioma cultures in serum-free neurobasal medium supplemented with EGF and FGF, growth factors that support neural stem cell growth, has been shown to retain the phenotypic, histological, and genetic features of the parental tumors; these include invasiveness, migratory, self-renewal, and tumor-initiating capacity, all features considered to be characteristic of the glioma GSCs (Lee et al. 2006).

Nevertheless, while switching from serum to serum-free culture conditions has been extensively described in the literature, there are concerns that this practice may induce major alterations in gene expression and phenotypic features that do not reflect the original tumor conditions (Gómez-Oliva et al. 2021). Finally, misidentification issues of established glioma cell lines distributed by the ATCC that emerged 20 years ago revealed that certain lines widely used in published studies were genetically non-distinct from each other, leading to confusion over their authenticity and origin (Timerman et al. 2014, Allen et al. 2016). Overall, this system has contributed significantly to understanding glioblastoma pathophysiology and represents a great and rapid tool for early drug discovery and development projects. Therefore, we selected this system to study the pharmacological effects of BMH-21 and screen for drug combinations. However, given the limitations described above and depending on the research question, results obtained by using this system should be interpreted with caution and validated using alternative models, such as patient-derived GSC lines.

GSC lines are derived from tumor tissue resected during surgery and directly cultured non-adherently as neurospheres in a serum-free, conditioned culture medium called neurobasal (Galli et al. 2004, Wenger et al. 2017). These cells can be maintained in vitro for a longer time, they are capable of indefinite self-renewal and differentiation, and they express Neural Stem Cells (NSC)-associated genes such as NESTIN and SOX2 that are known to promote NSC phenotypes (Hattermann et al. 2016). They are tumorigenic, mimic the invasive behavior of GBM, and are highly resistant to therapeutic treatments, and thus better reflect the clinical picture (Pollard et al. 2009). Nevertheless, GSC lines are not as widely available as they often cannot be isolated, expanded, and distributed without ethical permission and/or material and transfer agreements. We, therefore, collaborated with a research group that has isolated and characterized a GSC panel derived from pediatric GBM, and this helped us validate our main findings obtained using the established GBM lines in Paper II. Due to the technical challenges and cost of the GSC cultures compared with the established GBM lines, as well as the cellular characteristics such as slower duplication times or neurosphere growth, the downstream applications that could be performed within the timeframe of the study were limited but enough to confirm the most important data shown in Paper II. Optimizations described in the literature have enabled high-throughput applications and drug screenings using GSC lines, which are anticipated to advance the field further and provide unexplored treatment strategy directions (Pollard et al. 2009, Johansson et al. 2020, Taylor et al. 2020).

3.3.2 *In Vivo* Models

Model organisms are used to overcome the limitations of in vitro, cell-based systems and mirror the physiological disease state more effectively. The most commonly used organism in the study of brain tumors is the **mouse** (*Mus musculus*), with the standard model to be the **xenograft transplantation** of human GBM cells into immunocompromised mice by subcutaneous or intracranial injection (Liu et al. 2021). However, the model has been extensively debated regarding its suitability to mimic the tumor microenvironment depending on the injection site, and the inflammatory responses are known to play a critical role in the

biology of the tumor (Janbazian et al. 2014, Gómez-Oliva et al. 2021, Liu et al. 2021). Given these limitations, combined with the technically challenging and resource-intensive procedures involved in mouse studies, we decided to use an alternative, non-murine model system that enables high-throughput investigation fast and cost-efficiently.

The model organism of choice is **zebrafish** (*Danio rerio*), which due to its considerable genetic, molecular, and histological homology with humans has emerged as an invaluable tool in many research fields, including developmental, toxicology, and cancer research (Driever et al. 1994, Dai et al. 2014, Hason et al. 2019). Zebrafish embryos are easily obtained and maintained with low cost, develop rapidly, and can be analyzed in multi-well plates, enabling high throughput applications (Sarmiento et al. 2022). Additionally, embryos can be genetically manipulated, and by being optically transparent, they can be monitored by live fluorescent microscopy. Importantly, they do not display functional adaptive immunity up to 2-3 weeks after fertilization, facilitating the transplantation of human tumor cells into zebrafish.

Embryos develop a functional nervous system within the first 48 hours, while the primary regions and subdivisions of the brain, cell types, gene expression, and differentiation patterns, connectivity, and signaling pathways are highly conserved (Reimunde et al. 2021, Sarmiento et al. 2022). Additionally, evidence shows that they start to display premature blood-brain barrier (BBB) function already at 72hpf (Zeng et al. 2017, Quiñonez-Silvero et al. 2020), making them suitable *in vivo* modeling of brain tumors.

There are several zebrafish patient-derived xenografts (zPDX) reported in the literature able to sufficiently recapitulate the human disease, where patient-derived or GSC GBM cells are microinjected into embryos a few hours up to 6 days post fertilization (Pudelko et al. 2018, Vargas-Patron et al. 2019, Almstedt et al. 2021, Ai et al. 2022, Larsson et al. 2022). The cells are fluorescently tagged by genetic manipulation or membrane dyes, and the engrafted tumors are visible within 24 hours post-injection (hpi), and the growth and response to treatments can be easily monitored.

