Exploring Mechanisms of Action in Combinatorial Therapy through Stability/Solubility Alterations: Advancing AML Treatment

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Abstract

Acute myeloid leukemia (AML) is an aggressive blood cancer with a poor prognosis. Although treatments like allogeneic hematopoietic stem cell transplantation and high-dose chemotherapy can potentially cure some younger patients, challenges such as relapse and treatment-related toxicities remain significant. Combination therapy has been a cornerstone in AML treatment, offering enhanced efficacy by leveraging the synergistic effects of multiple agents. However, high toxicity levels and the genetic heterogeneity of AML complicate the identification of effective and universally applicable drug regimens. To address these challenges, we introduce Co-PISA (Combination-Proteome Integral Stability Analysis), an innovative method designed to study drug-target interactions specifically within combination therapies. Co-PISA detects changes in protein stability that occur only when two drugs are used together, revealing cooperative mechanisms that single-drug treatments cannot achieve. We applied this method to two highly effective and low-toxicity drug combinations in AML, previously introduced in our group: LY3009120-Sapanisertib (LS) and Ruxolitinib-Ulixertinib (RU). Co-PISA utilizes advanced proteomic tools to investigate both primary and secondary target effects, providing a deeper understanding of how combination therapies influence multiple signaling pathways to overcome resistance. Furthermore, we propose a novel concept termed "conjunctional inhibition", where the combined action of drugs induces biological responses that cannot be replicated by individual agents. This approach shifts the paradigm in drug development and offers new directions for more effective treatments in AML and other complex diseases.

Introduction

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy characterized by the clonal expansion of immature myeloid cells in the bone marrow and peripheral blood, resulting in disrupted hematopoiesis. Despite advancements in treatment, the prognosis remains poor, particularly for older adults, with long-term survival rates below 15% for patients over 60 years old[1]. Combinatorial approaches, such as venetoclax with azacitidine (VenAza) or azacitidine with cytarabine (AzaCyt), have shown promise in improving response rates, particularly for older and less fit patients who may not tolerate high-dose regimens. These combinations leverage synergistic effects to target AML cells more effectively [2,3]. However, challenges such as resistance to these combinatorial therapies emphasize the need for further research to optimize treatment strategies and improve patient outcomes.

Combination therapy has long been a cornerstone of cancer treatment, particularly in hematologic malignancies. This approach often demonstrates superior efficacy compared to monotherapy, leveraging the synergistic or additive effects of different agents to combat complex diseases [4]. On the other hand, recent studies have demonstrated that co-targeting both cancer-specific targets and associated drug-escape mediators can effectively reduce drug resistance in cancers [5]. However, this approach faces several limitations. High toxicity levels often accompany multi-drug regimens, leading to severe side effects that limit therapeutic options [6,7]. Moreover, AML, as a genetically heterogeneous disease, is characterized by each patient's leukemia being driven by distinct mutations and epigenetic alterations. This patient-specific nature of AML makes it challenging to find a "one-size-fits-all" combination therapy [8,9]. The process of identifying optimal drug combinations is further complicated by the vast number of potential therapeutic agents, requiring extensive preclinical and clinical evaluation to assess synergistic effects, dosage optimization, and toxicity management [10]. Additionally, the molecular mechanisms underlying successful combination therapies remain poorly understood, hindering the rational design of new treatment strategies [11].

Recent studies have explored novel methods for elucidating drug-target interactions. One such approach is the study of drug-induced shifts in protein conformational stability, which can provide insights into the mechanisms of action of therapeutics [1213]. Thermodynamic-based methods have been developed on this principle and are emerging as promising strategies for drug target identification, particularly because they do not require prior knowledge of the protein [14]. The thermal shift assay leverages the principle that drug binding affects a protein's thermodynamic stability, allowing researchers to observe changes in the protein's solubility and stability upon drug treatment [15].

