



University of Turin

Department of Medical Sciences

PhD Biomedical Sciences and Oncology (XXXVI cycle)

Curriculum: Advanced Immunodiagnostics

Enhanced Apoptotic Responses in Richter's Syndrome Cells Through Combined Inhibition of CDK9 and BCL-2

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SSD: MED/03

A.A. 2023/2024

Abstract

Apoptosis inhibition through anti-apoptotic protein overexpression is a common characteristic of hematological cancers. One of the key regulators of apoptosis is cyclin-dependent kinase 9 (CDK9) due to its action on short-lived proteins of the BCL-2 family. Therefore, characterizing these molecules in the context of Richter's syndrome (RS) is crucial for identifying disease vulnerabilities. This study shows, for the first time, that direct inhibition of CDK9 suppresses MCL-1 expression, leading to the rapid reactivation of the intrinsic apoptotic pathway in RS cells. Furthermore, combining a CDK9 (CDK9i) and BCL-2 inhibitors (BCL-2i) offers a promising strategy to overcome resistance in refractory tumors. Utilizing four distinct patient-derived xenograft models of RS (RS-PDX) alongside U-RT1, the unique RS cell line available, we demonstrate that CDK9 inhibition reduces phosphorylation of the carboxyl-terminal domain (CTD) serine 2 and 5 of the RNA polymerase II (RNAP II) catalytic subunit B1 (Rpb1), resulting in a temporary transcriptional halt that depletes the cellular reservoir of MCL-1. As shown by flow cytometry and biochemical analyses, CDK9 inhibiting one of the latest and most specific small molecules, AZD4573 (CDK9i), in combination with Venetoclax induces robust apoptotic responses in all RS cells, without significantly modulating other anti-apoptotic molecules, including BCL-2, either in monotherapy or in combination. The CDK9/MCL-1 interplay is further elucidated by molecular silencing, which mirrors the results of pharmacological inhibition. Finally, treating RS-PDX mice with the combination of CDK9i and Venetoclax results in a marked inhibition of tumor growth, accompanied by in vivo evidence of activation of the apoptotic pathway.

Collectively, these findings highlight a novel potential use of CDK9i in combination with Venetoclax for some types of aggressive lymphomas.

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Introduction

1. Intrinsic apoptotic machinery

1.1. Mitochondrial stress response

Most mitochondrial proteins are encoded by nuclear DNA and serve as sensors for mitochondrial dysfunction, with their loss initiating quality control mechanisms. As key regulators of cell death, mitochondria mediate the removal of damaged or unnecessary cells in response to internal or external signals. ¹ Historically, cell death was categorized as either apoptosis (programmed) or necrosis (accidental). Today, persistent disruptions to cellular homeostasis are collectively termed regulated cell death (RCD). Apoptosis, mediated by caspase proteins, is a form of RCD. ¹ Among the 12 cell death classes defined by the Nomenclature Committee on Cell Death (NCCD), mitochondria are directly involved in intrinsic apoptosis through outer mitochondrial membrane permeabilization (MOMP). ¹

1.2. Overview of intrinsic apoptosis

Apoptosis is a genetically regulated form of cell death responsible for the programmed elimination of cells, ensuring normal development and homeostasis in eukaryotes. The best-characterized form of mitochondrial involvement in this process is intrinsic apoptosis, an energy-dependent mechanism that eliminates cells without releasing harmful components into the extracellular space. ² Morphologically, it features cell shrinkage, chromatin condensation, nuclear fragmentation, and the formation of small

vesicle-bound fragments.³ Intrinsic apoptosis is essential for normal tissue development and homeostasis.⁴ The pivotal event is the formation of MOMP, mediated by protein complexes, which releases pro-apoptotic factors from the intermembrane space. This triggers a cascade of signaling events culminating in cell death.¹ The intrinsic apoptotic pathway is regulated by the B-cell lymphoma 2 (BCL-2) family proteins, which localize principally to the outer mitochondrial membrane (OMM) and control events that either promote or inhibit MOMP.¹

1.3. BCL-2 protein family: structure and function

The BCL-2 protein family was first identified in the late 1980s through studies of chromosomal translocations in follicular lymphoma (FL).⁵ These proteins generally possess four conserved BCL-2 homology (BH) domains—BH1, BH2, BH3, and BH4—composed of eight α -helical fragments linked together.⁶ The highly conserved BH domains form the functional core of BCL-2 family molecules. Based on their homology and function, the BCL-2 family proteins in mammals are classified into three subfamilies: multidomain anti-apoptotic proteins (AAP), multidomain BH3 pro-apoptotic proteins (PAP), and BH3-only pro-apoptotic proteins.⁷ Over 25 BCL-2 family members are identified based on their sequence homology to the four BH domains (Figure I).⁸⁻¹⁰ The AAPs, as BCL-2 or B-cell lymphoma extra-large (BCL-xL), commonly expressed in cells, share sequence homology particularly within four regions, known as BH1–BH4¹¹, preventing the activation of intrinsic apoptosis. Pro-apoptotic proteins, such as the extensively studied BAX, BAK, and BOK¹, promote apoptosis and exhibit sequence

homology in the BH1, BH2, and BH3 regions, with minimal similarity at BH4. Notably, the BH3 domain is essential for the dimerization of pro-apoptotic and anti-apoptotic proteins and is critical for the pro-apoptotic functions of BCL-2 family members ¹². Furthermore, BH3-domain-only proteins, such as BAD, BID, NOXA, HRK and PUMA, exhibit sequence homology solely within the BH3 domain. ¹³ These proteins facilitate apoptosis via two distinct mechanisms: one inhibits anti-apoptotic proteins within the BCL-2 family, and the other activates the pro-apoptotic proteins BAX and BAK. ¹⁴

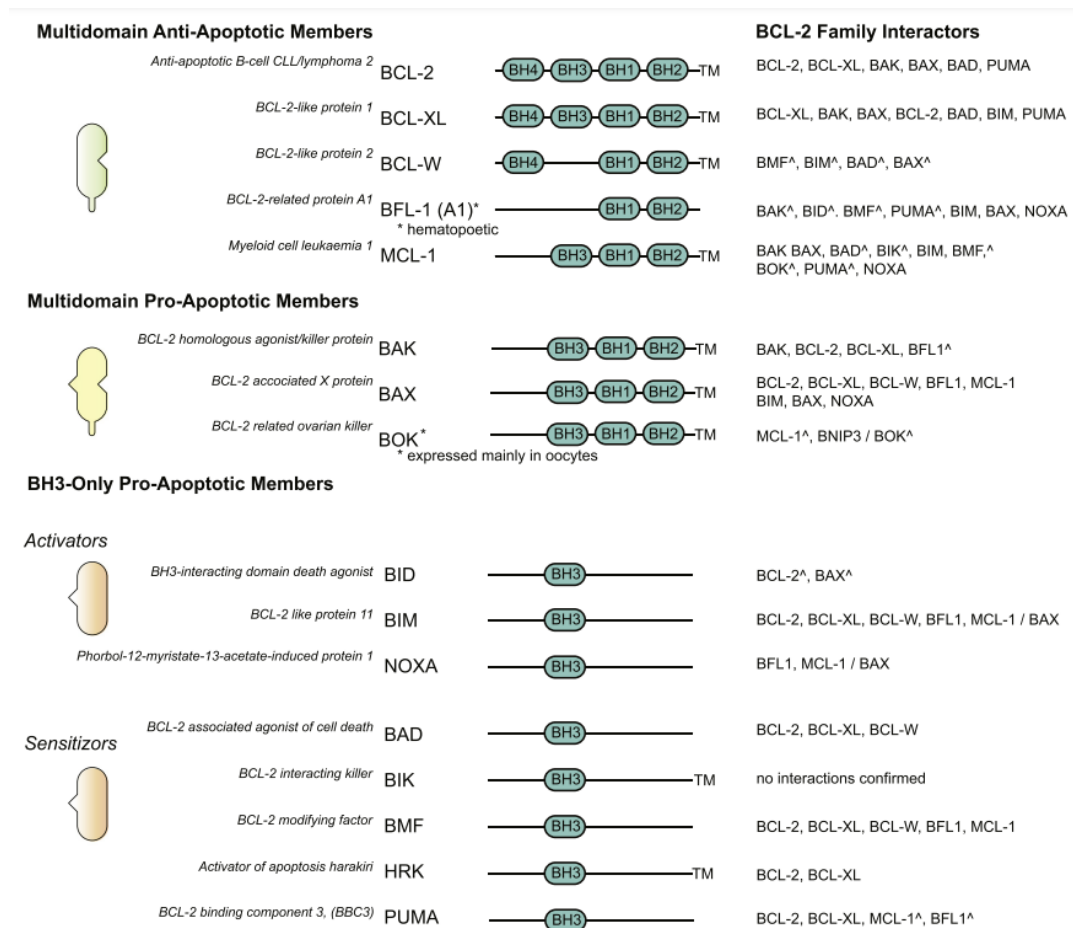


Figure I: BCL-2 protein family. The BCL-2 family regulates both mitophagy and cell death, with all members containing one or more BCL-2 homology (BH) domains, and some also possessing a transmembrane (TM) domain. Based on their sequence homology to BCL-2 and their roles in cell death, these members are classified into three subfamilies. The multidomain anti-apoptotic proteins interact with pro-apoptotic proteins through a hydrophobic BH3 binding pocket formed by the BH1-BH3 domains. Multidomain pro-apoptotic proteins homo-oligomerize to form pores in the outer mitochondrial membrane (OMM), while BH3-only pro-apoptotic proteins can induce cell death either by directly binding to BAX or by sensitizing cells to apoptosis through sequestration of anti-apoptotic proteins. ¹⁰

1.4. Anti-apoptotic proteins of the BCL-2 family

Historically, the AAPs is based on their ability to inhibit apoptosis, as well as the presence of BH4 and transmembrane domains that anchor them to cellular membranes.¹⁵ This subgroup comprises BCL-2, BCL-xL, and less extensively studied, tissue-specific proteins such as myeloid cell leukemia-1 (MCL-1), BCL-2-related protein A1 (Bfl-1), and BCL-2-like 2 (BCL-w).^{16,17} These AAPs inhibit MOMP by binding to and blocking the activity of multidomain pro-apoptotic proteins and BH3-only cell death activators. By influencing mitochondrial membrane permeability, AAPs are essential in the regulation of apoptosis.¹⁸ The defining feature of this class of proteins is the presence of the BH4 domain, which plays a critical role in their anti-apoptotic function. It ensures stable binding by shielding the BH3 domain, thereby blocking the pro-apoptotic activity of PAPs.¹² Its deletion leads to the complete loss of anti-apoptotic function and can even generate a pro-apoptotic mutant. However, even when the BH4 domain is absent, BCL-2 retains its ability to bind and form dimers.¹² Moreover, the BH4 domain interacts with other effector molecules and apoptosis regulators beyond the BCL-2 family, influencing pathways such as angiogenesis, autophagy, and apoptosis.¹⁹

1.5. Mechanisms of MCL-1 regulation and stability

MCL-1 expression is intricately regulated at multiple levels, including transcriptional, post-transcriptional, and post-translational mechanisms.²⁰ A variety of cytokines and growth factors, such as vascular endothelial growth factor (VEGF) and IL-6, can upregulate MCL-1 expression through autocrine signaling loops, thereby activating

diverse signaling pathways.^{20,21} Among these pathways, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway plays a crucial role by inhibiting MCL-1 protein degradation, which leads to its accumulation.²² Similarly, activation of the NOTCH1 pathway induces IL-6 production, further enhancing MCL-1 expression.²³ Additionally, MCL-1 levels are modulated by IL-15 and IL-22 via the JAK/STAT3 and phosphatidylinositol 3-kinase (PI3K) pathways, respectively.^{24,25} MCL-1 expression is also subjected to suppression by numerous miRNAs, including miR-26a²⁶, miR-15a, miR-101, and miR-197.²⁷ Given that MCL-1 is a short-lived protein with a half-life of approximately 30 minutes^{28,29}, its stability is tightly regulated by several E3 ubiquitin ligases, such as MULE³⁰, SCFFbw7³¹, APC/CCdc20³², and SCFB-TrCP³³, which tag MCL-1 for proteasomal degradation. Conversely, de-ubiquitinases, like USP9X³⁴ and USP13,³⁵ stabilize MCL-1 protein levels by counteracting this degradation process. The PEST domain of MCL-1, enriched in proline (P), glutamic acid (E), serine (S), and threonine (T), contain several critical phosphorylation sites, including Thr92, Thr163, Ser64, Ser155, and Ser159. Phosphorylation at these sites by kinases such as CDK1/2, GSK3, JNK, and ERK has been shown to influence MCL-1 interaction with various E3 ligases, thereby affecting its ubiquitination and stability.^{36,37}

1.6. Intrinsic apoptotic signal transduction

The multidomain pro-apoptotic proteins can form pores by oligomerizing and inserting into the outer mitochondrial membrane (OMM), thereby triggering MOMP.¹ The OMM is transiently associated with BAX in its inactive form, either as a monomer or dimer.

BOK is primarily found in the cytosol, while BAK is localized at the OMM, anchored via its transmembrane domain.^{1,9} BAK is likely maintained in its inactive form through interactions with anti-apoptotic MCL-1 and BCL-xL. The process of BAX and BOK translocating and stabilizing at the OMM is thought to be triggered by conformational changes that occur when BH3-only proteins bind to a unique groove created by the BH1, BH2, and BH3 domains.³⁸ When pro-apoptotic BH3-only proteins bind to the hydrophobic groove, they undergo a conformational change, which displaces a transmembrane domain and allows their attachment to the OMM.³⁸ The pro-apoptotic BH3-only protein family consists of diverse proteins that all share a common feature: the BH3 domain.¹ This group includes BAD, BIK, BMF, HRK, and PUMA, which bind to and neutralize anti-apoptotic BCL-2 proteins, lowering the apoptosis threshold and increasing cell sensitivity to apoptotic signals. Furthermore, activator BH3-only proteins such as BID and BIM, interact with BAX, leading to its oligomerization and subsequent OMM pore formation.¹ BID serves as a unique BH3-only protein that connects extrinsic apoptotic signals to the intrinsic apoptosis pathway. This role is facilitated by caspase-8-mediated cleavage of BID, its subsequent localization to mitochondria, and interaction with cardiolipin (CL), which promotes the formation of pores by BAX and BAK.^{39,40} MOMP, along with morphological alterations of the inner mitochondrial membrane (IMM), coordinates the release of apoptogenic proteins that are typically contained within the mitochondrion.¹ Upon pore formation, cytochrome C (cyt C), second mitochondria-derived activator of caspase (SMAC/DIABLO), high temperature requirement protein A2 (HTRA2), which interact with cytoplasmic inhibitor of apoptosis

(XIAP), and apoptosis-inducing factor (AIF), involved in mitochondrial protein import, are all released from the intermembrane space.¹ Apoptogenic factors, once they escape from the mitochondria, actively contribute to the activation and progression of apoptosis. Specifically, SMAC/DIABLO undermines the inhibitory effect of XIAP on caspases, which enhances the apoptotic process.⁴¹ Cyt C binds with Apaf-1 in the cytosol, facilitating the assembly of the apoptosome with caspase-9.⁴² This assembly activates caspase-9, which subsequently cleaves pro-caspases 3 and 7. The activation of these executioner caspases leads to DNA fragmentation, formation of apoptotic bodies, and the overall execution of the apoptotic cell death program.¹

1.7. Heterogeneity of lymphomas and focus on Richter's Syndrome

Malignant lymphomas represent a diverse group of cancers with varying malignancy levels, originating from lymphoid tissue cells, such as lymphocytes and histiocytes, at different developmental stages⁴³. Lukes et al.⁴⁴ defined malignant lymphoma as “a neoplastic proliferative process of the lymphopoietic portion of the reticuloendothelial system, that involves cells of either the lymphocytic or histiocytic series in varying degrees of differentiation, that occurs in an essentially homogeneous population of a single cell type.” Histologic involvement in lymphomas can present as either diffuse (uniform) or nodular, with affected areas ranging from regional to systemic (generalized) distribution. Despite these patterns, the disease process is fundamentally multicentric, involving multiple sites simultaneously⁴³. NHL (Non-Hodgkin Lymphomas) are a heterogeneous group of lymphoproliferative malignancies that are much less

predictable than Hodgkin's lymphomas and have a far greater predilection to disseminate to extranodal locations. Nearly 25% of NHL cases arise in extranodal locations, with most involving both nodal and extranodal sites⁴³. The most common NHL subtypes by far in developed countries are diffuse large B-cell lymphoma (DLBCL, about 30%) and FL (about 20%), while other subtypes each represent less than 10% of cases⁴⁵. In this work, the focus is placed on Richter's Syndrome, a rare and aggressive transformation seen in a subset of chronic lymphocytic leukemia (CLL) patients, which represents a minor subpopulation within NHL cases.