In Paper II, we employed an orthotopic glioblastoma model published by Pudelko et al. (Pudelko et al. 2018). Established GBM cells have been engineered to express luciferase and a fluorescence tag, so we can monitor the tumor growth by live microscopy and, following the treatment's endpoint, quantify the luminescent signal emitted by lysing the embryos and incubating them with a luciferase substrate. Embryos are injected within the first 6hpf, and 24hpi, embryos with successful tumor engraftment are screened and subjected to 48- or 72-hour treatment with chemical compounds in a 96-well format. Embryos are then imaged with automated fluorescent microscopy, and tumor development is followed up every 12 hours up to the treatment endpoint. Subsequently, embryos are lysed, and the luminescence assay is performed. The model has obtained valuable quantitative and qualitative information on tumor growth and GBM cells' response to the treatments of choice.

While zPDX has been very efficient and advantageous *in vivo* approach, there are certain limitations to be taken into consideration. One important aspect is that the fish is maintained at

33-34ºC, which could affect the injected tumor cells (Sarmiento et al. 2022). For that reason, it is important to monitor GBM cell viability *in vitro* under different temperature conditions and proceed with the most suitable line. Additionally, the preparation and microinjection of GBM cells are technically challenging, include multiple tests, and require rigorous optimization. The procedure often results in variable microinjection success rate and embryo survival, which, together with variations in tumor engraftment and development among embryos, affect the assay's reproducibility. An advantage is that embryos can be microinjected simultaneously, and experiments are easier to repeat, producing multiple technical and biological replicates and thus increasing the statistical power. Moreover, the assay is performed within five days post fertilization, and while it can be prolonged, an ethical permit is required. Finally, the lack of an adaptive immune system during the first weeks post-fertilization, while advantageous for xenotransplantation, creates a different immune microenvironment and limits the use of the model in studies focused on tumor microenvironment interactions.

3.4 Early Drug Efficacy And Safety Testing Using The Zebrafish Model

3.4.1 BBB Penetration Model

BBB is a biological barrier preventing xenobiotics from entering the brain parenchyma and introduces a major obstacle to brain drug delivery (Kadry et al. 2020, Mo et al. 2021). It is a lining formulated by microvascular endothelial cells surrounding the brain and spinal cord cerebral capillaries. It has distinct morphological and structural features, such as tight junctions that seal the paracellular space between neighboring endothelial cells and prevent penetration of bulky (>400 Da), polar molecules into the brain (Stanimirovic et al. 2015). Additionally, it regulates the active transport of nutrients or block potentially harmful substances, including P-glycoprotein (P-gp) efflux pumps that effectively return substrate substances to the blood circulation. It is estimated that less than 1-2% of small molecules can penetrate the CNS, depending on their physicochemical and structural characteristics (Fleming et al. 2013, Mo et al. 2021).

BBB is considered a critical limiting factor in drug development against brain tumors, even if its integrity is disrupted in such pathological conditions; evidence shows that the disruption is highly heterogeneous, preventing the even delivery of effective concentrations to the tumor (Mo et al. 2021). Extensive research has been conducted on developing relevant *in vitro*, *in vivo*, and computational models to predict drug permeability at early drug development stages (Stanimirovic et al. 2015). While *in vitro* models provide an adequate early evaluation of BBB permeability, they are generally labor-intensive and display heterogeneity and variable barrier tightness. In contrast, computational software-based on quantitative structure-activity relationship (QSAR) and/or physicochemical descriptors can provide an initial evaluation of chemical libraries time- and cost-efficiently (Alsenan et al. 2021). The majority of the algorithms available calculate a probability score for each compound and classify compounds as BBB+ (permeable) or BBB-(non-permeable). Even if there are some deviations between different algorithms when predicting compounds with low permeability, this approach is

acceptable to high accuracy rates (Alsenan et al. 2021). However, the need for *in vivo* pre-clinical evaluation of candidate compounds remains.

Zebrafish is increasingly used as a BBB-permeability model, given the notable anatomical and structural similarity of its BBB to that of humans (Stanimirovic et al. 2015). In detail, similarities have been observed in the neurovascular cellular composition, tight junction protein expression, and active transport mechanisms (Fleming et al. 2013, Kulkarni et al. 2017, Reimunde et al. 2021). Notably, the expression of certain tight-junction proteins indicative of BBB maturations has been observed already at 3dpf, (Jeong et al. 2008, Kulkarni et al. 2017, Zeng et al. 2017), however, it is considered not fully functional at this point with data indicating that size-dependent exclusion of compounds takes place after 10dpf (Fleming et al. 2013).

The majority of these models include drug administration to adult zebrafish or embryos and, depending on the study, collection of head/brain tissue and/or trunk tissue and blood samples. The samples are, after homogenization and preparation, subjected to liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis to detect the compounds of interest. However, the protocols are highly variable, and there is no standardized method.

In Paper II, BBB permeability of BMH-21 has been an important question to ensure effective penetration and, thus, suitability to be used for the treatment of high-grade tumors. Considering the parameters discussed above, we initially approached this question by computational prediction tools, such as the algorithm developed by Gupta *et al* (Gupta et al. 2019). Subsequently, we evaluated the predictions in vivo by developing an adult zebrafish BBB model to ensure optimal BBB functionality, where we treated zebrafish with the maximum tolerated doses (MTD) of the compounds in question for four hours. Following treatment, fish were anesthetized and sacrificed. Brains were surgically removed, and trunk tissue was collected for normalization purposes. After homogenization, the samples were analyzed by LC/MS/MS, together with water tank samples from each treatment group. As technical issues occurred with the trunk tissue homogenates that prevented their subsequent analysis, we normalized the concentrations found in the brain tissue with the drug concentration measured in the water tank and calculated a relative permeability score. While optimization is ongoing, the model could be highly informative and potentially superior to similar rodent models, given the cost and time required in the latter case.