In this work, we explore how drug combinations influence protein stability, facilitating target deconvolution and enhancing therapeutic outcomes in AML. Central to this study, we introduce Co-PISA (Proteome Integral Stability Assay for Combinations), an innovative method designed to investigate drug-target interactions specifically within the context of combinatorial therapies. The Co-PISA method captures drug interactions by detecting changes in protein stability that occur specifically when a combination of drugs is used, as compared to single-drug and control samples. We applied this approach to two recently identified drug combinations with demonstrated high efficacy and low toxicity in AML patients [16]. This technique uses advanced proteomic tools to

explore both primary and secondary target effects, providing a comprehensive understanding of how combination therapies can modulate multiple signaling pathways to overcome treatment resistance. This research extends beyond traditional drug target identification by examining how these interactions impact cellular behavior at a systems level, thereby addressing current limitations in understanding the mechanisms behind successful drug combinations. Moreover, we introduce a novel molecular mechanism termed 'conjunctional inhibition,' where the combined action of drugs produces biological responses that are unattainable by individual compounds. This innovative perspective transforms our understanding of drug interactions and paves the way for more effective therapeutic strategies in AML and other complex diseases.

Results

To investigate how combinatorial drug treatments affect protein stability, we used the CoPISA method, an extension of the PISA assay tailored for combination therapies. In the CoPISA assay, we quantify protein solubility by measuring the integral under each protein's melting curve (Sm), enabling precise solubility measurements without the need for curve fitting. The CoPISA workflow involved treating cells with each drug individually (A and B), as well as in combination (AB), alongside untreated controls. lysates or intact cells were then subjected to thermal stability assays across a temperature gradient to assess shifts in Sm under each condition (**Figure 1**). Relative changes in Sm compared to the control (ΔSm) provided quantitative insight into drug-induced effects on protein stability. These shifts, indicating either stabilization (increased Sm) or destabilization (decreased Sm), are denoted by "+" or "-" signs in **Figure 1**.

The CoPISA approach enabled a detailed analysis of drug-target interactions across eight experimental states, revealing distinct impacts on protein stability. In one state, some proteins were unaffected by any treatment, showing no changes in stability compared to controls. Two states involved Independent Inhibition (A or B), where treatment with either drug A or drug B alone led to specific alterations in stability profiles, indicating individual effects on protein stability. These proteins could suggest a synergy mechanism in combinations by targeting two proteins from different or identical biochemical pathways. Four conditions demonstrated shared protein targets across all three treatments (AB∩A, A∩B, A∩B∩AB, AB∩B), highlighting stability effects common to these drug interactions. Additionally, there was a unique condition where certain proteins were exclusively targeted during combination treatment (AB), meaning they required the simultaneous presence of both drugs to produce significant shifts in stability. We refer to this as Conjunctional Inhibition. This cooperative effect can be framed within logical gate theory[17], suggesting an "AND" gate mechanism—both drugs must be present to induce inhibition. This pattern indicates that these proteins may possess multiple binding sites or complex allosteric regulation modulated only by both drugs, providing insights into the underlying mechanisms of this drug combination. Conversely, if a protein is targeted in the presence of A, B, or AB, it aligns with an "OR" gate model, representing an overlap of effects across treatments. This mechanism offers an additional explanation for synergy in drug combinations, where the effects are enhanced when used in tandem compared to monotherapy.