1.8. Principal AAPs in lymphomas

Disruption of intrinsic apoptotic pathways is a key characteristic of lymphomas⁴⁶, and defects in the regulation of BCL-2 family members contribute to cancer development, expansion, and therapy resistance through the abnormal overexpression of pro-survival proteins.^{47,48} Moreover abnormal expression of AAPs play a significant role in the development of various human cancers, including leukemias, lymphomas, and solid tumors.⁴⁹ As described, BCL-2, along with MCL-1 and BCL-xL, is one of the primary AAPs and is commonly overexpressed in NHL, often due to genetic abnormalities⁵⁰, such as in mantle cell lymphoma (MCL) and DLBCL, where it is frequently amplified.⁵¹ Similarly, high BCL-2 protein expression is observed in nearly all patients with chronic lymphocytic leukemia (CLL) who have a 13q14 deletion⁵²⁻⁵⁴ or in CLL patients with a hypomethylated *BCL-2* gene.⁵² The involvement of MCL-1 in lymphoma pathogenesis has been confirmed in transgenic mouse models⁵⁵, and emerging evidence suggests that MCL-1 protein is

significantly overexpressed in aggressive B-NHL subtypes, DLBCL (84% of cases), Burkitt lymphoma (BL; 89% of cases), and grade 3 FL (100%).⁴⁹ In contrast, other B-NHL subtypes like MCL, marginal zone lymphoma (MZL), and small lymphocytic lymphoma (SLL) exhibit lower and less frequent MCL-1 expressions.^{56,57} Research into the molecular mechanisms underlying aberrant MCL-1 expression in DLBCL has shown that elevated MCL-1 levels are more prevalent in the ABC subtype, driven by gain or amplification of the MCL-1 locus (1q21) and the constitutive activation of the STAT3 pathway.⁵⁸ Of note, the downregulation of MCL-1 levels restores the effectiveness of these anti-cancer drugs in several cancer cell lines.^{59,60} Although BCL-xL is frequently expressed in lymphomas, its overexpression—and subsequent resistance to BCL-2 inhibitors—is mainly induced by pro-survival signaling from the lymph node microenvironment rather than by genetic mutations. Furthermore, BCL-xL is essential for platelet maturation, and its targeted inhibition has been associated with the development of thrombocytopenia.⁴⁹

1.9. Strategic use of BH3-mimetics in overcoming apoptosis resistance

There are several approaches to induce intrinsic apoptosis in cancer cells for therapy⁴⁷, with small-molecule inhibitors being the most widely used and the most promising.⁶¹ The discovery that BH3-only proteins trigger apoptosis has specifically led to the development of BH3-mimetic inhibitors targeting pro-survival members, offering significant potential in cancer treatment.^{62,63} The mechanism of action is based on the selective binding to the hydrophobic groove of AAPs, thereby preventing them from sequestering pro-apoptotic members such as BAX and BAK. This disruption leads to the

release of these pro-apoptotic factors, which then oligomerize, leading to the formation of MOMP.⁴⁷ The first BH3-mimetic, Navitoclax (ABT-263), inhibits BCL-2, BCL-xL, and BCL-W and was the first to be tested in patients⁶⁴ demonstrating effectiveness in CLL patients in clinical trials, but dose-limiting thrombocytopenia—due to the dependence of platelets on BCL-xL for survival⁶⁵—hampered the progression of this agent in the clinic.⁶⁶ To address previous challenges, ABT-199 (Venetoclax, Ven) was developed as a selective BH3-mimetic targeting BCL-2, effectively inducing apoptosis in malignant cells.⁶⁷ Venetoclax has shown high efficacy in relapsed or refractory CLL patients.^{68,69} Owing to its clinical success, Ven has been approved worldwide for CLL and acute myeloid leukemia (AML) treatment.^{62,63,70,71} However, resistance to Ven has emerged, particularly due to the BCL-2 G101V mutation, which reduces drug affinity for BCL-2 by 180-fold.⁴⁷ Abnormally elevated MCL-1 expression drives tumorigenesis and worsens prognosis, leading to the development and pre-clinical assessment of several MCL-1 inhibitors, including S63845⁷², A-1210477⁷³, AMG176⁷⁴, and AZD5991 (MCL-1i).⁷⁵ S63845 was explored in pre-clinical studies for its in vitro and in vivo potential in hematological malignancies like multiple myeloma (MM), AML, Chronic myeloid leukemia (CML), and blastic transformation (BT).⁷² It showed moderate effectiveness, either alone or with kinase inhibitors, in certain solid tumors, including breast and prostate cancer^{72,76}, as well as in small cell lung carcinoma (SCLC) cell lines with high MCL-1 and low BCL-xL expression.⁷⁷ However, its potency in ongoing clinical trials remains limited. AMG176 has shown effectiveness in various hematological malignancies⁷⁴ and in certain solid tumor cell lines, including breast and non-small cell

lung cancer.⁷⁸ MCL-1i, another MCL-1 specific BH3-mimetic, has proven effective in treating MM in mouse models, with its efficacy enhanced by co-treatment with the proteasome inhibitor bortezomib.⁷⁵ Its structure enables high-affinity binding to the BH3-binding groove of MCL-1, specifically interacting with key residues such as Arg263, which facilitates electrostatic interactions, and Met231, whose structural flexibility accommodates the macrocyclic conformation of MCL-1i, while Ala227 indirectly stabilizes the groove by minimizing steric hindrance and optimizing ligand accommodation. These interactions collectively disrupt the binding of PAP like BAK, leading to the activation of the apoptotic pathway through rapid MOMP, cytochrome c release, and caspase activation, resulting in apoptosis in cancer cells (Figure II).⁷⁵ Of note, the combination of an MCL-1 inhibitor with a BCL-2 inhibitor was shown to be tolerable in mice, providing a synergistic response that was able to overcome drug resistance in models of DLBCL.⁷⁹ To date, six MCL-1 inhibitors have reached clinical trials, though some were halted due to on-target cardiac toxicity⁸⁰, a risk anticipated from genetic studies indicating MCL-1's essential role in cardiomyocyte survival.^{81,82} Other malignancies depend on BCL-xL for survival, prompting the development of BCL-xL-specific BH3-mimetics. WEHI-539 was the first, followed by A-1155463 and A-1331852, which showed promising antitumor activity.⁸³⁻⁸⁵ However, no BCL-xL inhibitors have been clinically approved, with trials progressing slowly due to concerns over platelet toxicity. The survival of many cancer cells depends on multiple pro-survival BCL-2 proteins, requiring combination therapy with multiple BH3-mimetics or a BH3-mimetic

plus standard anti-cancer agents that increase BH3-only proteins.⁶² However, the tolerability of such treatments must be carefully determined.

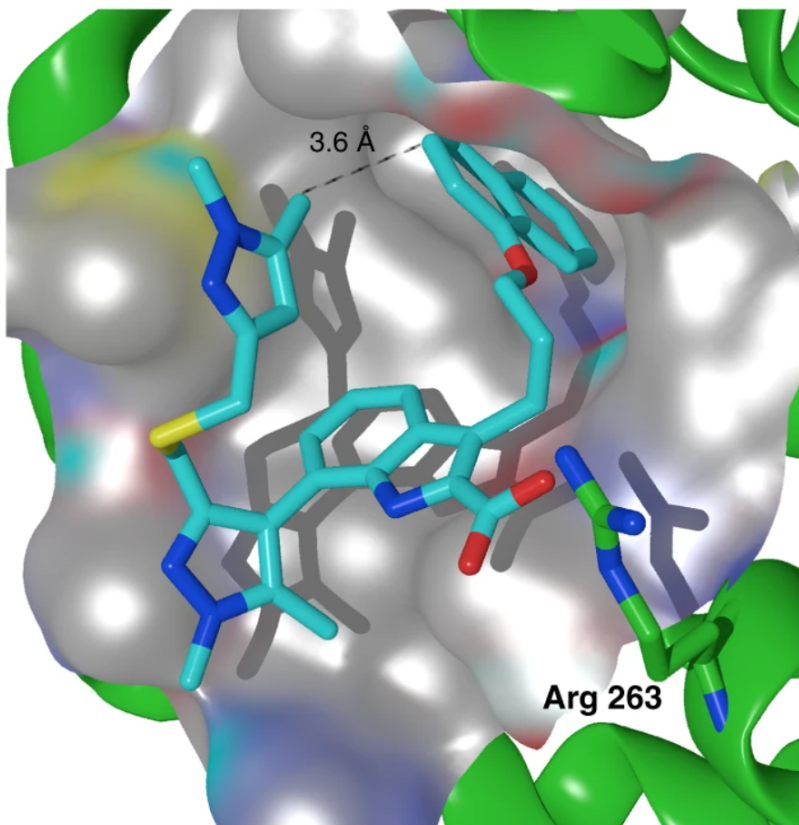


Figure II: MCL-1i Crystal Structure. Crystal structure of MCL-1i bound to MCL-1 (gray) (PDB: 6FS1). The ligand carbon atoms are shown in cyan. The macrocyclic conformation of MCL-1i fits into the BH3-binding pocket of MCL-1, with key interactions involving residues Arg263 and Ala227, stabilizing the inhibitor within the binding site.⁷⁵

2. CDK9 as a central regulator in transcriptional elongation and a target for cancer therapy

Protein kinases regulate numerous essential eukaryotic processes through phosphorylation, impacting cellular functions such as signal transduction, cell cycle progression, and gene expression. Dysregulation of these kinases—particularly cyclin-dependent kinases (CDKs), a family of Ser/Thr kinases activated by cyclin binding—has been closely linked to disease, especially cancer, where CDKs drive unchecked cell

proliferation. CDKs are functionally divided into two main groups: those that control cell cycle phases (e.g., CDK1, CDK2, CDK4, and CDK6) and those involved in transcriptional regulation (such as CDK7 and CDK9).⁸⁶⁻⁸⁸ CDK9, in particular, plays a critical role in sustaining cancer cell survival by supporting the transcription of genes required for continuous protein synthesis, thereby enhancing the cellular machinery needed for tumor growth and resistance to therapeutic stress.⁸⁹ In particular, the elongation phase of gene transcription relies on Positive Transcription Elongation Factor b (P-TEFb), a key regulatory complex composed of CDK9 and a cyclin subunit (typically cyclin T).⁹⁰ P-TEFb phosphorylates the carboxyl-terminal domain (CTD) of Rpb1, the largest subunit of RNAP II, enabling the transition from transcriptional pausing to productive elongation. Without P-TEFb, RNAP II remains paused, resulting in the production of only short RNAs.⁹⁰⁻⁹² Once considered secondary, elongation is now recognized as a highly regulated and dynamic stage of transcription.⁹³ After RNAP II initiates transcription and synthesizes 20-50 nucleotides⁹², it often pauses at promoter-proximal regions of most mammalian genes, potentially to increase gene accessibility⁹⁴, enable rapid activation, integrate signals, or couple elongation with 5' end-capping.^{95,96} Pausing is mediated by DRB sensitivity inducing factor (DSIF) and negative elongation factor (NELF), and is overcome by P-TEFb, which phosphorylates NELF and DSIF, releasing NELF and converting DSIF into a positive elongation factor.^{97,98} P-TEFb phosphorylates the Rpb1, primarily at Ser2, essential for coupling transcription elongation with pre-mRNA processing.^{99,100} This phosphorylation serves to recruit factors for chromatin modification, elongation, splicing, and 3' end processing.^{101,102} Additionally, P-TEFb's role in relieving RNAP II

pausing is linked to controlling transcriptional initiation, limiting new transcription rounds when CDK9 is inhibited.^{103,104} P-TEFb has also been implicated in the development of lymphoma, particularly in DLBCL¹⁰⁵, where the dysregulation of *MYC* is essential in its pathogenesis and involves either a *MYC* rearrangement mutation, most commonly involving its translocation into heavy or light-chain immunoglobulin loci (5-15%), or gene amplification (30-50%).¹⁰⁶ These changes often confer aggressive clinical courses.^{86,105} As a general transcription factor, *MYC* directly interacts and recruits P-TEFb to its promoter and other target genes to mediate RNAP II pause release.⁹⁵ Inhibition of CDK9 by selective inhibitors or by genetic knockdown negatively regulated *MYC* and *MCL-1* expression, and induced apoptosis in primary and transformed cells from this lymphoma, providing an attractive therapeutic strategy.¹⁰⁶ Given the significance of CDKs, a first generation of pan-CDK inhibitors was developed, including Flavopiridol (Alvocidib), which was tested in clinical trials.¹⁰⁷ Despite its limited efficacy and high toxicity, it achieved complete remission rates of 36-68% in AML.¹⁰⁸ Seliciclib, the second pan-CDK inhibitor, demonstrated improved selectivity but ultimately failed in clinical settings due to limited efficacy and toxicity.¹⁰⁹⁻¹¹² Subsequently, to directly target the bottleneck phase of elongation, increase specificity while reducing off-target effects, and decrease nonspecific cytotoxicity, selective CDK9 inhibitors such as CDKI-73¹¹³, TG02¹¹⁴, and BAY1143572 (Atuveciclib) have been developed and are currently under clinical evaluation for advanced cancers¹¹⁵⁻¹¹⁸. Additionally, CDK9i, a potent CDK9 inhibitor ($IC_{50} < 4$ nM), selectively targets CDK9, downregulates *MCL-1*, and induces apoptosis in hematologic cancer cell lines. CDK9i's potency is attributed to its key

interactions with residues such as Cys106, Asp167, Asn154, and Ala166 in the CDK9 binding pocket, which enhances its affinity (Figure III). Its transient inhibition, with a dissociation half-life of 16.2 minutes, allows for controlled target engagement, reducing off-target effects and toxicity.¹¹⁹ It has shown efficacy in AML models, both alone and in combination with Venetoclax. CDK9i is currently in phase I trials for hematological malignancies (NCT03263637).¹²⁰

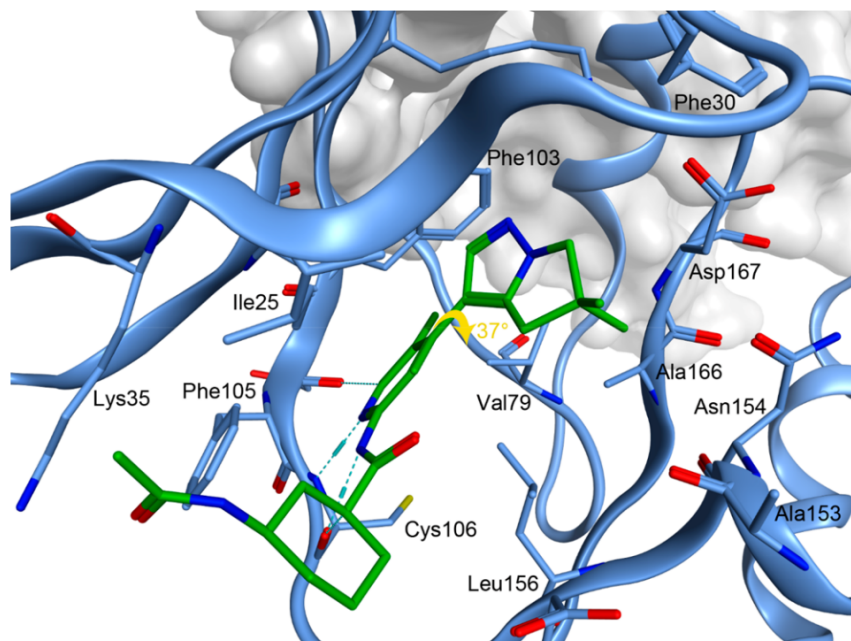


Figure III: CDK9i Crystal Structure. Crystal structure of CDK9i bound to the CDK9 (blue) and cyclin T1 (gray) complex (PDB: 6Z45). The ligand carbon atoms are shown in green. The biaryl pyrazole-pyridine moiety of compound 24 occupies the ATP-binding pocket, with key interactions involving residues Cys106, Asp167, Asn154, and Ala166, stabilizing the inhibitor within the binding site. These interactions enhance CDK9i's potency and selectivity for CDK9, contributing to its efficacy in downregulating MCL-1 and inducing apoptosis in hematologic malignancies. The transient binding of CDK9i also supports its role in minimizing off-target effects by allowing rapid dissociation from CDK9.¹¹⁹

3. Richter Syndrome

3.1. Definition of RS

The progression of CLL or SLL into a more aggressive lymphoid malignancy is referred to as Richter syndrome, also known as Richter transformation.¹²¹ In 1928, Richter first documented this condition, referring to it as "reticular cell sarcoma" in the context of lymphocytic leukemia.¹²² Forty years later, Lortholary and colleagues coined the term "Richter transformation" to describe this clinicopathological condition.¹²³ The aggressive lymphoma that develops in Richter syndrome exhibits a distinct histology compared to the underlying CLL. RS is not the same as the accelerated phase of CLL or prolymphocytic transformation. Over time, the associated lymphoid malignancies have been identified as DLBCL, Hodgkin lymphoma, lymphoblastic lymphoma, plasmablastic lymphoma, and composite lymphoma.^{124,125} According to the WHO classification, Richter syndrome (RS) is generally characterized by two distinct forms: the DLBCL variant, which constitutes the majority of cases (95%), and the much rarer Hodgkin lymphoma (HL) variant, identified in a small proportion of cases (0.5–5%).¹²⁶⁻¹²⁹

3.2. Clonally related RS

RS can be classified into two distinct types based on its clonal relationship to the pre-existing CLL: clonally related and clonally unrelated. The clonally related type, which represents a true transformation from the preceding CLL phase, is characterized by identical immunoglobulin heavy- and light-chain rearrangements in both the CLL and lymphoma cells (Figure V).^{130,131} Clonally related RS is identified in the majority (~60-

80%) of DLBCL cases. This clonal relationship is crucial for prognosis: patients experiencing true transformations, where the RS is clonally related to the initial CLL, typically face a more severe outcome, with a median survival of about 12 months. In contrast, patients with clonally unrelated RS have a prognosis similar to those with de novo DLBCL, enjoying a median survival of nearly 65 months. Furthermore, the clonally unrelated DLBCL variant tends to respond to treatment as effectively as de novo DLBCL, with a significantly longer median survival of about 5 years, compared to the 8–16 months seen in those with clonally related DLBCL.^{132,133} Clonally unrelated RS, whether of the DLBCL or HL variant, is essentially a secondary lymphoid malignancy arising in the context of CLL. The exact mechanisms driving the development of an aggressive lymphoma that is clonally unrelated to CLL cells remain unclear. However, it is thought that this may be partially linked to the state of immune dysregulation commonly observed in patients with CLL.