3.5 Zebrafish As A Screening Model For Drug Toxicity

Pre-clinical safety testing is a fundament part of drug development to prevent the entry of compounds with toxicity potential into clinical trials. Traditionally, most pre-clinical safety studies are conducted primarily in rodents; however, in line with the 3R principles (replacement, reduction, and refinement) of animal testing, zebrafish is becoming increasingly popular as it can provide a robust pre-clinical safety model and bridge the *in vitro* and *in vivo* testing (Cassar et al. 2020). It is highly used to address embryonic and fetal developmental toxicity (EFD) as it rapidly covers the organogenesis process and enables visceral observation due to its transparency (Miyawaki 2020). In EFD assay, the embryos are treated with the

desired compounds, and following exposure, visual examination of the skeleton, viscera, and external surface is performed. During the organogenesis period, more features can be observed, such as the heart morphology, heartbeat and the presence of cardiac edema, face and body shape, and somites. Specific models for neuronal, liver, kidney, and cardiac toxicity have also been developed and mostly involve transgenic lines and other reporter genes that can further highlight organ-specific morphological and/or functional changes by fluorescence microscopy (Zon et al. 2005). For example, by employing the transgenic *tg(elavl3:eGFP)* line, post-mitotic neurons can be fluorescently visualized and used in neurotoxicity studies (Park et al. 2021). Additionally, using a line with heart-specific expression, the morphology of zebrafish heart can be assessed and subjected to additional evaluations, such as heart rate measurements (Peng et al. 2013). Both lines are being evaluated for Paper II to evaluate potential BMH-21-induced neuro- and cardiotoxicity. We also performed the EFD assay to obtain the lethal dose (LD) and maximum tolerated dose (MTD) and observed fish morphology during 48- and 72-hour treatments before proceeding to subsequent analyses.

3.6 Ethical Considerations

Early translational cancer research, comprised by the stages of basic and pre-clinical research, is of utmost importance in drug discovery, and its contribution to the development of improved treatment options for cancer patients has been critical over the past decades. In contrast to the clinical stages of drug development, early translational research does not involve experimentation in human subjects; however, there are still important ethical considerations that need to be taken into account during the development of the research hypothesis and the design and implementation of the experimental part, especially in regard to the research models that need to be employed.

During the development of the research hypothesis, one has to evaluate the potential benefits and costs. A research project should aim to provide new knowledge and scientific advances that can potentially move clinical research forward to cover the unmet medical needs in cancer therapy. Even if cancer treatment represents a highly unmet medical need, survival rates and treatment outcomes vary significantly across various cancer types, highlighting the need to equally distribute financial and human recourses across cancers with the lowest survival rates, such as brain tumors and especially GBM. This work aimed to explore a novel cellular target for drug development, identify new chemical modulators and apply existing ones to uncurable cancers such as gliomas, revealing new dependencies of this cancer type and providing a proof of concept for future treatment strategies that could make the transition from the bench to the bedside. Thus, translational research in glioma is considered highly justified.

The second level of ethical considerations regards the research project's design and implementation. Different research models can be employed to answer specific scientific questions, from mammalian cell lines and organoids to patient tissue material and animal models, and the selection should be carefully considered based on the availability for specific cancer types, the suitability, and the financial and ethical constraints that the model might have.

The projects described within this Thesis work are primarily based on established cancer cell lines distributed by ATCC to evaluate all the biological aspects surrounding the scientific questions that have been set. Additionally, pediatric patient-derived cells have been used to evaluate drug response and include the factor of tumor heterogeneity and cancer stem cell-associated phenotypes. These cells were donated from children diagnosed with a high-grade tumor who underwent brain surgery; signed informed consent forms were obtained by the parents. The material is covered by regional ethical approval of the Review Board of Gothenburg. Due to ethical constraints, cells could not be transferred and tested on site; therefore, all experiments were performed at the site where cell cultures were initiated.

Since a major part of the project is the drug response of glioma cells to specific drugs, it is crucial to mimic the microenvironment and the physiological conditions of the tumor so that we can validate the findings obtained *in vitro*. Therefore, we employed an orthotopic glioblastoma model in zebrafish embryos, a model that does not require ethical approval, given that the whole experimental pipeline will be performed in zebrafish embryos younger than five days. Toxicity assessment employed embryos up to 72hpf and lies within the same category. However, 83-days-old adult zebrafish were used for the BBB permeability model, an experiment covered by ethical permit. The experimental work has been carefully designed based on the 3R guidelines (Replace, Reduce, Refine) and performed according to the regional guidelines for animal experimentation.

4 SUMMARY OF RESEARCH PAPERS

4.1 Paper I: The Antimalarial Drug Amodiaquine Stabilizes p53 through Ribosome Biogenesis Stress, Independently of its Autophagy-Inhibitory Activity

Drug repurposing, that is, the identification of new uses for approved drugs, has attained increasing popularity, providing the opportunity to assign new indications to 'old drugs' while shortening the clinical development stage and the high budget associated with it (Ashburn et al. 2004). In this study, we identified that the FDA-approved antimalarial drug Amodiaquine (AQ) inhibits Pol I activity by degrading the catalytic subunit of the enzyme, inducing nucleolar stress and stabilizing p53 in the absence of DNA damage, in a similar fashion to BMH-21.