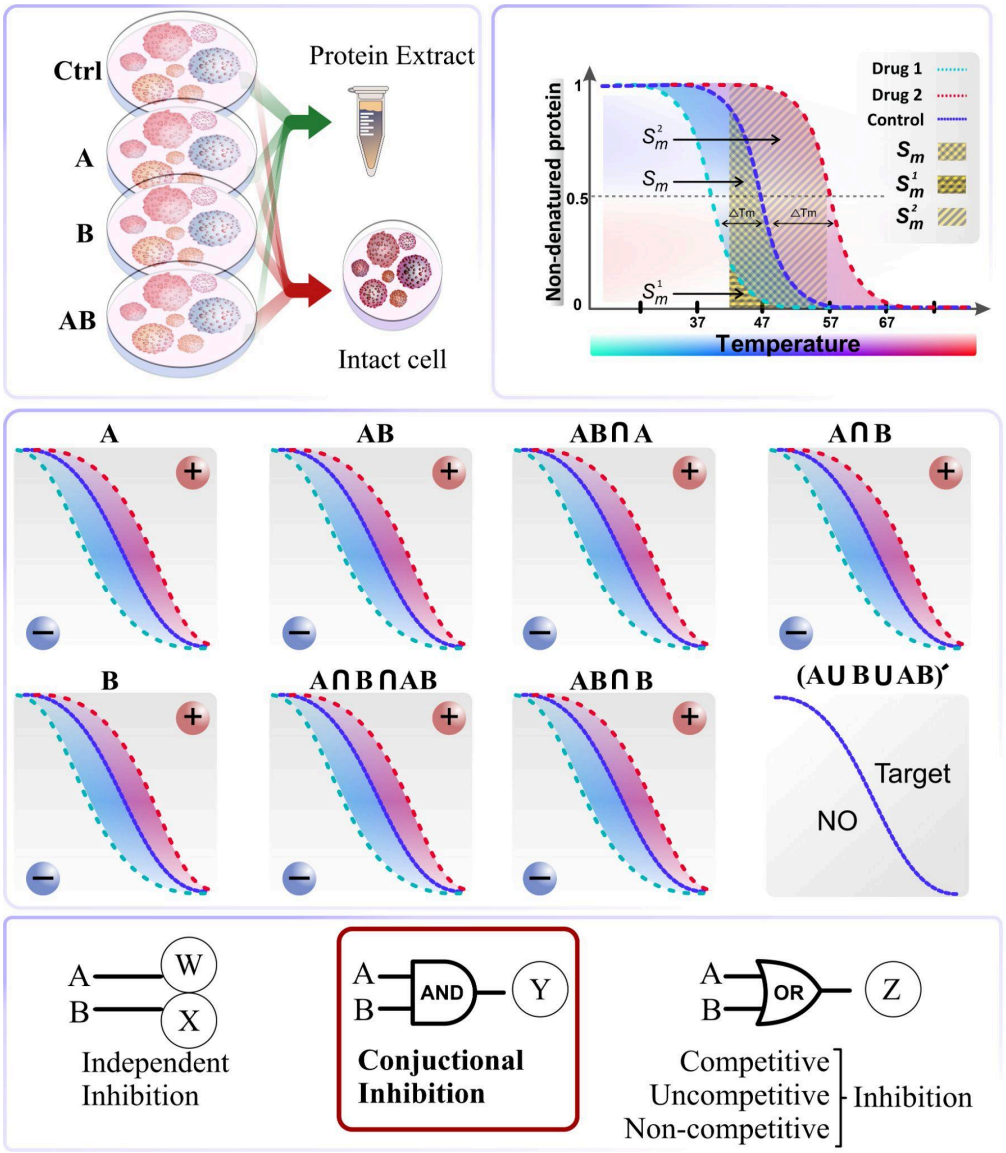

Figure 1: CoPISA workflow and possible states for target discovery in combinatorial therapy.

CoPISA Analysis of LY3009120-Sapanisertib and Ruxolitinib-Ulixertinib in AML cell lines

Using CoPISA, we identified protein stability patterns across single and combination treatments in AML cells treated with LY3009120-sapanisertib (LS) and ruxolitinib-ulixertinib (RU), across both lysate and intact cells conditions. We examined intact cells and lysate samples, identifying both potential primary and secondary targets to assess the intracellular effects of these treatments. Across the different experimental conditions, the analysis revealed distinctive patterns of protein targeting, reflecting the cellular responses elicited by each therapeutic approach.

In the LS-treated samples, significant shifts in protein stability were observed across the single and combination treatments under both intact and lysate extract conditions, as displayed in **Figure 2A** and **2B**. In the lysate extract condition (**Fig. 2A**), treatments with LY3009120,

Sapanisertib, and the LS combination resulted in 520, 432, and 704 significantly altered proteins, respectively, with 309 proteins common across all treatments, visually marked by a black halo around the respective data points. In contrast, under the intact condition (**Fig. 2B**), treatments with LY3009120, sapanisertib, and the LS combination yielded 381, 348, and 206 significantly altered proteins, respectively, with an overlapping set of 480 proteins common to all treatments. These results suggest that, while combination therapy with LS induces a greater number of stability alterations in the lysates, the intact cells condition demonstrates a more restrained, yet still substantial, set of commonly affected proteins, indicative of treatment-specific stability responses in both cellular states.