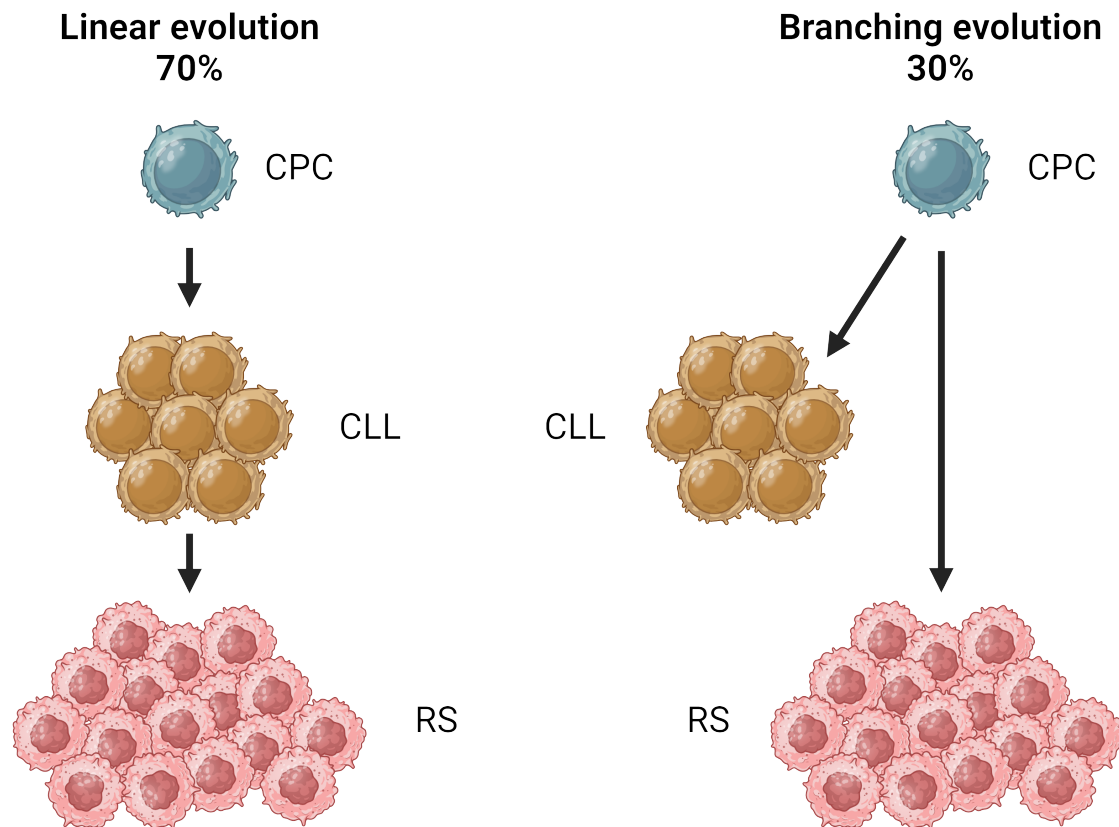


Figure V: Clonal evolution models of Richter syndrome. In most cases, Richter syndrome (RS) progresses through a linear evolutionary path, where diffuse large B-cell lymphoma (DLBCL) marks the final stage of the CLL clone's development. In a smaller proportion of cases, RS seems to result from a divergent evolutionary process, with chronic lymphocytic leukemia (CLL) and DLBCL both emerging from an earlier common precursor cell (CPC). ^{134,135}

3.3. Histopathology of RS transformation

RS transformation predominantly affects lymph nodes (60-70%) but can extend to extranodal sites such as the gastrointestinal tract, tonsils, and bone marrow (10% each).

^{131,132} The DLBCL variant features large B-cell clusters resembling centroblasts or immunoblasts. ¹³¹ CLL-derived RS often shows numerous proliferation centers and high counts of prolymphocytes and paraimmunoblasts, though lacking definitive DLBCL characteristics. ^{136,137} New criteria help distinguish accelerated CLL from RS by focusing

on large B-cells and diffuse growth patterns, reclassifying up to 20% of RS cases as accelerated CLL.¹³⁷ Biopsy confirmation is critical, with PET/CT SUVmax aiding in biopsy decisions, as RS lesions typically exhibit SUV values similar to de novo DLBCL.

During transformation, CLL cells often lose CD5 and CD23, with CD5 in ~30% and CD23 in ~15% of cases, while transformed cells express CD20, a key target for immunotherapy.

¹³¹ Most DLBCL transformations (90-95%) exhibit a post-germinal center phenotype (IRF4+), with a minority (5-10%) showing a germinal center phenotype (CD10/BCL6+).

^{131,132} HL transformation occurs in two types: Type 1, mirroring classical HL with CD30+/CD15+/CD20- Hodgkin-Reed-Sternberg cells, and Type 2, with Hodgkin-like cells in CLL lacking reactive infiltrates and expressing CD30+/CD20+/CD15-.^{124,138} About 40-50% of HL-RS cases are clonally related to CLL, while others (~20% DLBCL, ~50-60% HL morphology) represent de novo lymphomas.^{131,139,140}

3.4. Richter's Syndrome incidence in CLL

The frequency of RS in CLL patients has been reported to range from 2% to 12% in studies conducted before the advent of targeted therapy.¹⁴¹ Several factors, including patient cohort selection, follow-up duration, biopsy vigilance, and the inclusion of both clonally related and unrelated patients, influence the reported incidence of RS. Notably, most incidence estimates are retrospective and derived from selected patient data.^{125,142}

Many studies fail to distinguish between clonally related and unrelated RS due to a lack of investigation into clonal relationships. The largest study on RS was conducted at MD Anderson Cancer Center in 2006, where out of 3986 CLL patients, 148 (3.7%) had

histologically confirmed RS, and 204 (5.1%) had possible RS.¹⁴³ A cohort study from the Mayo Clinic involving 1641 newly diagnosed CLL patients reported an RS incidence of 2.3% (37/1641).¹²⁶ Interestingly, a retrospective Italian study from 1999 found that RS occurrence was significantly higher in patients under 55 years of age (5.9%) compared to those over 55 years (1.2%).¹⁴⁴ The incidence of RS in patients treated with novel targeted agents has been reported to range from 4% to 20%, particularly in those with TP53-disrupted CLL clones, who are at a higher risk of transformation.¹⁴⁵ Incidence in these cohorts typically refers to clonally related RS. Despite the advent of targeted therapy, the frequency of RS does not appear to be decreasing, with the highest occurrence observed in patient's refractory to treatment.^{145,146}

3.5. Pathogenesis of RS

The pathogenetic mechanisms of RS are still unclear, with genetic predisposition, impaired immunosurveillance, and potential carcinogenic effects of CLL treatment being investigated. Research is limited by RS rarity, complicating prospective studies, though larger cohorts have recently been studied thanks to global collaborations.¹⁴⁷ DLBCL transformation remains the most studied form of RS, with somatic mutations in hematopoiesis causing dysregulation and transformation being key to RS pathophysiology.¹⁴⁸ Although no RS-specific mutations have been identified, researchers have detected somatic mutations in genes regulating proliferation and apoptosis in a subset of RS patients, despite the broad mutation spectrum and absence

of RS-unique mutations.¹⁴⁵ Two studies have also uncovered common genetic pathways that CLL cells follow during clonal transformation to RS (Figure 6). One of these studies, conducted by Chigrinova et al., identified two distinct RS subgroups: one characterized by *TP53* inactivation and/or *CDKN2A/B* loss, often coupled with *MYC* activation (50%), and another with trisomy 12, frequently associated with *NOTCH1* mutations (28%).¹⁴⁷ Fabbri et al. confirmed these findings, showing clonal linearity during RS transformation, with CLL genetic alterations persisting in RS along with new lesions.¹⁴⁹ *TP53* disruption, found in 55% of transformations, involves a tumor suppressor critical for DNA damage response and cell cycle arrest.¹³² *TP53* disruption, a key negative prognostic marker in clonal DLBCL, often coexists with *MYC* activation and *CDKN2A/B* loss, driving malignancy.¹⁴⁹⁻¹⁵¹ *MYC* deregulation, seen in 40-60% of clonal transformations, results from genetic lesions or *NOTCH1* mutations, delineating distinct RS subgroups, especially in cases with trisomy 12.^{132,152,153} Murine models confirm that *TP53* and *CDKN2A/B* loss induces RS-like transformation via the BCR pathway, with *NOTCH1* mutations independently driving RS.^{154,155} Nadeu et al. identified new RS driver genes, primarily affecting cell cycle regulators (89%) and chromatin modifiers (79%), with *MYC* and *NOTCH* pathways altered in only 2 of 19 cases.¹⁵⁶ The study also revealed a novel single base substitution-replication timing (SBS-RT). SBS-RT signature, unique to RS cases treated with alkylating agents, suggesting RS evolves from multiple sub-clones, with selective enrichment post-therapy. Mutation-guided therapies are crucial for managing clonal evolution in high-risk CLL.^{157,158}

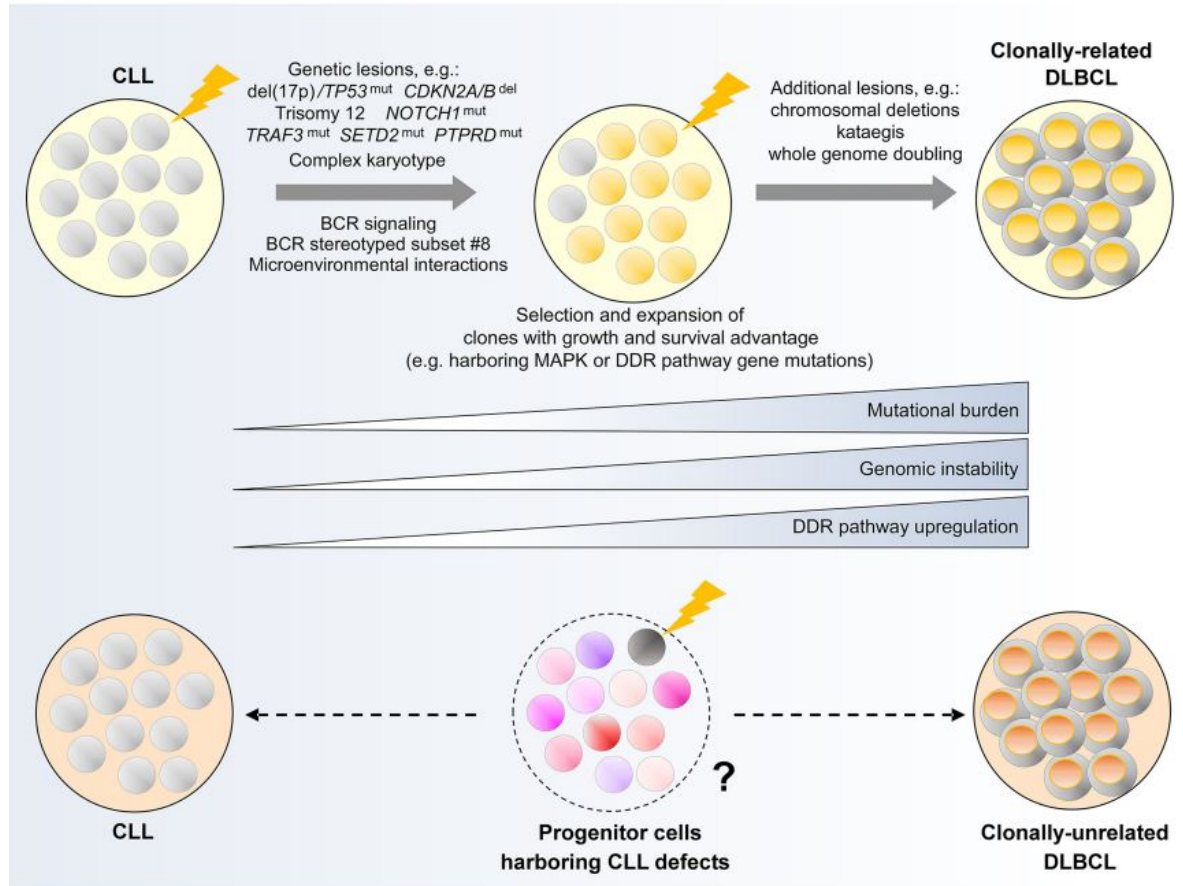


Figure 6: Clonal Evolution Driving the Transformation of CLL into DLBCL in Richter Syndrome. (Upper panel) Genetic alterations and biological mechanisms involved in the progression of clonally-related Richter syndrome. (Lower panel) A proposed model depicting a potential evolutionary trajectory for clonally-unrelated Richter syndrome. MAPK, mitogen-activated protein kinase; DDR, DNA damage response. ¹⁵⁸

3.6. RS risk factors

Despite several studies, no clear risk factors for RS in CLL patients have been identified, with small, selected cohorts and retrospective designs limiting these efforts.^{125,159} A 2008 pilot study by Rossi et al. involving 185 CLL patients identified factors predisposing to RS, including:

1. *CD38* expression ($\geq 30\%$)
2. Stereotyped B-cell receptor
3. IGHV4-39 gene usage
4. Telomere length < 5000 base pairs
5. Lymph node size > 3 cm
6. Absence of del13q14

Polymorphisms in *BCL2*, *CD38*, and *LRP4* genes have also been linked to increased RS risk.¹⁶⁰⁻¹⁶² *NOTCH1* mutations predict RS development, whereas *SF3B1* mutations do not.¹⁶³ CLL patients with unmutated IGHV gene face higher RS risk.^{126,132,164} Inactivating lesions in *CDKN2A*, *CDKN2B*, and *TP53* often co-occur in RS, with BCR stimulation driving proliferation. BCR and CDK4/6 inhibitors have shown synergistic effects in treating RS with these abnormalities.¹⁵⁴ Advanced stage and large lymph nodes at CLL diagnosis are also associated with a higher transformation risk.^{125,159} The relationship between CLL treatment and RS remains unclear. Alkylating agents and purine analogs may trigger RS, with a 3-fold increased risk observed in combination therapy.¹²⁶ No significant RS rate increase was noted with single-agent treatments.^{142,165} Variability in RS frequency across studies likely reflects differences in patient selection and follow-up, leaving the impact

of CLL therapy on RS risk uncertain. Ethically, prospective studies comparing untreated and treated patients are challenging due to the necessity of treatment for prognosis improvement.

3.7. Clinical presentation and progression of RS

The clinical presentation of RS in CLL patients is variable and nonspecific, requiring vigilant monitoring by physicians. Key indicators of potential transformation include the onset of new B symptoms, asymmetric enlargement of lymph nodes, and a sudden increase in lactate dehydrogenase (LDH) levels.¹⁶⁶ RS can also manifest with rapidly progressing lymphadenopathy, cytopenia, or involvement of extra-nodal sites. However, these clinical findings are only 50–60% specific for RS, necessitating a biopsy for definitive diagnosis.^{160,167} RS prognosis is generally poor, with a median survival of 8-16 months in clonally related cases.^{132,143} Prognosis varies based on clinical and biological factors, which have been used to develop a prognostic score including performance status, LDH levels, platelet count, tumor size, and prior therapies (Figure 7).¹⁴³ Patients are stratified into risk groups with corresponding median survival times ranging from 1 to 13 months.¹⁴³ Key factors affecting survival include *TP53* status, treatment response, and performance status. Low-risk patients show good response to treatment with a 5-year survival rate of 70%, while high-risk patients have a median survival of only 4 months.¹³² Clonally unrelated RS presents with a significantly better prognosis, akin to *de novo* DLBCL, with a median survival of 62 months.¹³² In contrast, the HL variant of RS shows a median survival of 10-20 months, indicating a high-risk disease.¹³⁸

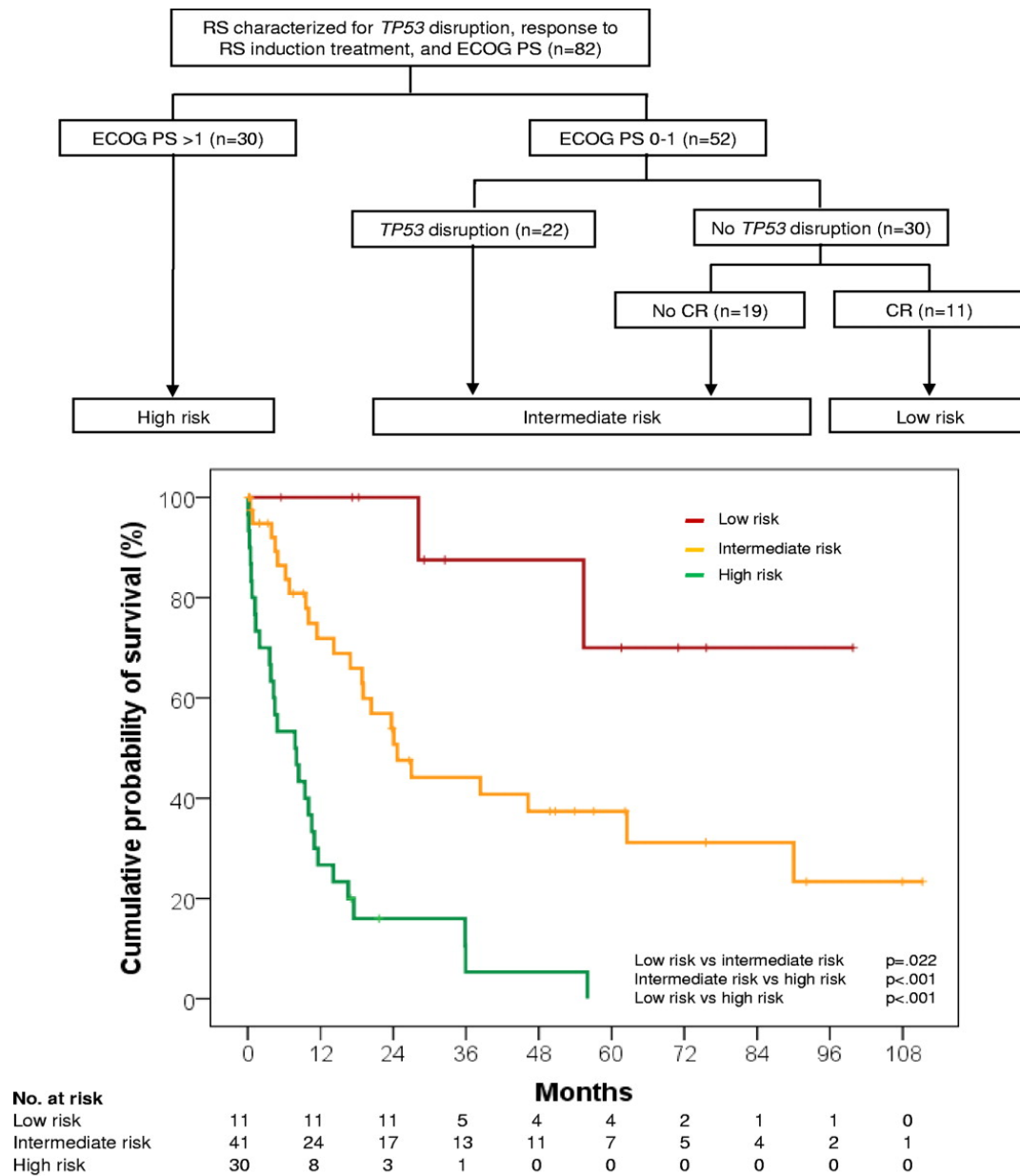


Figure 7: Risk-Based Classification of RS and Kaplan-Meier Survival Estimates Across Categories. (Upper panel) Patients with an Eastern Cooperative Oncology Group performance status (ECOG PS) greater than 1, regardless of their TP53 status or response to RS induction therapy, fall into the high-risk group (Events/N, 28/30; median survival, 7.8 months; 95% CI, 2.5-13.1 months). Those with an ECOG PS of 1 or lower, but either carrying TP53 disruption or failing to achieve complete remission (CR) after RS induction therapy despite being TP53 wild type, are classified in the intermediate-risk group (Events/N, 23/41; median survival, 24.6 months; 95% CI, 15.8-33.4 months). Meanwhile, patients who have an ECOG PS of 1 or lower, no TP53 disruption, and achieve CR after RS induction treatment belong to the low-risk group (Events/N, 2/11; median survival not reached; 5-year survival, 70%; 95% CI, 34.4%-100%).¹³²

3.8. Therapeutic strategies and advances

RS in CLL patients is commonly treated with regimens designed for aggressive B-cell lymphomas or de novo DLBCL, involving chemo-immunotherapy and stem cell transplantation (SCT). However, these treatments often cause severe toxicities and short remission periods. For example, the hyper-CVAD regimen, which includes cyclophosphamide, vincristine, doxorubicin, and dexamethasone, shows a 41% response rate with a median survival of 10 months.¹⁶⁸ Adding methotrexate and ara-C with rituximab increases the response rate to 43%, but toxicity remains high, with a 22% mortality rate.¹⁶⁹ Less intensive regimens, such as R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone), offer a better balance between efficacy and toxicity, achieving a 67% response rate in a phase II trial, with a median progression-free survival (PFS) of 10 months and overall survival (OS) of 21 months.¹⁷⁰ Retrospective studies on R-CHOP in RS patients report a 50% response rate and a median survival of 15 months.¹³⁸ Platinum-containing regimens like OFAR (oxaliplatin, fludarabine, ara-C, rituximab) achieve a 40% response rate, but result in only a 3-month remission and a median survival of 6-8 months, with notable hematotoxicity and infections.^{171,172} Adjustments in the OFAR2 trial do not significantly reduce these toxicities, with an overall response rate (ORR) of 39% and a median OS of 7 months.¹⁷² Chemo-immunotherapy remains a frontline treatment for the DLBCL variant of RS, but is associated with severe toxicities and short remissions (3-15 months).^{171,173} SCT is considered for younger, chemo-sensitive patients post-remission, with better long-term outcomes, such as a 5-year OS of 58% reported in patients undergoing allogeneic SCT.