AQ is structurally related to Chloroquine (CQ), another clinically used antimalarial drug with immunomodulatory applications. The compounds belong to the family of 4-aminoquinolines, and together with other family members, they exhibit autophagy inhibitory capacity. However, we showed that CQ neither inhibits rDNA transcription nor induces nucleolar stress at the concentration range tested, even though it showed some effect in much higher concentrations tested (>30μM). This could potentially explain the consistently superior anti-proliferative effect observed by AQ in a broad panel of cancer cell lines. Notably, the most significant difference in GI50 was observed in colorectal cancer (CRC) cell lines, a cancer type considered dependent on RiBi (Bruno et al. 2017).

We then focused on characterizing the specificity of the mechanism observed and investigated whether the degradation of RPA194 could follow autophagy inhibition. Interestingly, inhibiting autophagy did not rescue the observed nucleolar effect, suggesting that Pol I inhibition and autophagy occur simultaneously and independently. Subsequently, we performed a transcriptomics analysis of cells treated with AQ, CQ, and BMH-21 to compare shared and unique transcriptional perturbations. AQ-induced transcriptional changes overlapped with most of those induced by CQ, while almost 35% of differentially expressed genes were shared between AQ and BMH-21, supporting the dual AQ mechanism proposed.

Despite the structural similarities between AQ and CQ, AQ has a p-aminophenol moiety that undergoes cytochrome P450-dependent bioactivation to a reactive quinonimine intermediate. The reactive metabolite generates protein adducts suspected to induce reported side effects such as agranulocytosis and idiosyncratic hepatotoxicity, which have limited AQ's clinical applications. Chemical compounds with similar protein adduction capacity have been shown to induce nucleolar stress (Wang et al. 2016). To assess the possible contribution of the reactive metabolite to the nucleolar impairment, we synthesized an AQ analog by removing an -OH group, rendering the molecule incapable of reactive bioactivation (DH-AQ). The analog showed RPA194 degradation, nucleolar stress, and p53 stabilization, although slightly less effectively than AQ, while abrogating the protein adduct formation. We also tested the nucleolar effects of N-desethylamodiaquine (DE-AQ), one of the main AQ metabolites following CYP450, which also showed comparable nucleolar effects to DH-AQ, indicating

that the activity is retained following CYP450 activity. Finally, by screening other structurally related to AQ compounds for Pol I inhibitory capacity, we identified aminopyroquine (ApQ), which induced RPA194 degradation at even lower concentrations than AQ. ApQ also contains a p-aminophenol moiety and potentially shares a similar toxicological profile with AQ, motivating us to synthesize an analog (DH-ApQ) by removing an -OH group, as done for AQ. The final product could still induce nucleolar stress, however, with a lower potency than its mother compound.

Discussion

Pol I is considered a promising target to inhibit the commonly upregulated transcription of rDNA in cancer. Several drug candidates have emerged over the last ten years, but only a few have reached the clinical development stage, showing variable results due to toxicity or off-target effects (Zisi et al. 2022). While accumulating preclinical evidence shows the efficacy and relevance of RiBi for tumors with certain genetic backgrounds, the lack of specific, clinically-available Pol I inhibitors limits the implementation of this therapeutic approach in a clinical setting.

In this study, AQ was found to degrade RPA194 and inhibit rDNA transcription, the first FDA-approved compound reported to have such a mechanism of action. AQ was also shown to share significant similarities with BMH-21, one of the most effective Pol I inhibitors with robust preclinical anticancer activity. While higher concentrations (~10fold) are required to achieve cellular effects comparable to BMH-21, the availability of a clinical alternative could be of potential interest in proof-of-concept studies. The compound was particularly effective in CRC cell lines, supporting previous data that also show an increased sensitivity of CRC to RiBi inhibitors.

The autophagy-inhibitory activity of AQ could enhance the anticancer effects observed by RiBi perturbators. In support of this notion, several studies have evaluated the co-administration of chemotherapeutic agents with autophagy inhibitors to prevent resistance development mediated by autophagy induction (Mohsen et al. 2022, Salimi-Jeda et al. 2022). Most importantly, there is evidence of autophagy induction following RiBi perturbation in solid tumors, making a molecule with such dual activity highly relevant to target these tumors (Liao et al. 2021).

Following thorough mechanistic characterization, we performed limited structure-activity relationship studies by synthesizing and testing closely related analogs to address the critical structural characteristics for Pol I inhibition and showed a few changes that can improve or decrease the potency. The findings support further chemical optimization that could potentially enrich the current chemical space of compounds Pol I inhibitors and provide new candidates for preclinical testing and, hopefully, clinical development.

4.2 Paper II: Small Molecule-Mediated Disruption of Ribosome Biogenesis Synergizes With FGFR Inhibitors to Suppress Glioma Cell Growth

High-grade gliomas are malignant primary brain tumors lacking treatment options that come with long-term survival, which for GBM rarely exceeds 14 months. In this study, we sought to understand whether HGG could be a suitable cancer type for RiBi-targeting therapy and evaluate the effects of Pol I transcription inhibition in glioma biology. We first showed a positive correlation between histological grade, RiBi rate, and worse clinical outcomes by conducting an *in silico* analysis of transcriptomics data from gliomas and normal brain samples within the TCGA and CGGA patient cohorts. Based on these findings, we hypothesized that high-RiBi tumors might be susceptible to Pol I blockade. Comparing a set of the most known investigational Pol I inhibitors for anticancer effects on glioma cells, we identified BMH-21 as the most potent in reducing cell viability with GI_{50}s at the nanomolar level. The drug triggered the downregulation of RPA194, prevented rDNA transcription, and induced nucleolar stress, cell cycle arrest, and apoptosis in a panel of established glioma cell lines, independently of the p53 status. It also showed robust anti-proliferative activity in zebrafish xenografts in low concentrations (<0.2 µM), reducing glioma cell mass size size by ~50%.