Similarly, in the RU-treated samples, a distinct pattern of stability alterations emerged across treatments with ruxolitinib, ulexertinib, and the RU combination, under both intact cell and lysate conditions, as shown in **Figure 2C** and **2D**. In the intact cells condition (**Fig. 2C**), ruxolitinib, ulexertinib, and the RU combination, resulted in 462, 618, and 487 significantly altered proteins, respectively, with a core set of 751 proteins commonly affected across all treatments, also highlighted by a black halo. In the lysate condition (**Fig. 2D**), treatment with ruxolitinib, ulexertinib, and the RU combination yielded 636, 956, and 241 significantly altered proteins, respectively, with 830 proteins common among all treatments. These findings highlight that, while individual treatments tend to produce extensive alterations in protein stability, the combination treatment exhibits a more selective pattern of stability changes, particularly under the lysate condition. Together, these observations underscore the differential impact of single and combined treatments on protein stability, with notable differences between intact and lysate extract conditions that may inform the distinct mechanistic effects of combination therapies on protein dynamics.

Building upon these findings, further analysis of protein solubility shifts in the LS- and RU-treated samples (**Figure 3**) reveals distinct intracellular effects of each combination therapy. In the samples treated with LS, the solubility of 177 and 107 proteins were significantly shifted in lysates and intact cells in the AND gate analysis, suggesting specific intracellular effects of the combination therapy. Also in the samples treated with RU, 77 proteins with significant stability shifts were identified as primary targets and 105 were in intact cell samples. Notably, each treatment condition included some overlapping proteins identified in both intact cell and lysate samples, suggesting shared mechanisms that may consistently affect cellular pathways across different cellular states.