^{133,174-176} However, SCT is feasible in only about 10-15% of patients due to age, performance status, and donor availability, with disease activity at transplantation being a critical outcome factor. The ABVD regimen (doxorubicin, bleomycin, vinblastine, dacarbazine) shows a 40-60% response rate, but patients often relapse, with a median survival of 4 years. ^{124,138} SCT is less commonly used for the HL variant due to its relatively better prognosis compared to the DLBCL variant. Despite chemotherapy's challenges, recent advancements with small molecule inhibitors that target tumor-specific molecular alterations have enhanced patient-tailored therapies, transforming CLL treatment. ^{177,178} Bruton's tyrosine kinase (BTK), vital in BCR and cytokine receptor signaling, is effectively targeted by inhibitors like ibrutinib, which shows good tolerability but limited efficacy, with partial responses in small cohorts and a complete response in only one patient. ¹⁷⁹⁻¹⁸¹ Similarly, acalabrutinib, an irreversible BTK inhibitor, is well-tolerated but shows limited effectiveness as monotherapy. ¹⁸² Overexpression or mutation of the nuclear export protein XPO1, observed in several hematological malignancies, including CLL and RS, makes it a promising therapeutic target. In a phase I study, Selinexor, an XPO1 inhibitor, showed partial responses in 2 of 5 refractory/recurrent RS patients, with a good tolerability profile. ¹⁸³⁻¹⁸⁵ CAR-T cell therapy, specifically targeting CD19, is emerging as a promising approach for hematological malignancies. In a study involving 24 patients, including 5 with RS, CAR-T therapy achieved an ORR of 71% at four weeks post-administration. ¹⁸⁶ A subsequent study involving 19 patients treated concurrently with ibrutinib reported an ORR of 83%, with 1-year OS and PFS rates of 86%. ^{187,188} Additionally, an Israeli study including eight

high-risk CLL patients with RS reported an ORR of 71% (5/8) following CAR-T therapy.¹⁸⁹ However, more studies are needed to confirm the efficacy of CAR-T therapy in RS.

3.9. Combinatorial strategies

The clinical efficacy of small molecule inhibitors in CLL has extended their use in RS treatment, though outcomes often remain suboptimal due to the complex genetic background of RS cells. Combining drugs that target different molecules or pathways may improve outcomes. BTK inhibitors, like Ibrutinib, have been tested with other agents. A 2015 phase 1b/II trial combining ibrutinib and ofatumumab in RS patients showed tumor burden reduction in 3 patients, with two achieving stable disease for a median of 10 months and one showing a partial response before progressing 5 months later.¹⁹⁰ Another study reported temporary disease reduction in an RS patient treated with ibrutinib and rituximab after multiple chemotherapy cycles.¹⁹¹ A further study involving 20 RS cases tested ibrutinib with nivolumab, an anti-PD-1 agent, showing a 65% ORR and two complete remissions, but treatment was halted due to severe side effects.¹⁹² The STELLAR trial (NCT03899337) in 2019 demonstrated the feasibility of adding acalabrutinib to R-CHOP, enhancing clinical activity.¹⁹³ Venetoclax, BCL2 inhibitor, showed promise in RS when combined with R-EPOCH, achieving a 62% ORR and a median survival of 19 months, though neutropenia and thrombocytopenia were notable side effects.¹⁹⁴ Despite advances, RS remains a challenging condition, with few ongoing trials and no established first-line therapy.¹⁹⁵ Understanding RS genetics and biology is essential to identifying new therapeutic targets.

3.10. Richter syndrome-Patient-derived xenografts

Our research team has succeeded, for the first time, in developing four distinct patient derived xenograft (PDX) models of Richter syndrome (RS9737, RS1316, IP867/17, and RS1050).¹⁹⁶ These models were created using neoplastic cells obtained from lymph node or peripheral blood biopsies of patients diagnosed with Richter syndrome. Three of these models (RS9737, RS1316, and RS1050) were developed from patients with a prior history of CLL who had shown resistance to standard chemo-immunotherapy, including R-CHOP and/or ibrutinib. Conversely, the IP867/17 model was derived from a patient who had not undergone any treatment. The neoplastic cells from these RS patients were subcutaneously injected into NOD/SCID gamma chain^{-/-} (NSG) mice, and the resulting tumor cells were re-implanted for at least 10 successive passages. All RS-PDX models demonstrated positivity for B-cell markers including CD19, CD21, and CD23, with CD5 expression uniquely present in RS1316, reflecting the characteristic phenotype of primary RS samples. Immunohistochemical analyses confirmed the strong expression of CD20, CD23, and PAX5, consistent with a DLBCL diagnosis. Each of the RS-PDX models retains a strong correlation with the primary RS clone, exhibiting consistent phenotypic and genotypic traits that remain stable over time. Notably, the majority of single or multi-nucleotide variations found in the four PDX models were already present in the original patient samples. These RS-PDX models display a complex array of somatic mutations, including point mutations or deletions on chromosome 17p leading to biallelic inactivation of the *TP53* gene. Additionally, the IP867/17 model features *MYC*

gene amplification in the 8q24 region. These RS-PDX models provide a valuable platform for elucidating pathogenic mechanisms and identifying key signaling pathways of therapeutic relevance. A promising therapeutic approach recently approved by the FDA for both solid and hematologic cancers involve the use of antibody-drug conjugates (ADCs). The effectiveness of these therapies relies heavily on their ability to selectively target neoplastic cells, thereby minimizing off-target effects. These advanced therapies combine the cytotoxic potential of a drug with the precision of a monoclonal antibody (mAb). To assess this approach, the preclinical effects of two ADC molecules were investigated in the four RS xenograft models, focusing on the molecules receptor tyrosine kinase-like orphan receptor 1 (ROR1) and CD37, which are highly and selectively expressed in these cancerous cells. ROR1 plays a crucial role during embryogenesis, but its expression diminishes during fetal development and is either absent or minimal in adult tissues.¹⁹⁷⁻¹⁹⁹ ROR1 expression has been documented in various solid and hematologic tumors, including CLL, making it a compelling target for tumor-specific therapy in RS.^{200,201} Vaisitti et al. examined the preclinical impact of the ADC VLS-101, which comprises UC-961, a humanized monoclonal immunoglobulin G1 antibody that binds to an extracellular epitope of human ROR1, linked to the antimicrotubular agent monomethyl auristatin E (MMAE).²⁰² Upon binding to the ROR1 receptor, VLS-101 is internalized and transported to lysosomes, where the drug is released, allowing it to inhibit cell cycle progression and trigger apoptosis. Ex-vivo studies using RS-PDX models have demonstrated that VLS-101 is highly effective in arresting the cell cycle and inducing apoptosis. These promising outcomes were also replicated in murine models,

where a reduction in tumor burden led to increased survival rates in the animals. Notably, VLS-101 exhibited high selectivity, with no clinical effects observed in RS9737, the only ROR1-negative model, and no toxic effects were detected in any of the treated mice. Building on these findings, a phase I clinical trial is currently underway to evaluate VLS-101 in RS and other hematologic malignancies (NCT03833180), though results are not yet available. The second target investigated by Vaisitti et al. in RS using ADCs is CD37, a leukocyte surface antigen of the tetraspanin family.²⁰³ This antigen is abundantly expressed in mature B cells and their malignant counterparts in leukemia/lymphoma. Due to its unique expression pattern in B cells and its absence in normal T cells, NK cells, and plasma cells, CD37 has been recently identified as a potential target for treating CLL and DLBCL.²⁰⁴⁻²⁰⁶ Surface expression of CD37 in both primary samples and RS-PDX models was found to be comparable to levels observed in FL and DLBCL. Flow cytometry and immunohistochemical staining revealed that two of the models (RS1316 and IP867/17) exhibited significantly higher CD37 expression compared to the other two models (RS9737 and RS1050). The efficacy of three different anti-CD37 ADCs was assessed, each utilizing amanitine as a payload—a toxin that, once internalized in CD37-positive cells, binds RNAP II, inhibiting its activity and thus cellular transcription. Treatment of RS cells ex vivo with the three different anti-CD37 ADCs resulted in a marked induction of apoptosis across all PDX models and significantly increased survival in mice treated with a single dose. Furthermore, each of the anti-CD37 ADCs demonstrated high specificity and selectivity, with no observed toxic effects. These findings highlight the preclinical effectiveness of anti-CD37 ADCs, positioning CD37 as a

promising therapeutic target for patients with RS. Additionally, recent research from our group has confirmed the preclinical efficacy of Duvelisib, a dual PI3K δ/γ inhibitor, and Venetoclax, a BCL-2 inhibitor, in RS-PDX models expressing the relevant target molecules.²⁰⁷ As described, the transient and selective inhibition of CDK9 resulted in downregulation of MYC and MCL-1 via the proteasome, inducing apoptosis in both primary and transformed DLBCL cells.¹⁰⁶ This finding, combined with the treatment of AML models with CDK9i and Venetoclax, which also showed induction of apoptosis¹²⁰, provided the rationale for testing this combination in our PDX models. Inhibition of CDK9, with concomitant downmodulation of MCL-1, together with BCL-2 inhibition by Venetoclax, resulted in a robust induction of apoptosis in almost all RS-PDX models, with greater apoptotic effects observed from the combination therapy compared to monotherapy. In in vivo models, the combination treatment led to a significant reduction in tumor volume and increased survival in the mice, particularly in RS1316, RS9737, and IP867. RS1050, which lacks BCL-2, was responsive only to treatment with CDK9i, with in vivo efficacy attributed solely to the action of CDK9i.²⁰⁷ These results underscore the importance of preventive molecular profiling in RS patients to enhance therapeutic efficacy, demonstrating the preclinical effectiveness of combining CDK9i and Venetoclax in treating RS.

3.11 Adaptation of CLL to RS: Shifts in apoptotic dependencies

RS represents the transformation of CLL into a high-grade and more aggressive lymphoma, characterized by a reduced dependence on BCL-2 and a shift towards

alternative anti-apoptotic mechanisms, notably MCL-1, BCL-xL and Bfl-1. In CLL, BCL-2 plays a central role in apoptosis inhibition, supporting cell survival.¹³⁵ However, as CLL progresses to RS, the cells adapt by downregulating BCL-2 and upregulating MCL-1 and BCL-xL, enabling RS cells to better resist apoptosis and contribute to a more aggressive, treatment-resistant phenotype. This adaptation reflects a shift in cellular survival strategies that helps RS cells evade therapies targeting BCL-2, such as Venetoclax, which are effective in CLL but often less so in RS. MCL-1 and BCL-xL stabilize mitochondrial function and enhance resilience under apoptotic stress, underscoring the need for alternative therapies that target these proteins. Inhibitors specific to MCL-1 and BCL-xL have shown potential in promoting apoptosis in RS, offering a promising therapeutic approach for counteracting the aggressive nature of this malignancy (Figure IV, as shown by Rigo et al., Cell Death and Disease, 2024).¹³⁵

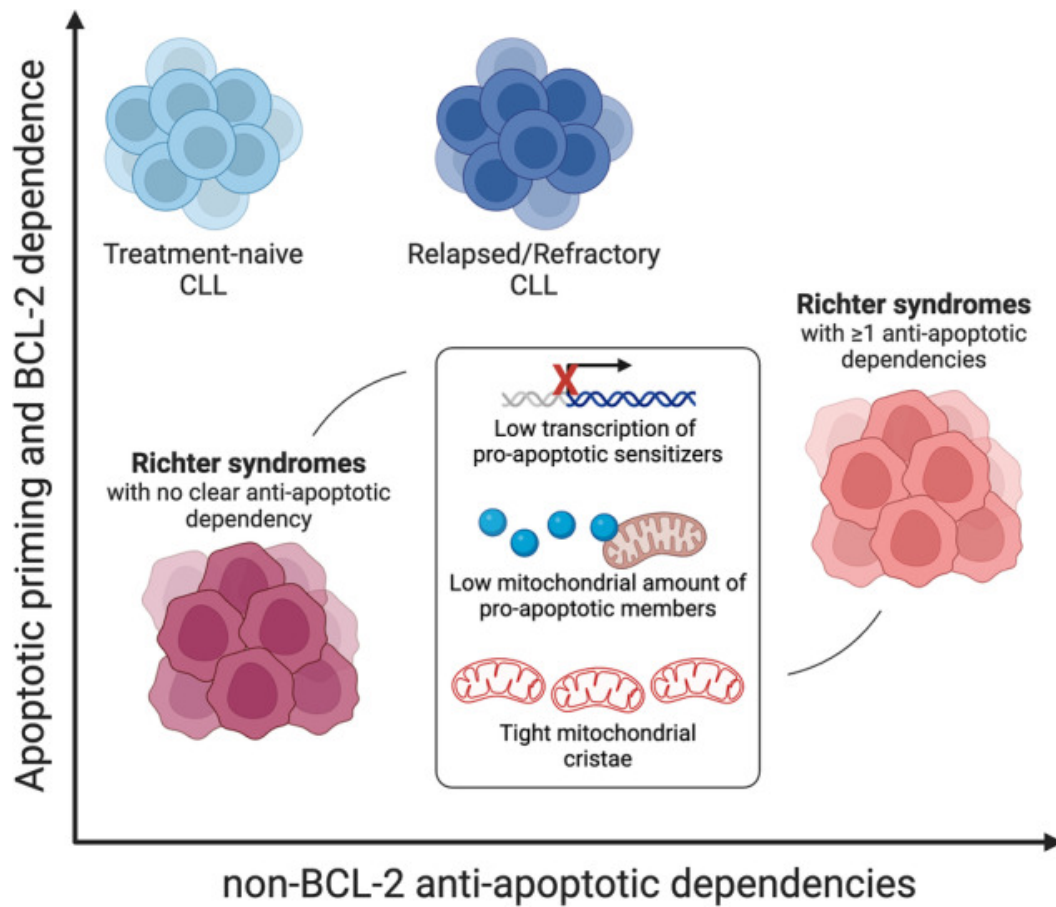


Figure IV: Transition from BCL-2 to BCL-xL during CLL to Richter Transformation. A BCL-2 loss is observed during the transformation of CLL into RS. CLL cells shift to BCL-xL dependence for survival. The figure outlines key stages: BCL-2 expression decreases, while BCL-xL increases, supporting cell proliferation. Moreover, reduced synthesis of pro-apoptotic members occurs, leading to their saturation and inhibition of intrinsic pathway activation. These changes are among the mechanisms underlying the apoptotic resistance CLL cells gain as they transform into RS, contributing to therapy resistance in RS.¹³⁵

Aim of the work

The intrinsic apoptotic pathway plays a pivotal role both in cancer onset and resistance to chemotherapy in different hematological malignancies, including RS. The aim of this project was to identify therapeutic vulnerabilities in the interplay between anti-apoptotic and pro-apoptotic members, trying to reactivate the apoptotic response through the identification of actionable pathways in RS cells.

This work stems from the observation that MCL-1 and BCL-2 are among the principal drivers of resistance in several hematological malignancies due to apoptotic escape and the accumulation of somatic mutations. Consequently, the direct targeting of BCL-2, along with the direct (MCL-1i) or indirect (through CDK9i) targeting of MCL-1, could represent a successful combinatorial strategy. By using four PDX models and the only RS cell line available, U-RT1, we explored the preclinical efficacy of CDK9i, a second-generation CDK9-specific inhibitor currently in phase I clinical trials for hematologic malignancies ¹²⁰, in combination with Venetoclax, a BCL-2 inhibitor approved worldwide for the treatment of CLL and AML ^{62,63,70,71}.