To evaluate the therapeutic window of BMH-21, we treated normal human astrocytes (NHA) with increasing concentrations. NHA showed a 7- to 10-fold higher GI_{50} value compared to the HGG lines, and they were generally more tolerant, as shown by evaluating p53 stabilization and apoptosis. Moreover, using zebrafish embryos for toxicity assessment, we identified the maximum tolerated dose to be 3 µM and the lethal dose 6 µM, indicating a satisfactory therapeutic window for BMH-21 to justify its further characterization. Importantly, our data suggested that BMH-21 is BBB permeable and thus highly relevant in treating brain tumors.

Given the importance of GSC in tumor heterogeneity, resistance and recurrence, we addressed the expression of stem-cell-associated genes following BMH-21 treatment. We observed a reduction of SOX2 at mRNA and protein levels, even though a higher dose was required in the presence of NSC complete medium compared with serum-based cultures.

To potentiate RiBi blockade monotherapy and hopefully prevent resistance development, we studied drug candidates that could synergize with BMH-21. We started with TMZ, the standard of care for glioma, showing that BMH-21 can potentiate the response to TMZ and synergistically kill glioma cells. To explore additional drug classes, we performed a high throughput drug synergy screen and revealed FGFR inhibitors as the top hits. By characterizing the mechanisms behind the synergy observed, we identified that FGFR1 protein levels are downregulated following BMH-21 treatment, while FGF2 is upregulated and translocates from the nucleolus to the cytoplasm. Involvement of the FGF2/FGFR1 axis appears to result in a positive feedback loop involving ERK1/2 phosphorylation, potentially allowing glioma cells to escape from ribosomal stress and growth inhibition. Combining RiBi and FGFR pathway inhibitors potentiated cell death by abrogating ERK1/2 phosphorylation and SOX2 upregulation. Importantly, these findings were validated using an independent cohort of pediatric patient-derived GSC cell lines obtained by children diagnosed with GBM: Whereas

not all five cell lines tested were equally sensitive to BMH-21, we could show synergistic effects across the whole cell line panel, and in most cases, we observed FGF2 upregulation and enhanced SOX2 downregulation.

Discussion

Although the association between nucleolar size and the degree of histopathological malignancy was reported 30 years ago, the importance of RiBi in glioma has remained elusive (Kajiwara et al. 1990). Several studies have shown increased sensitivity of glioma cells to the inhibition of enzymes responsible for the *de novo* nucleotide biosynthesis, such as IMPDH and DHODH, needed to sustain high rDNA transcription rates; together, these studies underline the potential dependency of GBM on elevated RNA synthesis and especially rRNA, with some of the studies showing apparent nucleolar stress phenotypes following treatments with such inhibitors (Wang et al. 2017, Kofuji et al. 2019, Wang et al. 2019, Lafita-Navarro et al. 2020, Pal et al. 2022, Shi et al. 2022). Additional supportive evidence was reported by Taylor *et al.*, where a cell viability-based drug screening conducted on glioma GSC lines identified ActD as one of the most potential hits. ActD was shown to effectively kill cells with most IC_{50}s to lie between 10-100nM. As discussed, increasing concentrations of ActD above 5-30 nM also inhibit Pol II and III, making it challenging to understand whether Pol I inhibition is the sole contributor to the cytotoxic effect observed. Nevertheless, the remarkable activity in inducing cell death and downregulating stem-cell-associated markers highlights transcriptional inhibition's relevance in GBM (Taylor et al. 2020).

Regardless of the increasing evidence on the importance of RiBi in Glioma, the direct targeting of rDNA transcription by Pol I inhibitors has not been reported to date. To explore this therapeutic avenue, we first searched for potential dependencies of Glioma on RiBi among broader patient cohorts, showing a consistent positive correlation between histological grades and RiBi-related gene expression activity, which were also associated with patient survival. These patterns aligned with and complemented findings from older reports that, while informative, displayed low statistical power due to small tumor sample sizes.

We then characterized the efficacy and toxicological profile of BMH-21, one of the most specific rDNA transcription inhibitors available, confirming its pharmacological effects on a panel of glioma cell lines for the first time. While the models used are extensively discussed in Chapter 3.3, it is essential to note the importance of GSC cells for treatment outcomes and the differences between the serum-grown glioma cell culture model with the patient-derived GSC cell lines grown in serum-free culture conditions. The main body of this work has been performed using serum-based cell cultures, known to promote cell proliferation and negatively affect the expression of stem cell-associated features, such as invasiveness, drug resistance, and tumorigenicity. These cells respond better to chemotherapeutic treatments partly due to rapid cell proliferation. On the contrary, GSCs grown in serum-free conditions proliferate slower, express drug efflux pumps, and in many cases display higher resistance to chemotherapy. Treatment response was indeed different in the case of BMH-21; while the mechanistic characteristics of BMH-21 were retained between the models, higher concentrations were