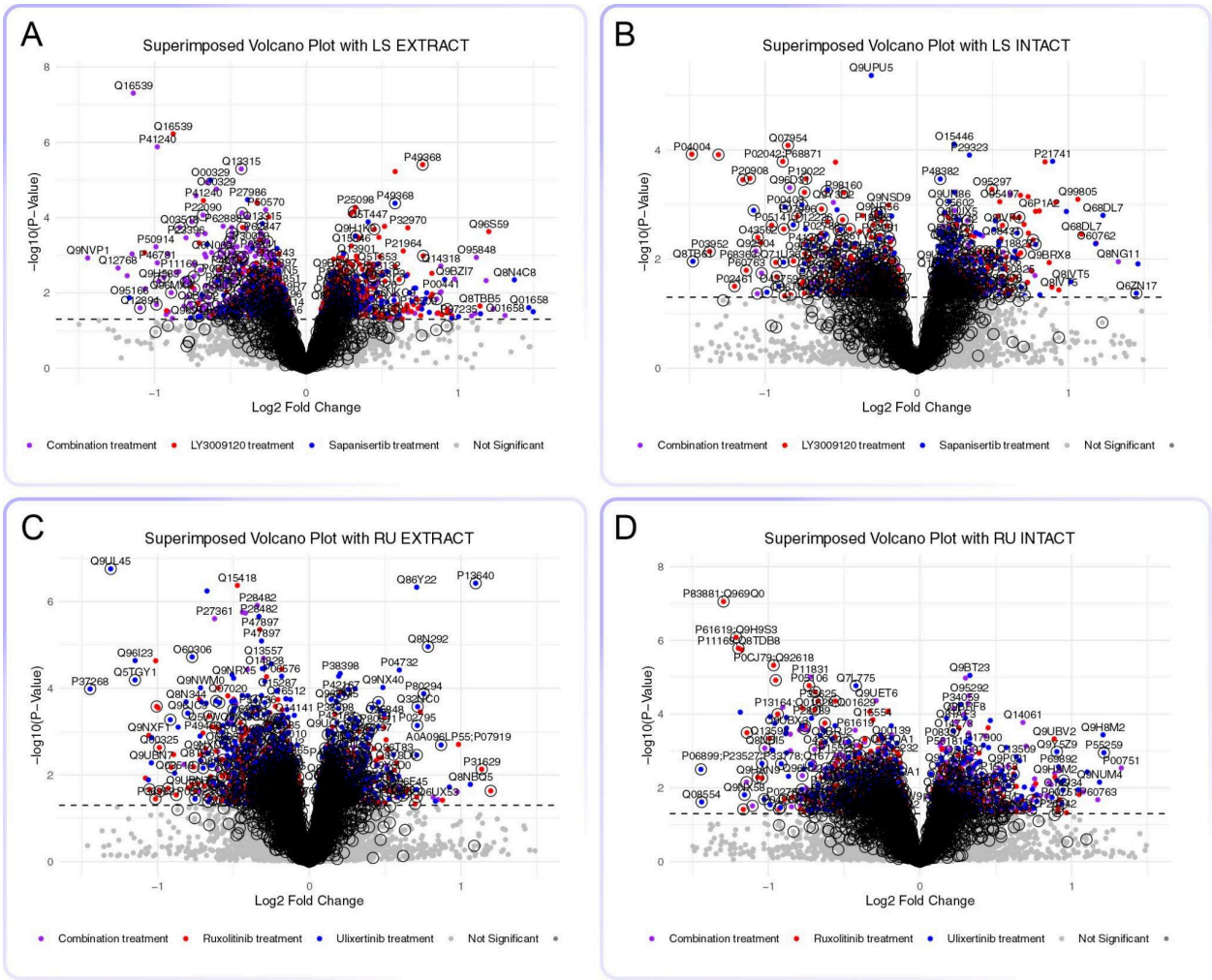

Figure 2. Volcano plots illustrate identified targets in the Co-PISA method for lysate extract and intact cell treatments with both drug combinations. Each plot is a superimposition of three comparisons between control and treatment groups, with stability shifts indicated by different colors: lysate extract samples (A) and intact cells (B) treated with LY3009120 (red), Sapanisertib (blue), and their combination, LS (purple); and lysate extract samples (C) and intact cells (D) treated with Ruxolitinib (red), Ulixertinib (blue), and their combination, RU (purple). Black outline circles highlight exclusive targets for each individual drug treatment.

Intersectional Target Analysis of Protein Stability Shifts in Lysate and Intact Cell Samples

The intersection analysis revealed proteins consistently targeted across both intact cell and lysate samples indicating a comprehensive coverage of the drug's actions on cellular pathways, potentially impacting multiple cellular processes. In samples treated with LY3009120, 11 proteins with significant stability shifts were consistently detected in both intact cell and lysate samples (**Figure 3**). Among these, the presence of mitogen-activated protein kinase suggests the involvement of MAPK signaling pathways in the therapeutic response. This indicates that

LY3009120 may inhibit key signaling processes, thereby limiting the proliferation and survival of AML cells [18]. The presence of procollagen-lysine dioxygenase points to potential effects on the tumor microenvironment, possibly altering leukemia cell interactions with surrounding tissues [19]. Additionally, the neutral amino acid transporter indicates that the drug may influence amino acid metabolism, which is critical for cellular growth [20]. Derlin-1 highlights a mechanism related to protein quality control disruption, which could increase endoplasmic reticulum stress in leukemia cells, contributing to cell death [21].

In samples treated with sapanisertib, two proteins were targeted in both the intact cell and lysate forms that are linked to ribosomal function and protein synthesis. Targeting these processes could restrict AML cell proliferation by disrupting their ability to synthesize proteins essential for survival [22]. The detection of histidine decarboxylase suggests an impact on histamine signaling pathways, potentially modulating immune responses and influencing the tumor microenvironment to enhance treatment efficacy [23].

Three proteins were targeted in the "OR" gate condition, common to both intact cell and lysate forms. The presence of plexin-C1 indicates that the treatment with any of LY3009120 or sapanisertib may affect cell signaling related to cell motility and adhesion, potentially influencing how AML cells migrate and interact with the bone marrow microenvironment [24]. Protein disulfide-isomerase TMX3 highlights a potential mechanism of endoplasmic reticulum stress induction, suggesting that the treatment may exploit this pathway to induce cell death in leukemia cells [25]. The detection of dolichyl-diphosphooligosaccharide-protein glycosyltransferase suggests that glycosylation processes may be altered, impacting protein stability and function, which could further contribute to the therapeutic effects of the treatment [25].