Materials and methods

5.1. Reagents and antibodies

For western blot analyses the following primary antibodies were used: MCL-1(D5V5L) (#39224S, dil. 1:1000), CDK9(C12F7) (#2316S, 1:1000 dilution), BCL-2(D55G8) (#4223S, 1:1000 dilution), BCL-xL (54H6) (#2764S, 1:1000 dilution), pRpb1 CTD (Ser2) (E1Z3G) (#13499S, 1:500 dilution), Rpb1 NTD (D8L4Y) (#14958S, 1:1000 dilution) all from Cell Signaling Technologies (Milan, IT). Anti-ERK (pan ERK) (#610124, 1:5000 dilution) was acquire from BD Biosciences (NJ, USA) and b-Actin (C4) (#sc-47778, 1:2000 dilution) from Santa Cruz Biotechnology (TX, USA). Secondary antibodies as α -Rabbit IgG HRP Linked Whole Ab (Cat. no GEHNA9341ML) and α -Mouse IgG HRP Linked Whole Ab (Cat. no GENA931-1ML) were obtained from Merk (Darmstadt, Hesse, DE). For half-life evaluation Cycloheximide (239763, 10 ug/mL) and MG-132 (Cat. no. M8699) were obtained from Sigma-Alrich (Milan, IT)

5.2. RNA extraction, RT and qRT-PCR

Total RNA was extracted using the Qiagen Kit and retrotranscribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit. qRT-PCRs were performed using MCL1 (Hs01050896_m1), BCL2 (Hs00608023_m1), BCL2L1 (Hs00236329_m1), CDK9 (Hs00176222_m1), and B2M (Hs00984230_m1) primers. For each gene, expression levels were calculated as the ratio of the number of copies of the target gene over 10^5 copies of β 2-microglobulin (B2M) (Δ Ct) and then normalized against Total RNA Control - ($\Delta\Delta$ Ct)- (Cat. no 4307281). All items were obtained from Eurofins Genomics.

5.3. Western blotting

Proteins were resolved by SDS-PAGE and transferred into a 0.2 μ m nitrocellulose Trans-Blot Turbo Transfer membrane using the Trans-Blot Turbo Transfer System (all from Bio-Rad). The optical density (OD) of the bands was determined with the Image Lab 3.0 software (Bio-Rad Laboratories). Protein levels were normalized using the β -actin and pan-ERK.

5.4. EX-vivo treatment of RS cells

RS cells, freshly purified from PDX-RS tumor masses and the U-RT1 cell line, were exposed to various drugs for the indicated time points, depending on the read-out being measured. Specifically, the following treatments were used: CDK9 inhibitor AZD4573 (3, 10, 30, 100, 200 nM; 6, 24, 48, and 72 hours); MCL-1 inhibitor AZD5991 (15, 50, 150, 500, 1000 nM; 6, 24, 48, and 72 hours); and Venetoclax (25 nM; 6, 24, 48, and 72 hours). All indicated concentrations were used for FACS staining, while RS cells were further treated with CDK9i (10, 30, and 100 nM) and Venetoclax (25 nM) and subsequently lysed for Western blot (WB) analysis. All treatments with CDK9i were followed by a washout at 6 hours, after which Venetoclax was re-added in combination treatments. All cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS), referred to as "complete medium," and penicillin/streptomycin (P/S, 100 IU/mL). Additionally, one of the following antibiotics was added: gentamicin (10 mg/mL), kanamycin (50 mg/mL), or tylosin (8 mg/mL).

5.5. Apoptosis evaluation

RS cells were cultured in vehicle (0.1% DMSO), CDK9i, MCL-1i, and Venetoclax, or their combinations (CDK9i + Venetoclax, MCL-1i + Venetoclax), for 6, 24, 48, and 72 hours. Viability was then assessed using the APC-Annexin V/PI staining Apoptosis Kit (ThermoFisher, Milan, IT). Additionally, both siRNA control-treated cells and those treated with siRNA targeting CDK9 were also analyzed for viability. All samples were analyzed by flow cytometry using the FACS Celesta (BD Bioscience, Franklin Lakes, NJ, USA).

5.6. Combination index calculation

The Combination Index (CI) for CDK9i + Venetoclax and MCL-1i + Venetoclax was calculated using the Bliss Independence model. The expected effect for each combination was determined with the Bliss formula: $E_{AB} = E_A + E_B - (E_A \times E_B)$, where E_A and E_B represent the individual drug effects. The observed combined effect was then compared to the Bliss-predicted effect, and CI was calculated as: $CI = E_{AB,observed} / E_{AB}$, with CI values indicating synergy ($CI < 1$), additivity ($CI = 1$), or antagonism ($CI > 1$).

5.7. IC₅₀

IC₅₀ values of CDK9i and MCL-1i in RS cells were calculated based on flow cytometry data after 24 hours of treatment at the six concentrations reported in the treatments section. Concentrations were expressed in molar (M) and converted to base-10

logarithmic values. To calculate the IC_{50} , a dose-response curve was used, specifically the log(inhibitor) vs. response - variable slope (four parameters) model, utilizing GraphPad 9.0.

5.8. Half-life evaluation

Proteins half-life were evaluated by treating RS cells, cultured in RPMI complete medium, with the protein synthesis inhibitor cycloheximide (CHX, 10 μ g/mL) at different time points (0h, 3h, and 9h), either alone or in combination with the proteasome inhibitor MG-132 (10 μ M for 9h). Protein degradation was monitored by measuring the reduction in protein levels over time using Western blot.

5.9. Gene silencing

Knockdown of CDK9 was performed in freshly isolated RS1316 cells at a density of 2×10^6 cells per nucleocuvette. Cells were nucleofected with a mixture of CDK9-specific siRNA (siCDK9, validated/pre-validated) or non-specific siRNA (siCtrl) targeting the sequence 5'-GCUGCUAAUGUGCUUAUCA-3' at a final concentration of 500 nM, obtained from Sigma-Alrich (Milan, IT), using the Lonza 4D Nucleofector Transfection System (Basel, Switzerland). Following nucleofection, RS1316 cells were cultured in complete medium and harvested at a density of 2×10^6 cells per well in 24-well plates after 24 hours for protein extraction or stained for apoptosis evaluation.

5.10. In vivo treatment

RS-PDX models were established and maintained as described by Vaisitti et al.¹⁹⁶. Briefly, RS cells (5×10^6) were resuspended in Matrigel (Becton Dickinson, Milan, IT), injected (double flank) into 8-week-old NOD/SCID/ γ -chain^{-/-} (NSG) mice, and left to engraft. When palpable masses were present (volume of ~ 200 mm), animals were randomly assigned to 4 different groups and treated by intraperitoneal injection with CDK9i Vehicle (Dimethylamine 2%, PEG400 30%, 1% Tween80 68%), CDK9i (30 mg/Kg/ ~ 2 h bis in die, AstraZeneca, Cambridge, UK) alone or in combination, for 2 non-consecutive days, spaced six days apart. Venetoclax (50 mg/Kg/Daily, Selleck Chemicals, TX, USA) alone or in combination, was administered by oral gavage for 10 consecutive days (DMSO 5%, Tween80 5%, PEG300 90%). All reagents are from Sigma-Aldrich (Milan, IT). During drug administration mice weight was monitored, and tumor masses regularly measured twice a week by caliper. Mice were euthanized the day after the last treatment. The Institutional Animal Care and Use Committee approved all the experiments involving mice. Mice were treated following the European guidelines and with the approval of the Italian Ministry of Health (authorization #664/2020-PR).

5.11. Hematoxylin and eosin staining

Hematoxylin and Eosin staining was used to assess the cellular morphology and tissue architecture of RS models, with RS1316 as a representative. After formalin fixation, sections were deparaffinized, rehydrated, and stained with hematoxylin to visualize nuclei in blue-purple, followed by eosin staining for pink cytoplasmic contrast. The

sections were then dehydrated, cleared, and mounted for microscopic examination. Slides were analyzed using an AXIO Lab.A1 microscope (Zeiss) with a Canon EOS600D camera, and images were captured with ZoomBrowserEX software (Canon).

5.12. Statistical analysis

Statistical analyses were performed using paired t-tests for the comparison of two groups, while two-way ANOVA was employed for comparisons involving more than two groups, followed by Tukey's multiple comparison test to identify specific group differences. Statistical significance was indicated with asterisks: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). All analyses were conducted using GraphPad Prism version 9.0, with a significance threshold set at $p < 0.05$.

Results

6.1. Expression of anti-apoptotic molecules in RS cells

The intrinsic apoptosis pathway is primarily regulated by pro- and anti-apoptotic proteins, such as MCL-1, BCL-2, and BCL-xL, which show variable expression across lymphoma subtypes.²⁰⁸ In the present study, we analyzed the expression levels of these key AAPs within the models under investigation. Recent data indicates that transformation from CLL to RS is accompanied by loss of dependence on BCL-2.¹³⁵ We therefore decided to characterize expression of anti-apoptotic proteins in RS-PDX models.¹⁹⁶ RNA-seq data obtained from the 4 PDX models showed that *MCL1* is the gene with the highest expression, while *BCL2* expression was generally lower (Figure 1A). As expected, based on previous results, *BCL2* was completely absent in RS1050, which overexpressed *BCL2L1*. We also determined that *CDK9* was invariably present in the four models. We confirmed these expression patterns both the mRNA and protein levels including a recently established RS cell line, U-RT1²⁰⁹, which exhibited reduced levels of MCL-1 at (Figure 1B-C). These findings indicate that MCL-1 is consistently expressed in RS cells despite genetic variability, prompting further exploration of the turnover dynamics of MCL-1 and other AAPs to clarify their role in RS.

6.2. Stability of apoptotic targets and CDK9 in RS cells

Protein turnover is influenced by several factors, including the absence of certain extracellular signals in ex vivo cultures, which can impact the viability of cells over time. As a result, the temporal window for studying critical survival molecules, such as AAPs,

is limited, necessitating focused evaluation of their stability within the experimental timeframe. To assess the stability of these molecules over time, we cultured RS cells for 6, 24, and 48 hours in complete medium and analyzed expressions of BCL-2, BCL-xL, MCL-1, and CDK9 by qRT-PCR and Western blotting (Figure 2A-B). Results indicate that, despite some fluctuations, AAPs were consistently expressed until the end of the cultures. Specifically, MCL-1, BCL-2, and CDK9 maintained relatively stable protein levels (Figure 2A), even though real-time analysis revealed variations at the transcript level. In contrast, BCL-xL displayed greater stability in its mRNA, but showed more variability at the protein level (Figure 2B). Importantly, none of these fluctuations at the protein level reached statistical significance, suggesting that, overall, the stability of these anti-apoptotic proteins was maintained despite the observed differences between transcript and protein levels. Moreover, these results demonstrate that, despite the intrinsic limitations of PDX models, they allowed us to effectively assess protein turnover under basal conditions providing us a foundation for subsequent studies aimed at determining the half-life of the proteins under investigation.

6.3. MCL-1 protein has a short half-life, and its expression is tightly regulated in RS cells

It is well-known that MCL-1 has a short half-life, undergoing degradation through the proteasome and continuous re-synthesis through active transcription, therefore exhibiting a tight regulation.^{36,210} These findings were confirmed in our experimental models by using the protein synthesis inhibitor cycloheximide (CHX, 10 ug/mL for 0h, 3h,

and 9h) alone or in combination with the proteasome inhibitor MG-132 (10uM for 9h), in U-RT1 and RS-PDX cells (Figure 3A-C). In these conditions, ribosome inhibition by CHX led to a significant accumulation of *MCL1* and *CDK9* transcripts at 3 hours in 3 out of 5 models, with *MCL1* accumulation persisting at 9 hours in RS1316 and IP867/17. On the contrary, *BCL2* and *BCL2L1* did not show significant changes (Figure 3A). qRT-PCR data were complemented by biochemical analysis under the same conditions, revealing a dramatic and significant downregulation of MCL-1 after 3 hours of treatment, more pronounced at 9 hours, in all the models. Upon proteasome inhibition with MG-132, MCL-1 levels were fully restored, indicating that its rapid degradation is mediated through the proteasome pathway. In contrast, none of the other proteins exhibited the same level of down-regulation, suggesting higher stability under basal conditions. Specifically, CDK9 demonstrated the greatest stability across all models at each time point, while BCL-2 and BCL-xL displayed more heterogeneous expression patterns. Notably, BCL-2 was nearly absent in RS1050, consistent with both transcriptomic data and previous findings.²⁰⁷ Additionally, BCL-xL did not show any recovery following MG-132 treatment, suggesting that its degradation is not mediated by the ubiquitin-proteasome pathway. It is important to note that under these conditions, MCL-1 is the only target that systematically declines to protein levels after 3 hours of treatment, demonstrating a short half-life. Moreover, these data show tight regulation of MCL-1 to maintain high levels necessary for RS apoptosis suppression. Together these data suggest that MCL-1 may be a good therapeutic target, both through specific inhibitors and by inhibiting its transcription through CDK9 inhibitors.

6.4. CDK9 Inhibition drives specific depletion of MCL-1 in RS cells, highlighting potential for therapeutic application

The elongation phase of RNAP II is regulated by the phosphorylation of serine 2 and serine 5 residues (Ser2, Ser5) in the CTD of its catalytic subunit, Rpb1, by CDK9, a component of the P-TEFb complex. This regulation establishes CDK9 as a central hub for transcriptional control of short half-life molecules such as MCL-1. In this context, we assessed the effects of CDK9i and MCL-1i on RS cells after 6 hours. Treatment of RS cells with CDK9i at 100 nM for 24 hours led to a transcriptional blockade, resulting in a significant reduction in the transcripts of all AAPs, including *MCL1*, *BCL2*, *BCL2L1*, and *CDK9* itself (Figure 4A). Notably, there was a five-fold decrease in *MCL1* transcript levels in the RS1316 model. Following CDK9i treatment, biochemical analyses revealed a specific shutdown of MCL-1, accompanied by the loss of the phosphorylated form of Rpb1, without significant impact on the other AAPs, which remained expressed at high levels except in RS1050 (Figure 4B). Despite the general decline in transcript levels, only MCL-1 showed a dramatic reduction at the protein level. In contrast, treatment with MCL-1i at 500 nM for 6 hours, used as a control as it was not expected to modulate any other targets, did not result in meaningful protein modulation, as anticipated. The only exception was observed in U-RT1 cells, which inherently express the lowest levels of MCL-1 among RS cells, where an accumulation of MCL-1 was noted post-treatment (Figure 4B).

These pharmacological findings substantiate that CDK9 inhibition specifically targets Rpb1 phosphorylation across all models, reinforcing our strategy to affect short-half-life

proteins such as MCL-1. Moreover, these results underline the critical role played by CDK9 in regulating RNAP II activity and highlight the transient stability of MCL-1. Collectively, the results connect acute CDK9 inhibition to the rapid depletion of MCL-1 in RS cells, emphasizing the potential of CDK9 inhibitors as therapeutic agents.

6.5. CDK9i and MCL-1i impair RS cells viability and induce reactivation of intrinsic apoptotic machinery

To further investigate the effects of CDK9 and MCL-1 inhibition, RS cells were treated as described above (CDK9i at 100 nM; MCL-1i at 500 nM for 6h) to evaluate induction of apoptosis. Western blot analysis of Caspase-3 and PARP cleavage, markers of apoptosis activation, demonstrated significant anti-tumor activity for both inhibitors, particularly in RS1316, RS9737, and IP867/17, with CDK9i being effective at doses fivefold lower than MCL-1i (Figure 5A). RS1050 also exhibited sensitivity, albeit with greater variability among replicates and more pronounced cleavage in response to CDK9i. Following these preliminary results on apoptosis reactivation, a significant decrease in cell viability was observed in nearly all RS cells treated with lower concentrations of MCL-1i (50-150 nM) (Figure 5B-C) and CDK9i (10-30 nM) (Figure 5D-E) for 6 and 24 hours and detected by flow cytometry. In contrast, U-RT1 cells, as shown by western blot analysis (Figure 5A), were completely unresponsive 6 hours post-treatment with both drugs at the tested doses. This was further confirmed by flow cytometry, where U-RT1 cells remained insensitive to both drugs at 6 and 24 hours across all tested doses (data not shown). Due to this lack of response, the treatment duration was extended to 48 and 72 hours (Figure

5B, C). At these extended time points, a significant induction of apoptosis was observed only following CDK9i exposure, while the cells remained entirely resistant to MCL-1i, consistent with the lower basal expression of the target (Figure 1B, C). To determine the optimal strategy for MCL-1 inhibition and to identify an approach that also targets U-RT1 cells, we subsequently focused determining the IC_{50} values and the lowest effective doses of the drugs. IC_{50} values for both drugs was calculated based on flow cytometry data obtained after 24 hours of treatment (Figure 5F). As expected, CDK9i demonstrated greater potency, with IC_{50} values of 1.85 nM for RS1316, 2.84 nM for RS9737, 2.85 nM for IP867/17, and 3.56 nM for RS1050, resulting in a mean of 2.775 nM across all models. In contrast, only RS1316 showed a significant IC_{50} for MCL-1i, with a value of 4.96 nM. Notably, U-RT1 cells remained insensitive to both drugs at all tested doses at this time point. Notably, CDK9 inhibition also significantly impacts cell viability, with effects observed at both low concentrations (10 and 30 nM) and across different time points. In conclusion, both drugs reactivated apoptosis, but CDK9i demonstrated superior potential compared to MCL-1i, showing efficacy even in cells that were initially insensitive and required prolonged exposure times. CDK9i achieved similar effects at a five-fold lower dose in biochemical assays and showed greater potency in reducing cell survival at lower concentrations. This dose-response profile makes CDK9i a more suitable option for combination therapies, offering increased flexibility in designing treatment strategies and enhancing therapeutic efficacy, particularly against resistant cells. These findings provide a strong rationale for further development and optimization of combinatorial approaches.

6.6. siRNA-mediated validation of CDK9 inhibition effects

To validate the results obtained from pharmacological inhibition and prove the molecular link between CDK9 and MCL-1, silencing CDK9 via siRNA was performed. Freshly purified RS1316 cells were nucleofected with a mix of CDK9-specific siRNAs at a final concentration of 500 nM and maintained in culture for 24 hours. Depletion of CDK9 transcripts led to a concomitant reduction in both CDK9 protein levels and, indirectly, MCL-1, in line with the pharmacological inhibition of CDK9 (Figure 6A). On the contrary, CDK9 silencing did not impact the expression of other targets, including BCL-2. The specificity of the targeting was confirmed by transfection with a control siRNA that did not result in any significant changes. Additionally, RS1316 cells nucleofected with siCDK9 exhibited the same apoptotic activation pattern previously described (Figure 5A, D, and E), with pronounced cleavage of Caspase-3 and PARP detected by Western blotting (Figure 6B) and flow cytometry (Figure 6C). This led to a significant reduction in cell viability compared to both untreated cells and the siRNA control. These results demonstrate that both pharmacological and molecular approaches converge on the specific depletion of MCL-1 downstream of CDK9 inhibition, while largely preserving the expression pattern of other AAPs. Furthermore, this inhibition mirrors the reactivation of intrinsic apoptosis in RS cells, strengthening the pharmacological data and suggesting that these findings can be further developed in a combinatorial approach.