needed to achieve GSC cell cycle arrest and death, with GI_{50}s ranging from 0.6-1.6 μM, compared with 0.2-0.7 μM for the serum-grown cell lines. Interestingly, while we used a serum-grown GBM line to perform the drug synergy screening, our monotherapy hits overlapped significantly with a recent GSC-based screen that also revealed several protein synthesis-targeting compounds and RiBi-inhibitory compounds (Johansson et al. 2020). Importantly, cell lines with increased sensitivity to BMH-21 shared common genomic alterations among established and GSC cell line panels, such as deletions of CDKN2A/B, PTEN, or RB1, frequently found in the classical molecular subtypes. Nevertheless, both models used are two-dimensional and could not provide information on the tumor microenvironment interactions, vascularization, and invasion. Emerging models that recapitulate the 3D dimensionality of GBM, such as 3D cerebral organoids from human pluripotent stem cells or patient-derived glioblastoma cells, could have been useful in validating the effects of BMH-21 (Joseph et al. 2021). However, they are also insufficient to recapitulate the issue of vascularization and the immunological aspects, making the zebrafish model a practical way to approach most of these aspects.

We also approached the role of RiBi in the GSC population and its involvement in the more aggressive phenotypes observed. Several studies have suggested the connection between RiBi and the stem-like state (Kajiwara et al. 1992, Mi et al. 2021), including the study mentioned above showing that ActD strongly increased SOX2 levels, in line with our findings showing BMH-21-induced SOX2 mRNA and protein expression. BMH-21 also downregulated FGFR1 protein levels; FGFR1 is associated with higher histological grade, vascularization, invasion, and resistance to irradiation. It is also expressed in CSCs, regulating crucial stem-cell-associated transcription factors, including SOX2 and ZEB1, upon FGF2 stimuli (Jimenez-Pascual et al. 2020). This response could be responsible for the synergistic mechanism we observed between BMH-21 and FGFR inhibitors, where glioma cells, following Pol I inhibition-induced FGFR1 and SOX2 downregulation, upregulate FGF2 possibly to maintain FGFR signaling, phosphorylate ERK and sustain cell growth and/or the expression of stem-cell-associated genes to overcome nucleolar stress. While further research is required to elucidate the exact signaling events, the suggested mechanism seems to be an important aspect of the cellular response to RiBi, and was validated in the GSC lines, were we confirmed both FGF2 induction, SOX2, and FGFR1 downregulation. However, as this data has been obtained by IF-based protein quantification only due to technical and time constraints, it would be informative for the main findings to be reproduced by additional methods such as IB. Overall, our study conceptualizes the implementation of RiBi as a target in the management of HGG and provides a thorough characterization of small molecule-mediated Pol I inhibition, raising hope for developing novel therapies against HGG.

4.3 Paper III: The Exon-Junction Complex Helicase EIF4A3 Controls Cell Fate via Coordinated Regulation of Ribosome Biogenesis and Translational Output

Eukaryotic initiation factor 4A-III (eIF4A3) is an adenosine 5′-triphosphate (ATP)–dependent RNA helicase and an eIF4A family member. It is part of the Exon Junction Complex (EJC) and is involved in posttranscriptional RNA splicing and Nonsense Mediated Decay (NMD) (Le Hir et al. 2016, Popp et al. 2018). Based on accumulated evidence, eIF4A3 expression is upregulated in cancer, a pattern we could validate by comparing cancer to normal publicly available RNAseq data. At the same time, it is positively correlated with worse prognosis in most cancer types represented in TCGA (Lin et al. 2018). The cancer connection and its involvement in NMD led to the development of chemical eIF4A3 inhibitors showing promising anticancer effects (Ito et al. 2017, Mizojiri et al. 2017). However, eIF4A3 has also been implicated in rRNA processing, but its exact role remained unknown, motivating us to investigate the potential non-NMD functions of eIF4A3 and its connection to RiBi (Alexandrov et al. 2011).

Following a releant computational approach to paper II, we classified all cancer types as low or high RiBi and noticed that eIF4A3 shows higher expression in high RiBi tumors. We also showed eIF4A3 nucleolar localization upon active Pol I activity, a pattern also observed in most cancer tissue samples analyzed by immunohistochemistry. *EIF4A3* knockdown affected nucleolar integrity, with a morphology indicating rRNA processing defects, analyzed by IF and TEM, while it induced p53 levels. The results were reproduced by both genetic ablation and chemical inhibition of eIF4A3, supporting a potential role of eIF4A3 in maintaining nucleolar integrity and in nucleolar stress-dependent p53 induction.

eIF4A3 levels have been associated with the onset of the Richieri-Costa-Pereira syndrome (RCPS), a pathology characterized by craniofacial alterations and limb developmental defects (Favaro et al. 2014, Mao et al. 2016). The defects could be rescued by p53 ablation in an eIF4A3 haploinsufficient mouse model, indicating an important p53 role, which, however, remains elusive (Mao et al. 2016). We confirmed that eIF4A3 knock down by shRNAs induced p53 accumulation at the protein level, which rescued when we ectopically re-expressed the helicase. An RNAseq analysis used to uncover eIF4A3 knockdown-mediated effects on U2OS cells showed enrichment of gene terms such as “p53 signaling”, “translation”, and “RNA metabolism”, while revealing an upregulation of rRNA processing-related genes, including components of the small subunit (SSU) processome. Comparing the transcriptome changes induced by eIF4A3 knockdown with those induced by low-dose ActD treatment, we observed shared perturbations of genes composing the p53 signaling signature. Moreover, both treatments were found to trigger comparable p53 and p21 induction, suggesting RiBi as a plausible mediator of *eIF4A3* knockdown-mediated p53 induction, a hypothesis further supported by the observation that NMD inhibition did not alter the p53 response observed by the depletion.