More importantly, four proteins are targeted in samples treated with the combination of LY3009120-sapanisertib , in both intact cell and lysate samples. The mitochondrial proton/calcium exchanger protein indicates that the combination therapy may modulate mitochondrial function and calcium homeostasis, which are crucial for maintaining apoptotic pathways in AML cells [26]. This modulation could potentially enhance the sensitivity of cancer cells to apoptosis. Additionally, the small integral membrane protein suggests that drug-induced changes in membrane dynamics could influence drug uptake and intracellular transport, enhancing therapeutic efficacy [27]. The presence of the ribosomal subunit protein uS2m implies that the combination may affect protein synthesis, disrupting AML cell proliferation. Lastly, the 8-oxo-dGDP phosphatase suggests an increased targeting of oxidative stress and DNA repair pathways, enhancing the therapeutic effects of the combination therapy by increasing the susceptibility of leukemic cells to DNA damage [28]. This integrated approach highlights the potential of LY3009120 and sapanisertib combination treatment to disrupt multiple cellular processes essential for AML cell survival.

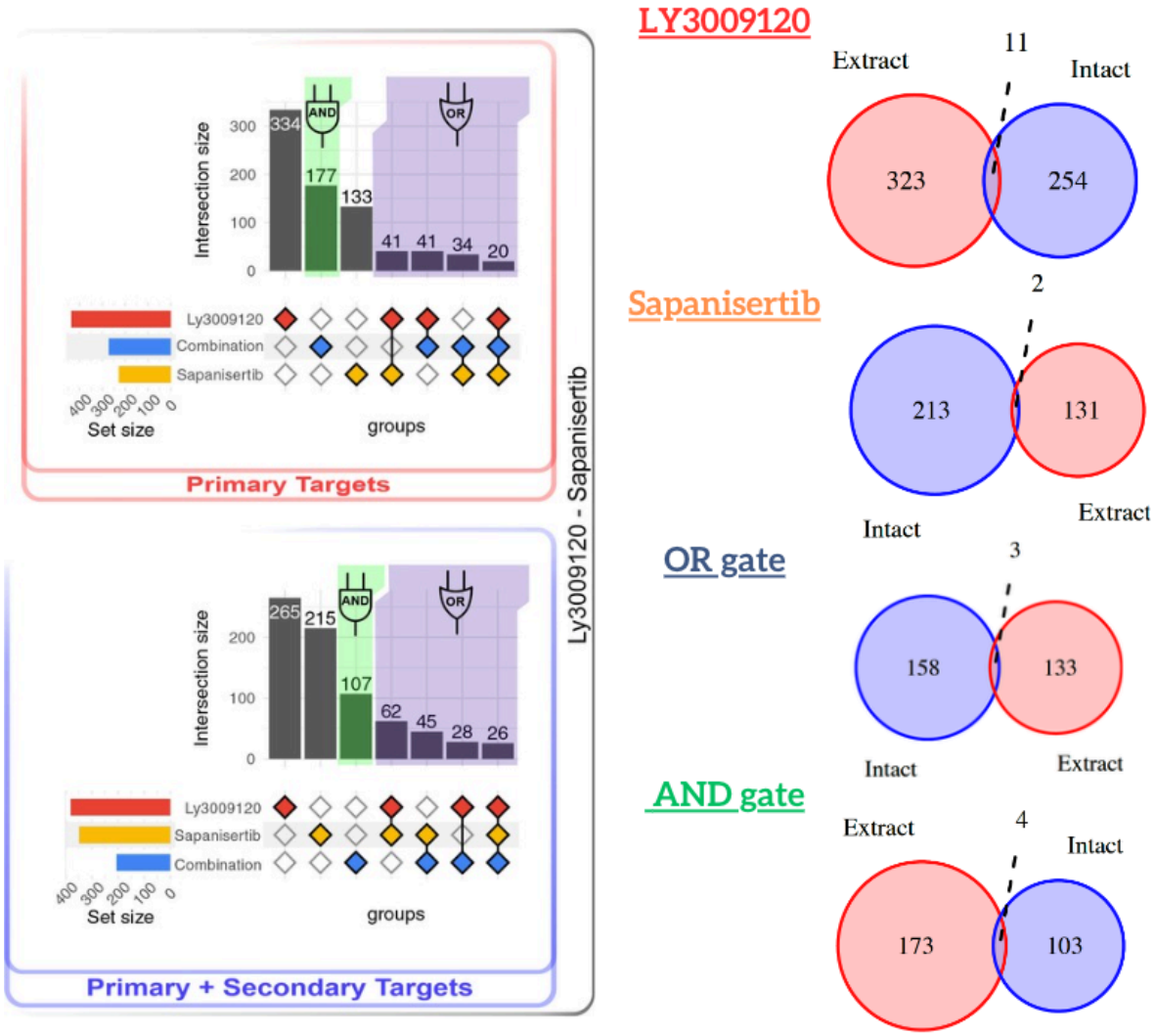

Figure 3: Upset plots display the number of identified target IDs in the lysate (representing primary targets) and intact cell samples (encompassing both primary and secondary targets) for LY3009120-Sapanisertib treatments. These are complemented by Venn diagrams that illustrate the intersection of protein IDs between the lysate and intact cell samples, highlighting both AND gate targets and OR gate targets.

In the ruxolitinib treated samples, seven proteins were targeted in both intact cell and lysate samples (**Figure 4**). Notable proteins such as RNA-binding protein and Dysferlin play significant roles in modulating cellular responses. RNA-binding proteins are essential for alternative splicing and regulation of gene expression, suggesting that ruxolitinib may impact splicing mechanisms critical for AML cell proliferation [29]. Dysferlin, on the other hand, is implicated in membrane repair and muscle cell integrity, indicating a possible role in maintaining cellular homeostasis during therapeutic stress [30]. The identification of these proteins emphasizes how ruxolitinib may alter gene expression and cellular responses, potentially enhancing its therapeutic effects in AML.