6.7. Synergy of CDK9 and BCL-2 inhibition in RS cells: rationale for in vivo testing

Given that MCL-1 is modulated by CDK9, and by inhibiting its synthesis using CDK9i, we demonstrated a generalized collapse in AAP synthesis, which partially explains the incomplete effect of the inhibitors on cell survival. These results prompted us to test CDK9i in combination with the BCL-2 inhibitor Venetoclax to block the main anti-apoptotic pathways. RS cells were treated with CDK9i at 10 and 30 nM combined with Venetoclax at 25 nM for 24 hours. Flow cytometry data showed a robust and significant decrease in cell viability in U-RT1 and RS1316 at both doses and enhanced efficacy in RS9737 and IP867/17. At variance with the other models and in line with the lack of BCL-2, RS1050 did not benefit from the combination of the two drugs, with results overlapping to the CDK9 monotherapy (Figure 7A). Similar results were obtained at the biochemical level, looking at Caspase-3 and PARP cleavage post-treatment. Both molecules were strongly activated across all models, particularly in U-RT1, and even enhanced in RS1316, where the cleaved form disappeared due to rapid apoptosis reactivation. Consistent with the flow cytometry data, RS1050 did not benefit from the combination treatment (Figure 7B). The phosphorylated form of Rpb1 was significantly inhibited at 10 nM and even more at 30 nM. The same impacted profile was highlighted for MCL-1, which remained consistently expressed following Venetoclax treatment, confirming the specificity of CDK9 targeting. BCL-2, BCL-xL, and CDK9, however, were regularly expressed in the other models that express them, except in RS1316, where the effect of CDK9i, both in monotherapy and in combination, significantly impaired cell viability, supported by biochemical and flow cytometry data (Figure 7B).

The combination index (CI) showed strong synergy between the drugs, particularly in U-RT1, with a CI value of 0.216 (CDK9i 10 nM + Ven 25 nM) and 0.223 (CDK9i 30 nM + Ven 25 nM), followed by RS1316 (0.538- 0.584), RS9737 (0.421-0.611) and IP867/17 (0.437 - 0.512). In contrast, CI values for RS1050 were 1.099 and 1.072, respectively, indicating lack of synergy between the two drugs in this model due to the absence of Venetoclax effects (Figure 7C).

In conclusion, the data demonstrates that CDK9i is effective at the lowest doses as monotherapy in PDX models, and its combination with Venetoclax is significantly effective in the resistant cell line. Moreover, Venetoclax ineffectiveness in RS1050, due to the absence of BCL-2, underscores the importance of profiling AAP molecules in RS patients to guide the selection of appropriate therapeutic strategies. Finally, these findings provide us with the rationale for testing the in vivo efficacy of the combinatorial treatment.

6.8. CDK9i and Venetoclax combination significantly reduces tumor progression in BCL-2 RS-PDX-expressing models

The efficacy of the CDK9i/Venetoclax drug combination was finally validated in vivo using both RS-PDXs and U-RT1 xenograft models. RS cells were subcutaneously injected in 8-week-old male NSG mice and left to engraft until tumor masses were approximately 200 mm³. Mice were then randomized in 4 groups and treated with Venetoclax (50mg/kg for ten consecutive days) or CDK9i (30 mg/kg, two doses administered the same day, and two cycles of treatment in total). At the end of the treatment, mice were euthanized and tumor masses recovered. In four out of five models, we observed that the combination

treatment was significantly more effective than the individual treatments, with a strong tumor growth reduction in RS1316, where tumors were no more detectable (Figure 8A). More precise measurements of tumor volume were taken at the end of the experiments, largely reflecting tumor growth (Figure 8B). U-RT1 demonstrated sensitivity to all treatments, with a significant reduction in tumor volume observed in the combination group compared to the vehicle (fourfold decrease) and Venetoclax groups (2.5-fold decrease). No significant difference was found compared to CDK9i alone, which was already effective. Similar outcomes were obtained for RS1316, where one of the combination-treated tumors went into complete remission, along with an average fourfold reduction in volume. For RS9737, tumor volume in the combination group was reduced fiftyfold compared to all other experimental groups. In IP867/17, tumor volume decreased threefold relative to the control group. Notably, in RS1050, the combination treatment had no effect, both in terms of tumor growth kinetics and tumor volume at the end of the experiment, as the results of CDK9i were comparable to the combination, while Venetoclax monotherapy showed results similar to the vehicle group (Figure 8B). Longitudinal time-course data were accompanied by histological analysis of one of the representative disease models, RS1316, where the combination treatment displayed significant areas of necrosis characterized by loss of cellular structure compared to the other experimental groups (Figure 8C). Although significant tumor reduction was observed across multiple RS models, survival studies were conducted on two models, RS1316 and RS9737 (data not shown). The results indicated that the combination treatment did not significantly extend survival beyond that of the vehicle or

monotherapy groups. These in vivo results confirmed that the combined use of CDK9i and Venetoclax is significantly more effective than individual treatments in reducing tumor progression in models expressing BCL-2. Although its promising translational application is supported, further optimization of the treatment schedule is required. Furthermore, these findings underscore the importance of targeted therapy tailored to the specific characteristics of the patient's Richter syndrome.

Discussion

This study provides preclinical evidence for the therapeutic potential of co-targeting CDK9 and BCL-2. Recent findings indicate that during the transformation of CLL into RS, there is a significant decrease in BCL-2 dependency, suggesting a deregulation of apoptosis in RS. Unlike CLL cells, which are characterized by maximal apoptotic priming and strong BCL-2 dependence, RS cells exhibit a substantial reduction in these traits. This transformation leads to progressive independence from BCL-2, rendering BCL-2 inhibitors like Venetoclax less effective as monotherapy.¹³⁵ These data suggest the need to explore the efficacy of combination therapies to overcome potential resistance mechanisms occurring during transformation.

In previous work, our research group investigated the potential of a combination approach involving Duvelisib, a dual PI3K- δ/γ inhibitor, with Venetoclax. This combination induces a strong synergistic apoptotic response in RS cells expressing PI3K- δ/γ and BCL-2. While assessing the expression of anti-apoptotic proteins in our RS-PDX models, an increased expression of MCL-1 was highlighted. The dual targeting of these pathways leads to GSK3 β activation, degradation of MCL-1 and MYC, and a subsequent increase in apoptosis, effectively overcoming resistance to BCL-2 inhibitors in RS models.

²⁰⁷ Based on these findings, we explored the expression of key AAPs in RS-PDX models as well as the U-RT1 cell line. RNA-seq data revealed that *MCL1* had the highest expression, while *BCL2* was generally expressed at lower (Figure 1A), with *CDK9* consistently expressed across all four models. In line with previous findings, *BCL2* was completely absent in the RS1050 model¹⁹⁵, which instead overexpressed *BCL2L1*. We

confirmed these expression patterns in ex vivo PDX cells and included the recently established RS cell line, U-RT1, which exhibited reduced levels of MCL-1 at both the transcript and protein levels (Figure 1B, C). These results suggest that the heterogeneity of RS cells leads them to favor other anti-apoptotic proteins, such as BCL-xL in the case of RS1050, or MCL-1, which is consistently expressed, indicating that it could therefore serve as a potential therapeutic target. We also noticed that, as is well known in the literature and as described in the introduction, MCL-1 has a short half-life due to rapid turnover regulated by phosphorylation, allowing quick adjustments in its levels to control cell survival and apoptosis.^{28,29} This observation laid the foundation for exploring a combined targeting strategy of MCL-1 and BCL-2, drawing inspiration from a similar approach applied in DLBCL by Moujalled et al., which was shown to be tolerable in murine models of DLBCL.⁷⁹ In this context, MCL-1 targeting can be directly achieved using a selective inhibitor as MCL-1i⁷⁵, or indirectly by targeting its transcription through specific CDK9 inhibition. The use of CDK9i, currently in phase I trials for hematological malignancies (NCT03263637),¹²⁰ validated the viability of the indirect approach for targeting MCL-1. Building on this speculation, we observed suppression of Rpb1 phosphorylation at 100 nM CDK9i, resulting in the downregulation of AAP transcripts. While mRNA levels for all AAPs decreased, only MCL-1 was downregulated at the protein level, unlike other stable molecules. Subsequent investigation of the two strategies—CDK9i as an indirect approach and MCL-1i as a direct approach—used in monotherapy revealed that both could induce significant apoptosis in RS cells, with CDK9i proving effective at lower doses in PDX models, while U-RT1 cells showed complete insensitivity

to MCL-1i. Apoptosis data further underscored a strong dose- and time-dependent response to CDK9i, which exhibited greater potency with IC50 values ranging from 1.85 to 3.56 nM, indicating its superior efficacy and potential for combination therapies. Pharmacological inhibition was subsequently validated at the molecular level using transient siCDK9 inhibition, where, after 24 hours, a reduction in CDK9 and MCL-1 levels was observed, with BCL-2 remaining unchanged (Figure 6A). This depletion effectively mimicked the pharmacological effects of CDK9 inhibition, both in terms of AAP expression and the apoptotic pattern, including Caspase-3 activation, PARP cleavage, and reduced cell viability (Figure 6B, C), reinforcing the pharmacological data for combinatorial strategies. The study later demonstrated that combining low doses of CDK9i with Venetoclax at a reduced concentration strongly activated caspase and PARP signatures as early as 6 hours (Figure 7B) and significantly reduced cell viability at 24 hours in ex vivo PDX models expressing BCL-2 and in U-RT1 cells (Figure 7A), where the CI value for the CDK9i 10 nM + Ven 25 nM combination was 0.216 (Figure 7C). In contrast, the CI value for both combinations in RS1050 was >1. The observed synergy suggested that this combination could be a potent therapeutic approach and provided the rationale for in vivo testing of this combinatorial therapy in our models. Finally, in vivo, the combination of CDK9i and Venetoclax proved significantly more effective than either treatment alone in reducing tumor growth in most models. In RS1316, the combination treatment caused such a strong reduction in tumor size that the tumor became impalpable (Figure 8A). Tumor volume measurements confirmed this finding, with U-RT1 also showing a marked response to the combination therapy (Figure 8B). However,

in RS1050, which lacks BCL-2 expression, the combination offered no additional benefit, yielding results similar to CDK9i alone, while Venetoclax had no effect (Figure 8B). Histological analysis of RS1316 tumors revealed extensive necrosis in the combination treatment group, indicating a more profound impact on tumor cell viability (Figure 8C). Considering the results obtained, some important considerations are warranted. The transient inhibition of CDK9 affects transcription broadly, potentially causing toxicity, which required reducing treatment frequency. Additionally, the *in vivo* response was limited in duration and highly dependent on continuous drug administration. A further limitation concerns the 20-25% of RS patients who do not express BCL-2; as demonstrated in our data, the addition of Venetoclax in RS1050 was ineffective, emphasizing the necessity of molecular profiling prior to treatment to ensure the presence of BCL-2 for effective therapy.

Conversely, these preclinical results indicate that the combination of CDK9i and Venetoclax holds significant promise for treating Richter syndrome, particularly in BCL-2-expressing models. Key advantages include increased efficacy compared to many other *in vitro* approaches, the ability to exploit vulnerabilities of short half-life proteins overexpressed in RS, such as MCL-1, MYC, and Bcl-1, and the capacity to force tumor cells to rely on less efficient anti-apoptotic pathways. Moreover, the combination's efficacy at lower concentrations reduces the need for high drug doses, potentially enhancing tolerability.

Overall, these findings indicate that co-targeting CDK9 and BCL-2 could be an effective strategy, allowing for reduced drug concentrations and providing an alternative to direct MCL-1 inhibition. This underscores the potential of combinatorial strategies to improve therapeutic efficacy in RS. Further research is needed to optimize this approach, offering new hope for the treatment of resistant lymphomas.

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Abbreviations Used

AAP = Anti-apoptotic protein
ADCs = Antibody-drug conjugate
AIF = Apoptosis-inducing factor
AML = Acute myeloid leukemia
Apaf-1 = Apoptotic Protease Activating Factor 1
AZD4573 = CDK9 inhibitor
AZD5991 = MCL-1 inhibitor
BAD = BCL-2-associated death promoter
BAK = BCL-2 homologous antagonist/killer
BAX = BCL-2-associated X protein
BCL-2 = B-cell lymphoma 2
BCL-2i = B-cell lymphoma 2 inhibitor
BCL-w = BCL-2-like 2
BCL-xL = B-cell lymphoma extra-large
Bfl-1 = BCL-2-related protein A1
BH = BCL-2 homology
BID = BH3-interacting domain death agonist
BIK = BCL-2 interacting killer
BL = Burkitt lymphoma
BOK = BCL-2 related ovarian killer
BT = Blastic Transformation
BTK = Bruton's tyrosine kinase
CDK1/2/4/6/7/9 = Cyclin-dependent kinase 1, 2, 4, 6, 7 and 9
CDK9i = Cyclin-dependent kinase 9 inhibitor
CI = Combination index
CLL = Chronic lymphocytic leukemia
CML = Chronic myeloid leukemia
CPC = Common precursor cell
Cyt C = Cytochrome C
CTD = Carboxy-terminal domain
DLBCL = Diffuse large B-cell lymphoma
ECOG = Eastern Cooperative Oncology Group
ECOG PS = ECOG performance status
ERK = Extracellular signal-regulated kinase
FCS = Fetal calf serum
FL = Follicular lymphoma
GSK3 = Glycogen Synthase Kinase 3
HL = Hodgkin lymphoma
IMM = Inner mitochondrial membrane

JAK = Janus kinase
JNK = c-Jun N-terminal kinase.
LDH = Lactate dehydrogenase
LRP4 = Lipoprotein receptor-related protein 4
mAb = Monoclonal antibody
MAPK = Mitogen-activated protein kinase
MCL = Mantle cell lymphoma
MCL-1 = Myeloid cell leukemia sequence 1
MCL-1i = Myeloid cell leukemia sequence 1 Inhibitor
MOMP = Mitochondrial outer membrane permeabilization
MM = Multiple myeloma
MZL = Marginal zone lymphoma
NOXA = Phorbol-12-myristate-13-acetate-induced protein 1
NSG = NOD/SCID gamma
NOTCH-1 = Neurogenic locus notch homolog protein 1
OMM = Outer mitochondrial membrane
ORR = Overall response rate
OS = Overall survival
P/S = Penicillin/Streptomycin
PARPs = Poly ADP-ribose polymerases
PAP = Pro-apoptotic protein
PDB = Protein data bank
PDX = Patient derived xenograft
PEST = Proline (P), Glutamic acid (E), Serine (S), Threonine (T) sequence
PET = Positron emission tomography
PFS = Progression-free survival
PI3K = Phosphoinositide 3-kinase
pRpb1 = Phospho-RNA polymerase II subunit B1
P-TEFb = Positive transcription elongation factor b
qRT-PCR = Quantitative reverse transcription polymerase chain reaction
R-CHOP = Rituximab, Cyclophosphamide, Doxorubicin, Vincristine, Prednisone
RCD = Regulated cell death
ROR1 = receptor tyrosine kinase-like orphan receptor 1
RNAP II = RNA polymerase II
Rpb1 = RNA polymerase II subunit B1
RS = Richter syndrome
SCLC = Small cell lung carcinoma
SCT = Stem cell transplantation
SLL = Small lymphocytic lymphoma
TPM = Transcripts per million
Ven = Venetoclax
WB = Western Blot
XIAP = X-linked inhibitor of apoptosis protein

Figures

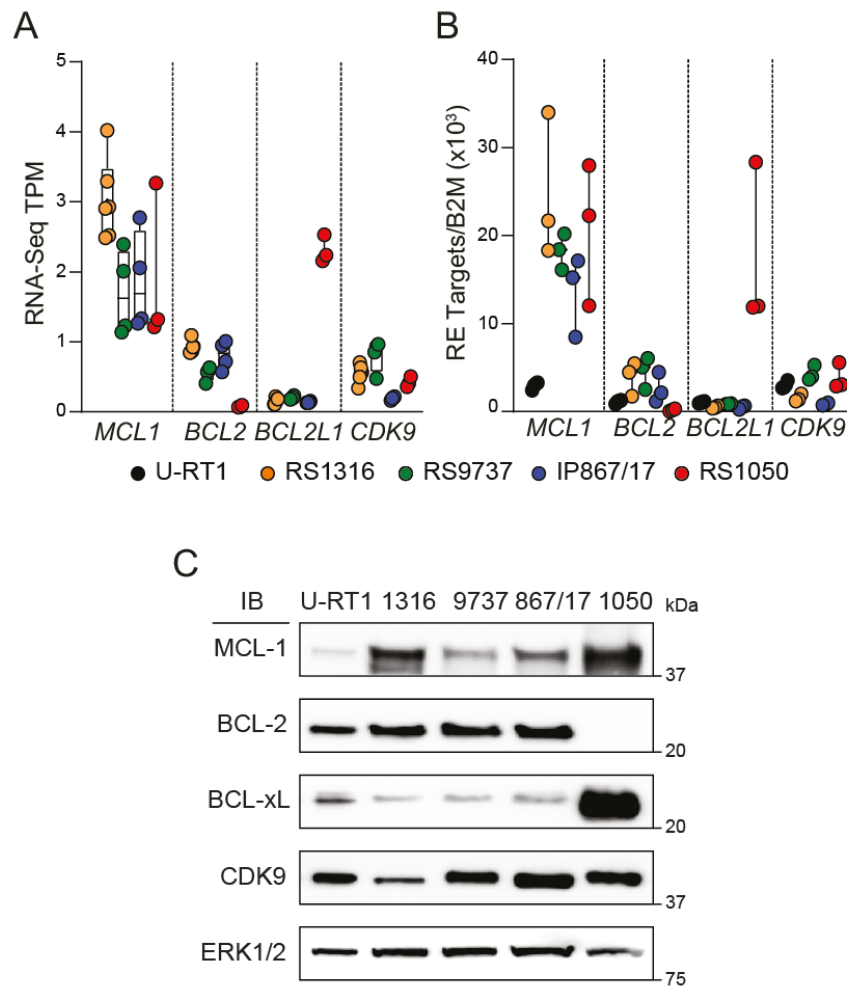


Figure 1: Differential expression of anti-apoptotic members across RS-PDX models and U-RT1 cell line. (A) RNA-seq and (B) qRT-PCR ($n=3$) analyses were performed on four RS-PDX models taken at different passages, illustrating differential expression of CDK9 and key anti-apoptotic proteins under basal conditions, with β -2-microglobulin (*B2M*) used as a control, and then normalized on Universal RNA (U-RT1: black dots; RS1316: yellow dots; RS9737: green dots; IP867/17: blue dots; RS1050: red dots). (C) Western Blot panel ($n=3$) of RS cells freshly purified from RS-PDX mice and U-RT1 cell line under basal condition showing expression of MCL-1, BCL-2, BCL-xL and CDK9 with ERK1/2 used as a loading control in western blot.