To investigate the role of eIF4A3 in RiBi, we quantified the levels of rRNA following *eIF4A3* depletion and observed a decrease in early formed rRNAs species. This effect was independent of Pol I activity, further supporting altered rRNA processing while suggesting a possible involvement in the SSU processome that executes early rRNA processing events. Indeed, RNA and cross-linking immunoprecipitation assays showed that eIF4A3 interacts with theU3 small nucleolar RNA (snoRNA), the main SSU snoRNA, alongside other early formed rRNA species such as 18S rRNA. Given the R loop resolution activity of several DEAD helicases, we reasoned that eIF4A3's role in SSU could be related to the clearance of excessive R loops, RNA-DNA formations that can be genotoxic *via* transcription-replication collision events. Indeed, we noticed a Pol I activity-related increase of R loops and γH2AX foci accumulation upon *eIF4A3* depletion, rescued by RNase H1 expression or Pol I inhibition by ActD. Based on these findings, we addressed whether p53 induction is RiBi stress-mediated and showed that depletion of the IRBC components rescues p53 stabilization, supporting that indeed, *eIF4A3* knockdown induced p53 is nucleolar stress-mediated and is dependent on both EJC and RNA binding domains of the protein as indicated by our mutational analysis.

Subsequently, we quantified the levels of protein synthesis upon *eIF4A3* depletion to detect changes following RiBi stress. Knocking down the helicase slightly affected protein synthesis; however, polysome profiling showed patterns indicative of translational arrest, with a higher 80S monosome peak, suggesting that any residual translation may be monosome-mediated. To understand the translational output upon *eIF4A3* knockdown in more detail, we performed Mass spectrometry (MS)-based proteomics and combined it with polysome-profile-associated RNASeq. We found that most differentially expressed proteins correlated with monosome-bound mRNAs, supporting active translation on 80S monosomes upon eIF4A3 knockdown. Most of these mRNAs/proteins were found to affect central homeostatic mechanisms such as cell cycle and apoptosis. Differential expression of cell cycle-related genes was validated by cell cycle analysis and could be rescued by concomitant knockdown of p53 underlying a direct p53 effect. In contrast, genes implicated in apoptosis were upregulated irrespective of the *TP53* status, and their protein products did not follow the RNA kinetics implicating an intermediate gene regulation level achieved at the ribosome (translational buffering) (Kusnadi et al. 2022).

An important gene found upregulated and associated with the 80S monosomes was *MDM2*. Interestingly, we observed unique *MDM2* isoforms following *eIF4A3* knockdown, which, based on our findings, are likely derived by EJC-mediated alternative splicing. Upregulation was found to be p53-dependent. Given the enhanced stability of p53 observed upon depletion of the helicase, we concluded that differential translation of MDM2 isoforms could affect the MDM2-p53 interaction and render this loop dysfunctional, sensitizing cancer cells to IRBC.

Discussion

This work has revealed previously unexplored functions of eIF4A3, a protein with increasing therapeutic interest in cancer therapy. The findings highlighted its involvement in RiBi and its importance in rRNA processing and nucleolar integrity, connecting the p53 induction with the nucleolar stress response and a differential translation pattern of MDM2. Moreover, molecular

interactions with SSU components are provided, highlighting the importance of the R loop resolution helicase activity in the early processing steps of rRNA.

Using an advanced multi-omics approach, we mapped the full spectrum of expression changes following eIF4A3 depletion at the mRNA and protein levels. Our observations introduce the concept of residual translation following eIF4A3 depletion-induced translational changes, documenting the 80S monosome-dependent translation of critical genes such as cell cycle regulators and apoptotic genes. Additionally, we thoroughly characterize the effects of eIF4A3 on cell cycle and cell death, defining the involvement of p53 and the expression patterns of the interactors involved. Finally, we supplement the current connection of the helicase to cancer by showing higher expression rates in tumor samples that positively correlate with tumors characterized by higher RiBi rates, worse prognosis, alongside its preferential nucleolar localization in more aggressive subtypes. These observations, together with our findings, underline a druggable dependency of cancer cells on eIF4A3 that holds clinical potential, especially for cancer types with upregulated RiBi and aberrant Pol I activity.

5 CONCLUSIONS AND POINTS OF PERSPECTIVE

Cancer therapy is one of the most creative yet challenging drug discovery fields. Intensive research has led to the emergence of targetable pathways and mechanisms, leading to the development of numerous chemical modalities that are being pre- and clinically investigated. In 2021 alone, 59 novel compounds got marketing authorization; 14 of them represented novel targets that have never been described (Avram et al. 2022). However, it is known that attrition rates during clinical development are high, with only a small percentage making it to approval (Moreno et al. 2013). These patterns are partly attributed to the challenges of cancer biology itself: heterogeneity, drug resistance, drug delivery issues, and generalized toxicity contribute to an increased risk-to-benefit ratio, mediocre efficacy, and often lethal side effects. Other contributing factors are the off-target effects, inadequately characterized mechanisms of action and poor design/modeling during preclinical testing.

The research conducted in the framework of this Thesis touches upon most drug discovery steps up to the early preclinical stage, contributing to several aspects of the biology and pharmacology of Ribosome Biogenesis, aiming at the development, characterization, and better understanding of RiBi-inhibitory anticancer drugs and their applications.

In Paper I, we identified a licensed drug with Pol I inhibitory capacity, with a similar mechanism to the first-in-class rDNA transcription inhibitor, BMH-21, and performed drug profiling *in vitro*. As the compound has been associated with rare side effects and given the 10fold higher concentrations recorded to exhibit comparable activity to BMH-21, we introduced structural changes to improve these features, generating new pol I inhibitors. We thus characterized a series of analogs on their mechanism of action and structural differences in relation to the efficacy observed, providing valuable structural insights into the drug family, as well as a pharmacophore to work with and optimize chemically, a task we did not have the technical capacity to complete. However, we could not address the signaling events behind RPA194 degradation, a mechanism that, almost ten years after its discovery, is still under investigation. Additionally, we did not perform early toxicity testing using *in vivo* models such as zebrafish or mice that could potentially provide better insights into the toxicological properties of the analogs. Validation and efficacy assessment *in vivo* could have also been included in this study to bring these compounds closer to the late preclinical phase. Finally, while we observed that CRC cell lines were more susceptible to RiBi inhibition, we could have focused on classifying which CRC types would be suitable for RiBi-targeted therapy by analyzing the genetic status of the cell lines used, expanding our panel, and exploring available databases with patient data to provide the basis for patient stratification in the future.

In Paper II, we used a characterized Pol I inhibitor and focused on the therapeutic applications of this approach. We detected a potentially exploitable vulnerability in HGG, brain tumors with dismal survival rates, which show elevated RiBi rates and, thus, could be preferentially sensitive to Pol I inhibition. Indeed, the strategy showed remarkable anticancer effects *in vitro* and zebrafish models within an acceptable therapeutic window. While additional Pol I

inhibitors such as ActD and CX-5461 were used as positive controls or comparators in certain experiments, we should have analyzed more compounds, including those from Paper I, in parallel with BMH-21 and gotten a better overview of the observed effects and reported modes of action. Importantly, it would be highly interesting to confirm our findings by testing the newest Pol I inhibitors currently under development, which are expected to show improved safety and efficacy profiles.

As tumor recurrence is almost inevitable in patients diagnosed with HGG, we focused on the possible resistance mechanisms to RiBi perturbation. We initially attempted to detect potential resistance development by exposing the cells to slowly increasing BMH-21 concentrations for longer periods without being able to obtain a resistant cell line with a significant difference in GI_{50}s values. Instead, we proceeded with a synergy drug screening to detect pathways whose concomitant inhibition with Pol I could lead to a better response; we revealed the FGFR pathway and explored the connections of FGF2, FGFR1, rDNA transcription, and the nucleolus, showing a potential mechanism of resistance development and a highly promising combinatorial treatment for HGG. Whereas we thoroughly characterized and validated these findings using both cell-based and zebrafish models, we did not employ a rodent model. This, together with the fact that none of the compounds tested are under clinical development, make this study conceptual rather than preclinical; regardless, we believe that this study holds translational potential and anticipate that the data produced will be of great interest to the teams working on the development of the next generations of Pol I inhibitors and RiBi-targeted chemotherapy.

In Paper III, we implemented a hypothesis-driven approach for target identification, where by analyzing literature observations and publicly available data, we identified a druggable helicase with anticancer potential. EIF4A3 is part of the EJC complex with documented roles in NMD and splicing. Reduced eIF4A3 expression or loss of function is responsible for severe developmental defects due to p53 induction and excessive apoptosis; however, the mechanism is not fully elucidated and hints toward non-NMD actions. We thus sought to investigate the functional connection behind these observations, revealing an overlooked yet critical role in rRNA processing and RiBi. Our detailed characterization of the cellular effects and responses upon eIF4A3 inhibition provides a better understanding of an emerging cancer vulnerability and has the potential to inspire future therapeutic strategies in oncology. However, chemical inhibitors are required to establish the pharmacological importance of this target in cancer therapy and demonstrate the proof of concept in cell lines and animal models suspected to display higher sensitivity to eIF4A3 inhibition. Currently, only two selective, ATP-competitive inhibitors are available, designed as probes to study NMD and optimized based on the NMD inhibitory activity. While both compounds were used in our study to validate the effects observed with siRNA and shRNA depletion, we have not evaluated the effect of cell viability on a broader cell line panel due to limited compound availability. Drug profiling and toxicity assessment using zebrafish would be an insightful early step to defining the therapeutic window and detecting preferentially susceptible cancer cell lines. We anticipate that this study will motivate the development of newer inhibitors to implement the proposed treatment strategy,

enable preclinical assessment, and, hopefully, the clinical development of an eIF4A3-targeted therapy against cancer.

Overall, the content of this work provides an in-depth evaluation of RiBi inhibition as an emerging target in cancer therapy and demonstrates three different strategies to approach drug discovery based on the targets of interest:

(I) by revisiting licensed drugs and searching for novel mechanisms and indications, a powerful, fast and cost-effective approach to detect pharmacophores of potential interest, which, however, offer limited chemical space,

(II) by characterizing available investigational drugs and probes to evaluate efficacy in certain disease states and reveal additional mechanistic implications, which can eventually lead to the design of optimized analogs suitable for clinical development

(III) by investigating targets with well or partially characterized properties, searching for alternative functions, and following functional characterization, leading a target-based drug discovery campaign.

Optimistically, the content of this work will support the ongoing efforts in establishing RiBi as a target in the cancer therapy map and develop enhanced medicines and regimens to reinforce the fight against cancer

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