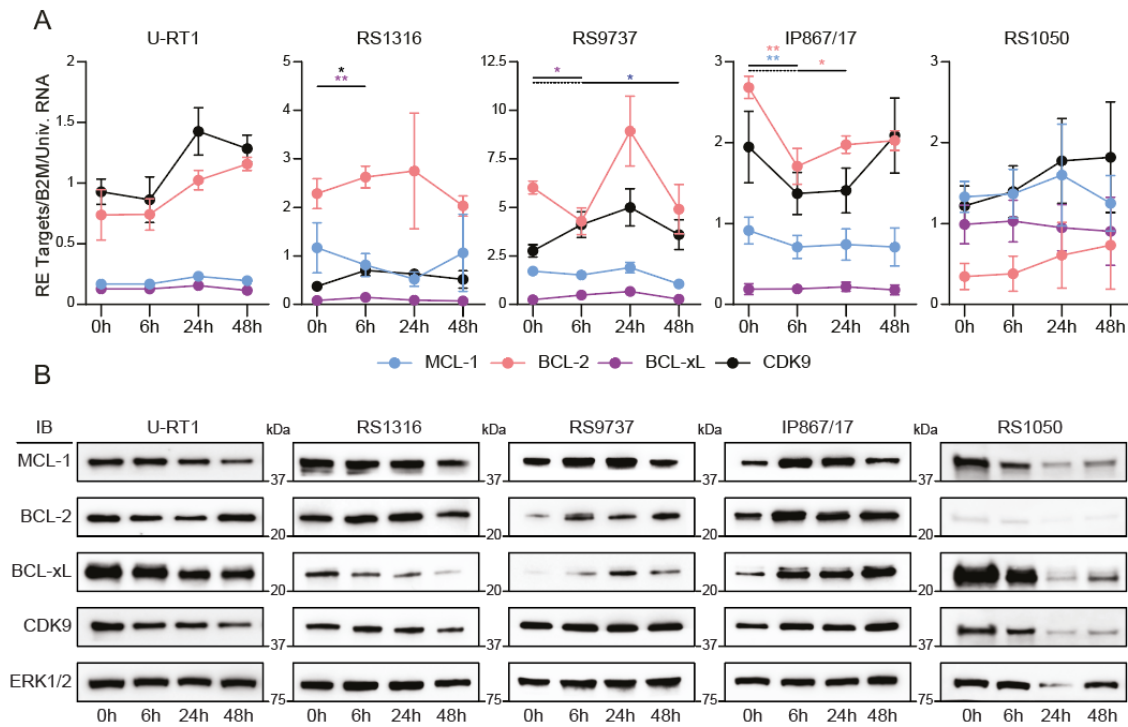


Figure 2: Stability of anti-apoptotic protein expression in cultured PDX models and U-RT1 cells over time. (A) qRT-PCR (n=3) analysis of *MCL1* (light blue), *BCL2* (pink), *BCL2L1* (purple) and *CDK9* (black) in RS cells freshly purified from RS-PDX mice and U-RT1 cell line after 6, 24, and 48 hours of culture in complete RPMI medium, with β -2-microglobulin (*B2M*) used as a control then normalized on Universal RNA and presented as mean $\pm$ SEM. (B) Western Blot (n=3) analysis of the same target over the same time points in RS cells freshly purified from RS-PDX mice and U-RT1 cell line, with ERK1/2 used as a loading control in western blot. The p-value was calculated using a multiparametric ANOVA test to compare treated groups with controls. Statistical significance was represented as $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

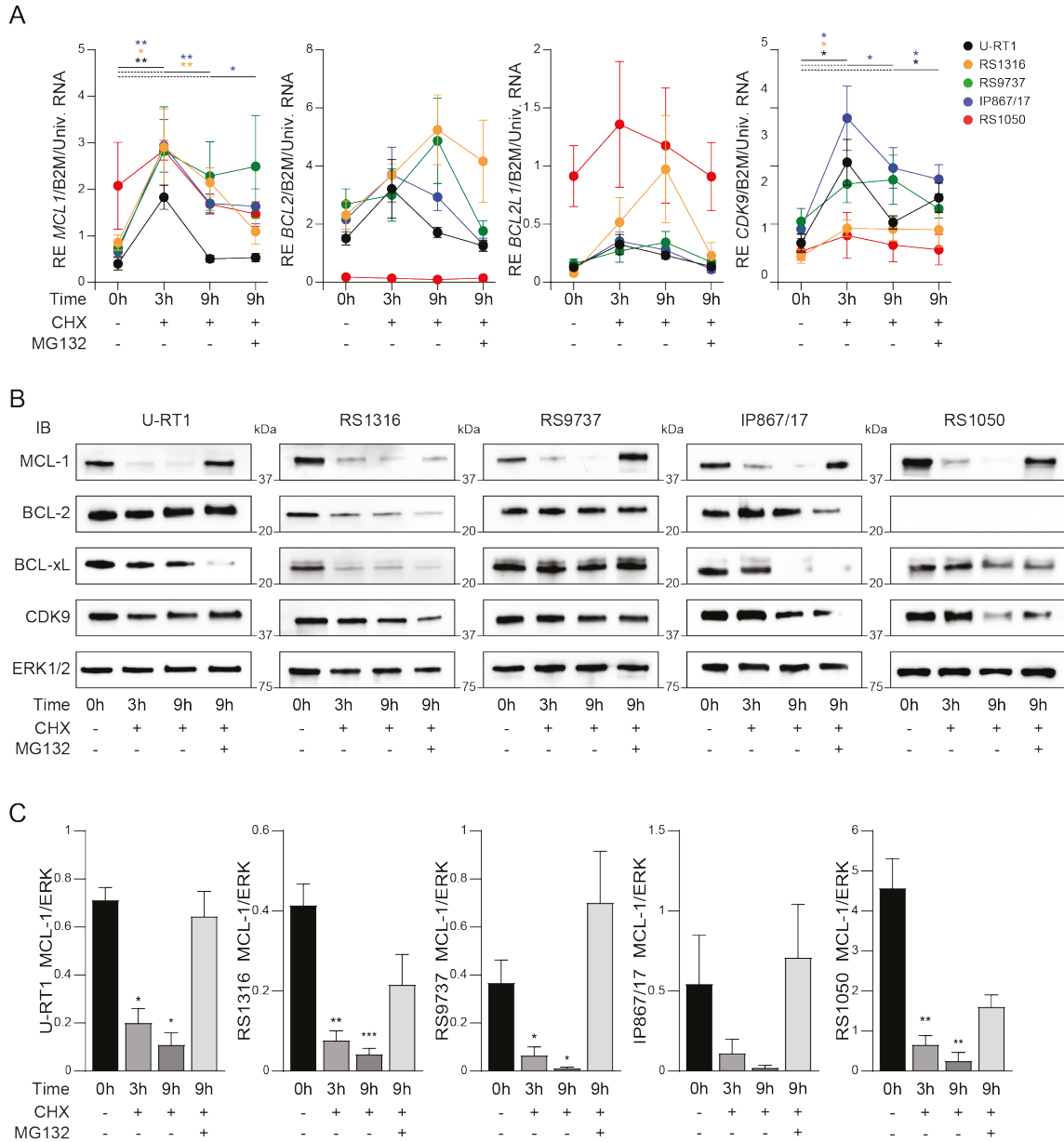


Figure 3: Regulation of MCL-1 degradation and stability in RS cells. Half-life evaluation of *MCL1*, *BCL2*, *BCL2L1* and *CDK9* using CHX+MG132 in (A) qRT-PCR (n=4) of freshly purified from RS-PDX mice and U-RT1 cell line, treated for 3h and 9h with CHX (10ug/mL) 9h with CHX (10ug/mL) + MG132 (10uM), with β -2-microglobulin (*B2M*) used as a control then normalized on Universal RNA and presented as mean $\pm$ SEM. (U-RT1: black lines; RS1316: yellow lines; RS9737: green lines; IP867/17: blue lines; RS1050: red lines). The RNA was extracted, quantified, and reverse-transcribed for qRT-PCR analysis. (B) Western Blot (n=3) analysis of the corresponding protein in the same experimental setting in RS cells freshly purified from RS-PDX mice and U-RT1 cell line, with ERK1/2 used as a loading control in western blot. (C) WB quantifications (n=3) were performed using ImageLab software and presented as mean $\pm$ SEM. The p-value was calculated using a multiparametric ANOVA test to compare treated groups with controls. Statistical significance was represented as $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

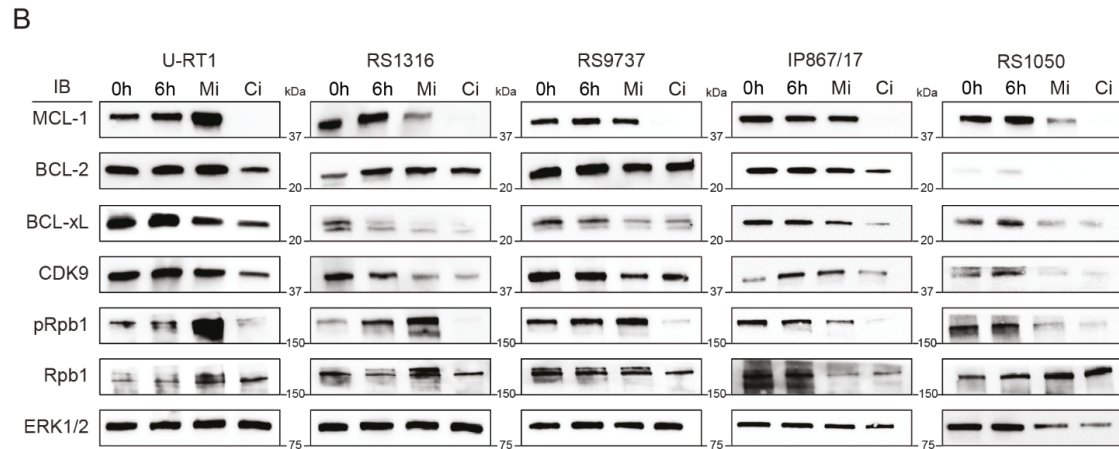
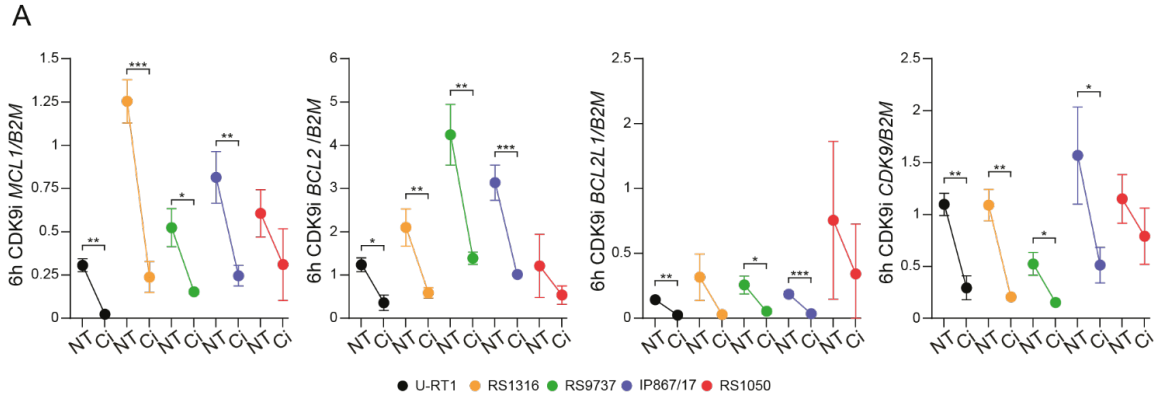


Figure 4: Impact of CDK9 and MCL-1 inhibition on transcript and protein expression in RS cells. (A) qRT-PCR analysis of AAPs and CDK9 mRNA expression in RS cells after 6 hours of treatment with 100 nM CDK9 inhibitor (CDK9i, Ci), with β -2-microglobulin (*B2M*) used as a control then normalized on Universal RNA and presented as mean $\pm$ SEM. (NT= non-treated cells) (U-RT1: black lines; RS1316: yellow lines; RS9737: green lines; IP867/17: blue lines; RS1050: red lines). (B) Western Blot (n=3) analysis of the corresponding protein expression in RS cells after 6 hours of treatment with 100 nM CDK9 inhibitor and 500 nM MCL-1 inhibitor (MCL-1i, Mi), with ERK1/2 used as a loading control in western blot. The p-value was calculated using a multiparametric ANOVA test to compare treated groups with controls. Statistical significance was represented as $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

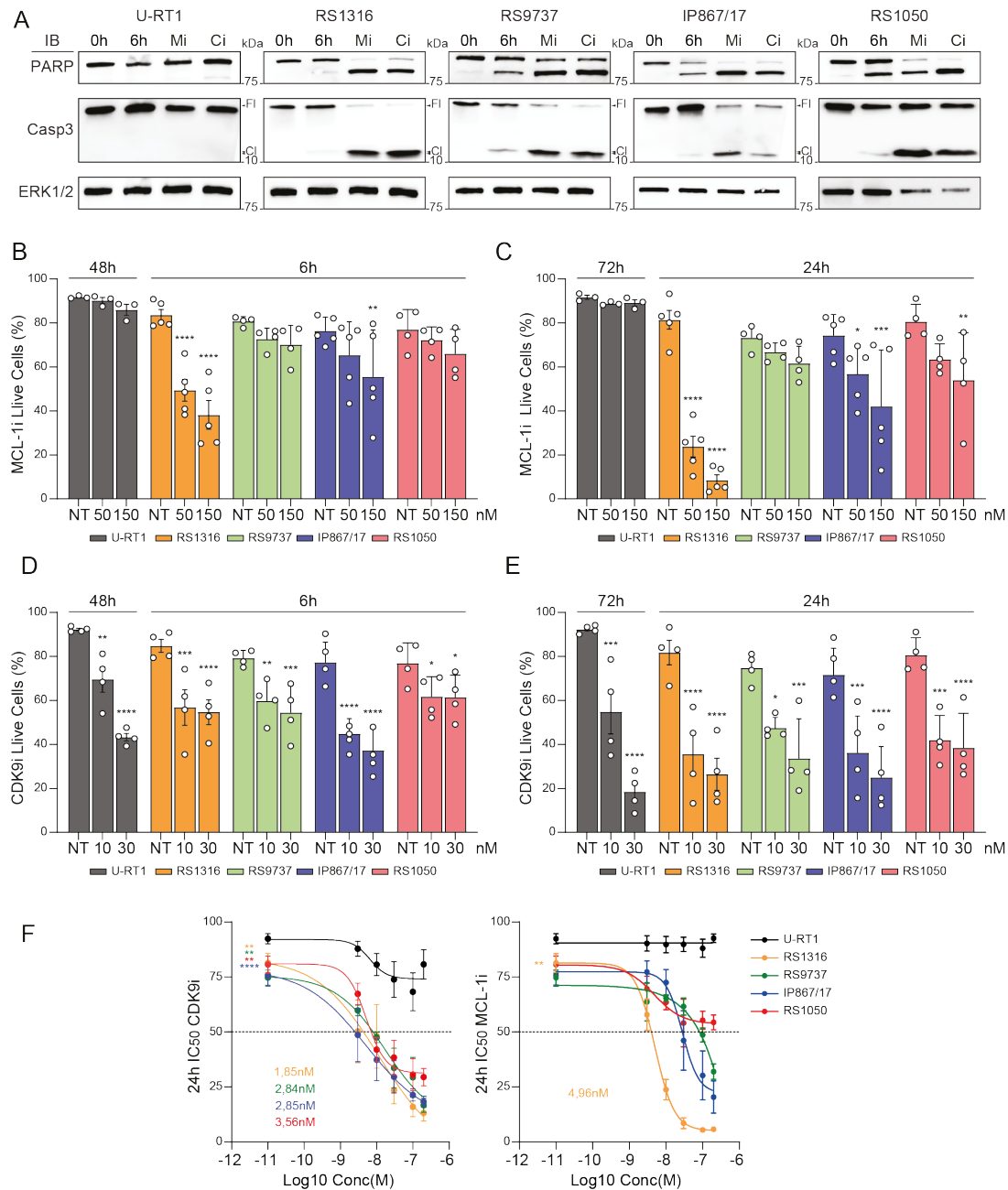


Figure 5: Effect of CDK9 and MCL-1 inhibition on apoptosis and cell viability in RS cells. (A) Western Blot analysis of Caspase-3 and PARP cleavage in U-RT1, RS1316, RS9737, IP867/17, and RS1050 cells following 6-hour treatment with 100 nM CDK9i (Ci) and 500 nM MCL-1i (Mi), with ERK1/2 used as a loading control in western blot. (B, C) Cell viability measured by Annexin V/PI staining in RS cells treated with MCL-1i (50-150 nM) for 6 and 24 hours. (D, E) Cell viability measured by Annexin V/PI staining in RS cells after treatment with CDK9i (10-30 nM) for 6 and 24 hours, with washout at 6 hours and presented as mean ± SEM. (U-RT1: gray histograms; RS1316: yellow histograms; RS9737: green histograms; RS1050: red histograms) (NT= non-treated cells). (F) IC₅₀ values of CDK9i and MCL-1i in RS cells, calculated based on flow cytometry data after 24 hours of treatment, with concentrations expressed in molar (M) and converted to base-10 logarithmic values and presented as mean ± SEM. (U-RT1: black lines; RS1316: yellow lines; RS9737: green lines; IP867/17: blue lines; RS1050: red lines). The p-value was calculated using a multiparametric ANOVA test to compare treated groups with controls. Statistical significance was represented as p < 0.05 *, p < 0.01 **, p < 0.001 ***, and p < 0.0001 ****.

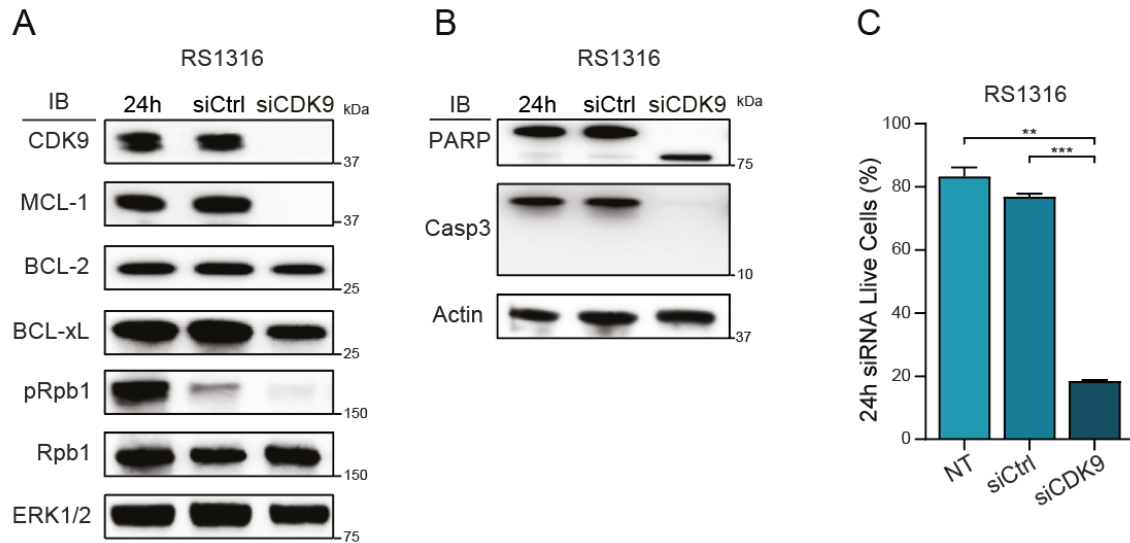


Figure 6: Validation of CDK9 inhibition using siRNA in RS1316 cells. (A) Western Blot analysis of AAPs, CDK9, pRpb1 and Rpb1 protein levels after nucleofection with CDK9-targeting siRNA (siCDK9, 500 nM) and control siRNA (siCtrl, 500 nM) for 24 hours, with ERK1/2 used as a loading control in western blot. (B) Western Blot analysis of apoptotic markers Caspase-3 and PARP cleavage in RS1316 cells nucleofected with siCDK9 compared to untreated and control siCtrl cells. Actin was used as a loading control in western blot. (C) Flow cytometry analysis of apoptotic activation using Annexin V/PI staining following siCDK9 nucleofection, compared to untreated (NT) and control siRNA cells and presented as mean $\pm$ SEM. The p-value was calculated using a multiparametric ANOVA test to compare treated groups with controls. Statistical significance was represented as $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

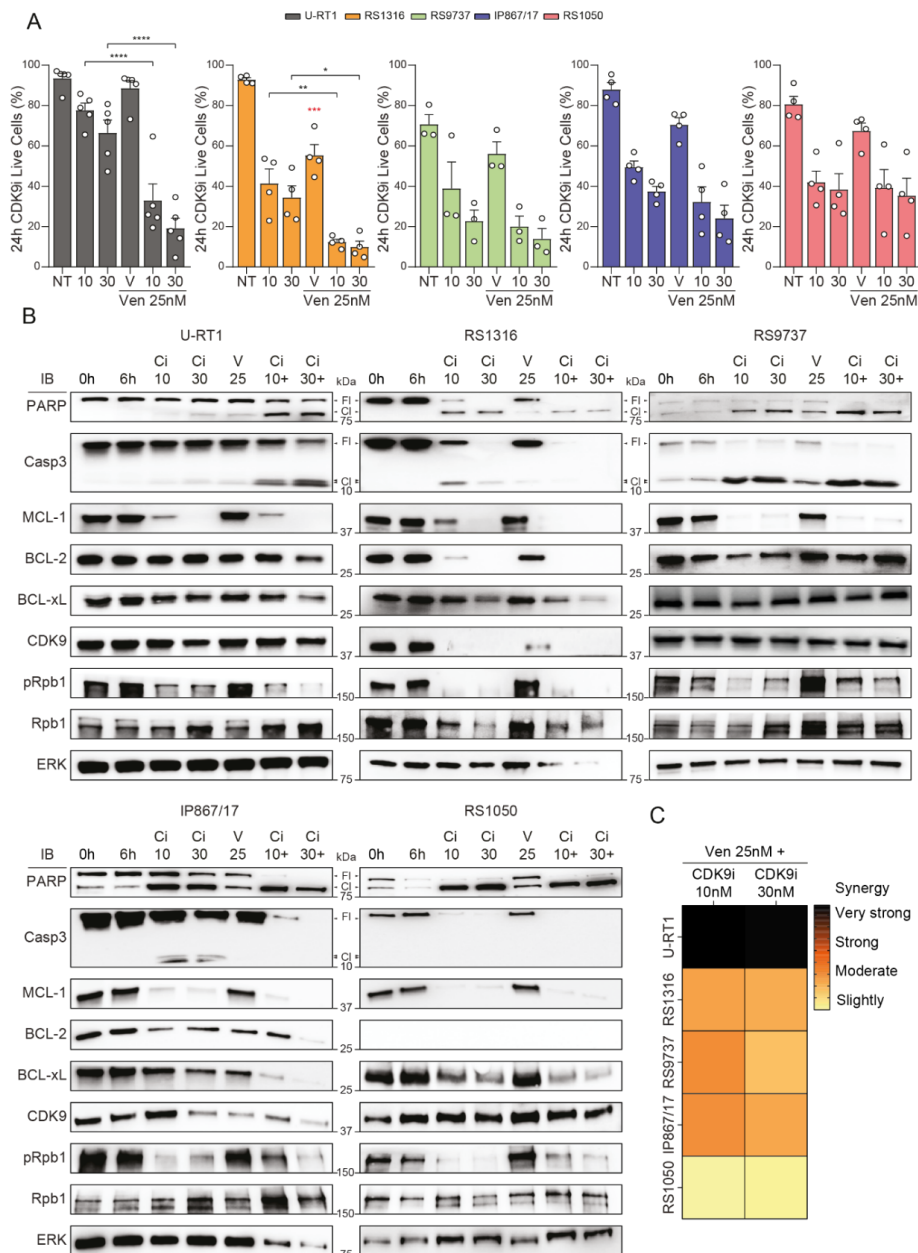


Figure 7: Effect of CDK9i and Venetoclax combination treatment on apoptosis and cell viability in RS cells. (A) Flow cytometry analysis of cell viability in RS cells treated with CDK9i (10 and 30 nM) in combination with Venetoclax (25 nM) for 24 hours, with washout at 6 hours and readdition of Venetoclax and presented as mean $\pm$ SEM. (U-RT1: gray histograms; RS1316: yellow histograms; RS9737: green histograms; IP867/17: blue histograms; RS1050: red histograms). (B) Western Blot analysis of Caspase-3 and PARP cleavage post-treatment in RS cells, with ERK1/2 used as a loading control in western blot. (C) Combination Index (CI) values for CDK9i and Venetoclax combination were calculated using the Bliss Independence model. Strong synergistic effect is defined in the interval 0-0.3 (black), strong 0.3-0.4 (dark orange), moderate 0.4-0.65 (orange), slight 0.66-1 (yellow). The p-value was calculated using a multiparametric ANOVA test to compare treated groups with controls. Statistical significance was represented as $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

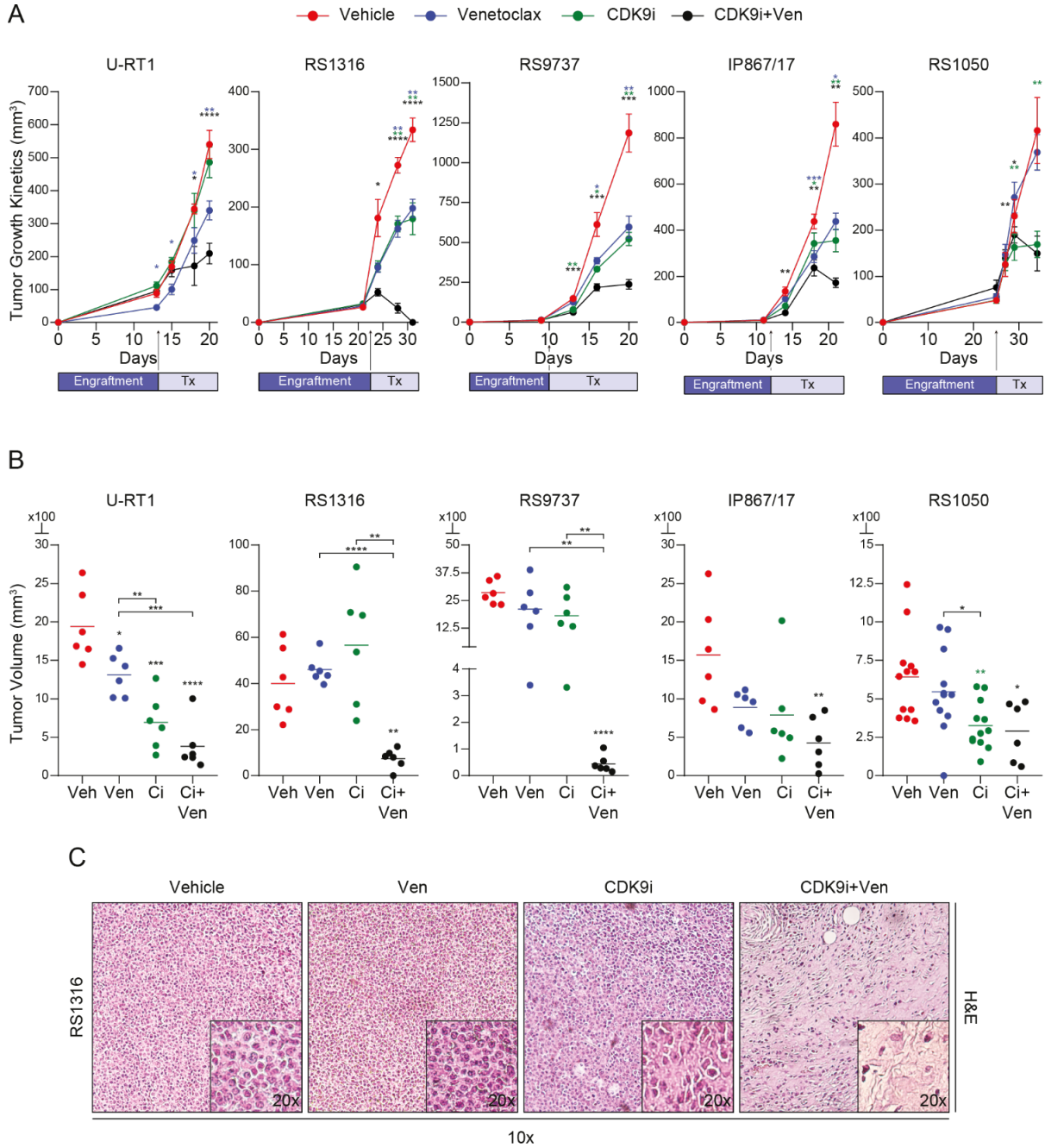


Figure 8: Combination of CDK9 and Venetoclax is highly effective in RS in vivo settings. U-RT1, RS1316, RS9737, IP867/17, and RS1050 cells were subcutaneously injected into 8-week-old male NSG mice and left to engraft. After palpable tumors were evident (tumor volume of 0.2 cm³) mice were randomized into 4 treatment groups, vehicle (red), CDK9i 30 mg/kg (green), Venetoclax 50 mg/kg (Blue), or their combination (black), treated for 10 consecutive days, and euthanized at the same time point. (A) Tumor growth kinetics were measured using a digital caliper four times during the treatment period, with measurements commencing differentially based on the engraftment period of each model and presented as mean $\pm$ SEM. (B) Tumor volume measurements were conducted at the end of the experiments. (C) Hematoxylin and eosin staining (H&E) of RS1316 tumors for histological analysis. The p-value was calculated using a multiparametric ANOVA test to compare treated groups with controls. Statistical significance was represented as $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***, and $p < 0.0001$ ****.

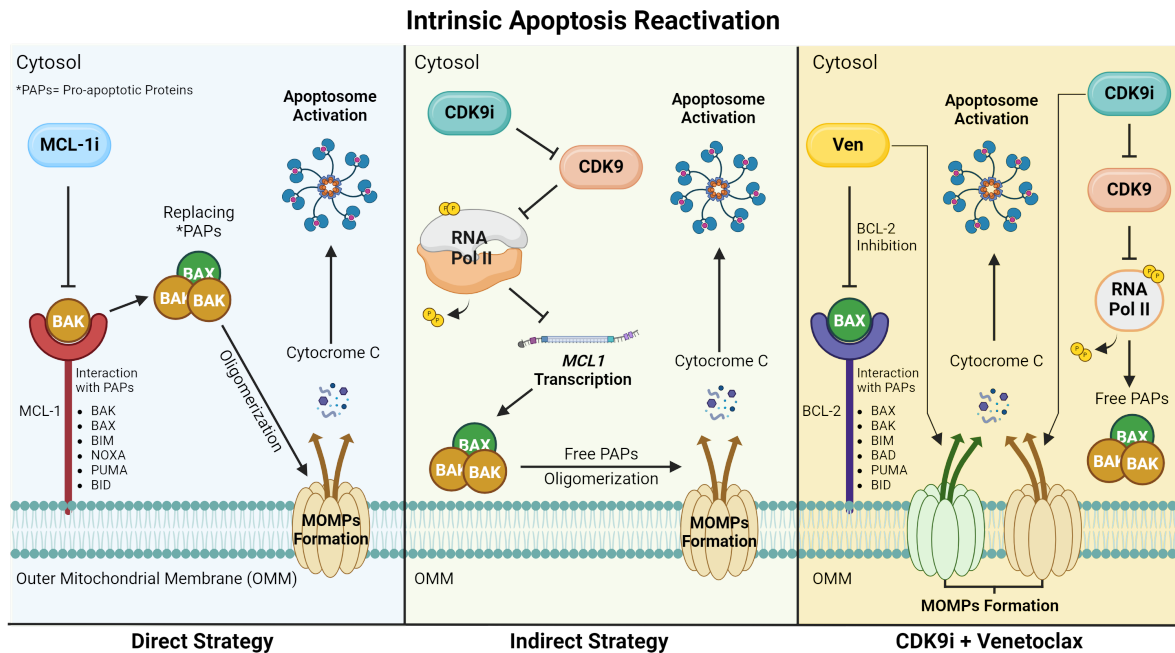


Figure 9: Representation of the molecular mechanism being the strategic combination of CDK9i and Venetoclax. CDK9i-mediated inhibition of CDK9/RNAP II results in a transient transcriptional blockade that affects the messenger RNA of *MCL1*, thereby lowering the threshold for apoptosis activation driven by BAK and BAX. Venetoclax treatment binds to BCL-2, triggering the release of these pro-apoptotic proteins, which leads to intrinsic apoptosis activation and further sensitizes BCL-2-expressing cells to CDK9i. This synergy promotes additional MOMP formation, causing substantial release of pro-apoptotic factors Cyt c, SMAC/Diablo, thereby enhancing apoptosis in RS cells.

Acknowledgements:

First, I'd like to thank my mentor, Prof. Dr. Tiziana Vaisitti, who guided me through my professional journey, and Prof. Dr. Silvia Deaglio for giving me the opportunity to complete my Ph.D. at the Molecular Biotechnology Center (MBC) Guido Tarone. Thanks to both for their solid scientific input and for the inspiring, challenging approaches.

I also want to thank my group colleagues: Amelia Fasci, Francesco Edoardo Vallone, Donatella Marsalla, Nahal Nabelsi, Matilde Micillo, Beatrice Pirajno, Alessandro Ragone, Gabriele Astolfi, Martina Migliorero and Giulia Omezzolli.

List of publications**(chronological order, 2020-to date, during PhD)**

- 1 Vaisitti T; Vitale N; Micillo M; **Brandimarte L**; Iannello A; Papotti MG; Jaksic O; Lopez G; Di Napoli A; Cutrin JC; Orlik C; Kulke M; Pahl A; Deaglio S. "Anti-CD37 α -amanitin-conjugated antibodies as potential therapeutic weapons for Richter syndrome" *Blood*, Set 2022, doi: 10.1182/blood.2022016211. PMID 35914223; PMCID PMC9523372
- 2 Audrito V; Moiso E; Ugolini F; Messana VG; **Brandimarte L**; Manfredonia I; Bianchi S; De Logu F; Nassini R; SzumeraCiećkiewicz A; Taverna D; Massi D; Deaglio S. "Tumors carrying BRAF-mutations over-express NAMPT that is genetically amplified and possesses oncogenic properties" *J Transl Med*, Mar 2022, doi: 10.1186/s12967-022-03315-9. PMID: 35272691; PMCID PMC8908704
- 3 Audrito V; Messana VG; **Brandimarte L**; Deaglio S: "The Extracellular NADome Modulates Immune Responses" *Front Immunol*, Aug 2021, doi: 10.3389/fimmu.2021.704779. PMID: 34421911; PMCID: PMC8371318
- 4 Audrito V; Messana VG; Moiso E; Vitale N; Arruga F; **Brandimarte L**; Gaudino F; Pellegrino E; Vaisitti T; Riganti C; Piva R; Deaglio S: "NAMPT Over-Expression Recapitulates the BRAF Inhibitor Resistant Phenotype Plasticity in Melanoma" *Cancers (Basel)*, Dec 2020, doi: 10.3390/cancers12123855. PMID: 33419372; PMCID: PMC7766175