For the Lysate and intact cells, 22 proteins were commonly bound to ulixertinib . Key proteins such as RNA polymerase II and mitogen-activated protein kinase are included in this group.

RNA polymerase II is crucial for transcribing mRNA, which highlights ulixertinib's role in regulating gene expression necessary for AML cell survival [31]. Furthermore, mitogen-activated protein kinase is involved in signaling pathways that control cellular growth and differentiation, indicating that ulixertinib may disrupt critical signaling pathways in AML [32]. The presence of these proteins suggests that ulixertinib targets transcriptional and signaling mechanisms, potentially enhancing the overall anti-leukemic activity of the combination therapies.

The "OR" gate, 21 protein targets are shared among various conditions in lysate and intact cells. Proteins such as C4b-binding protein and proto-oncogene tyrosine-protein kinase Src are significant members of this group. C4b-binding protein is involved in the regulation of the immune response, indicating that these therapies might modulate immune pathways to enhance anti-leukemic effects [33]. Additionally, Src kinase plays a role in signaling pathways that regulate cell growth and survival, suggesting that targeting this protein may help in overcoming resistance mechanisms in AML [34]. The presence of these proteins illustrates how treatment of Ruxolitinib and ulixertinib can simultaneously affect immune and growth signaling pathways, potentially leading to improved treatment outcomes in AML.

Finally, the one protein targeted in the lysate and intact cell treated with the combination of ruxolitinib and ulixertinib is NADH dehydrogenase. This protein is essential for mitochondrial function and energy production, proving that combination therapy enhances mitochondrial bioenergetics, potentially leading to increased apoptosis in AML cells. The involvement of NADH dehydrogenase in cellular respiration underscores its potential as a therapeutic target to improve treatment efficacy [35]. Moreover, the shared presence of this protein across conditions indicates that maintaining mitochondrial function could be crucial for the conjunctional effects observed in combination therapies.

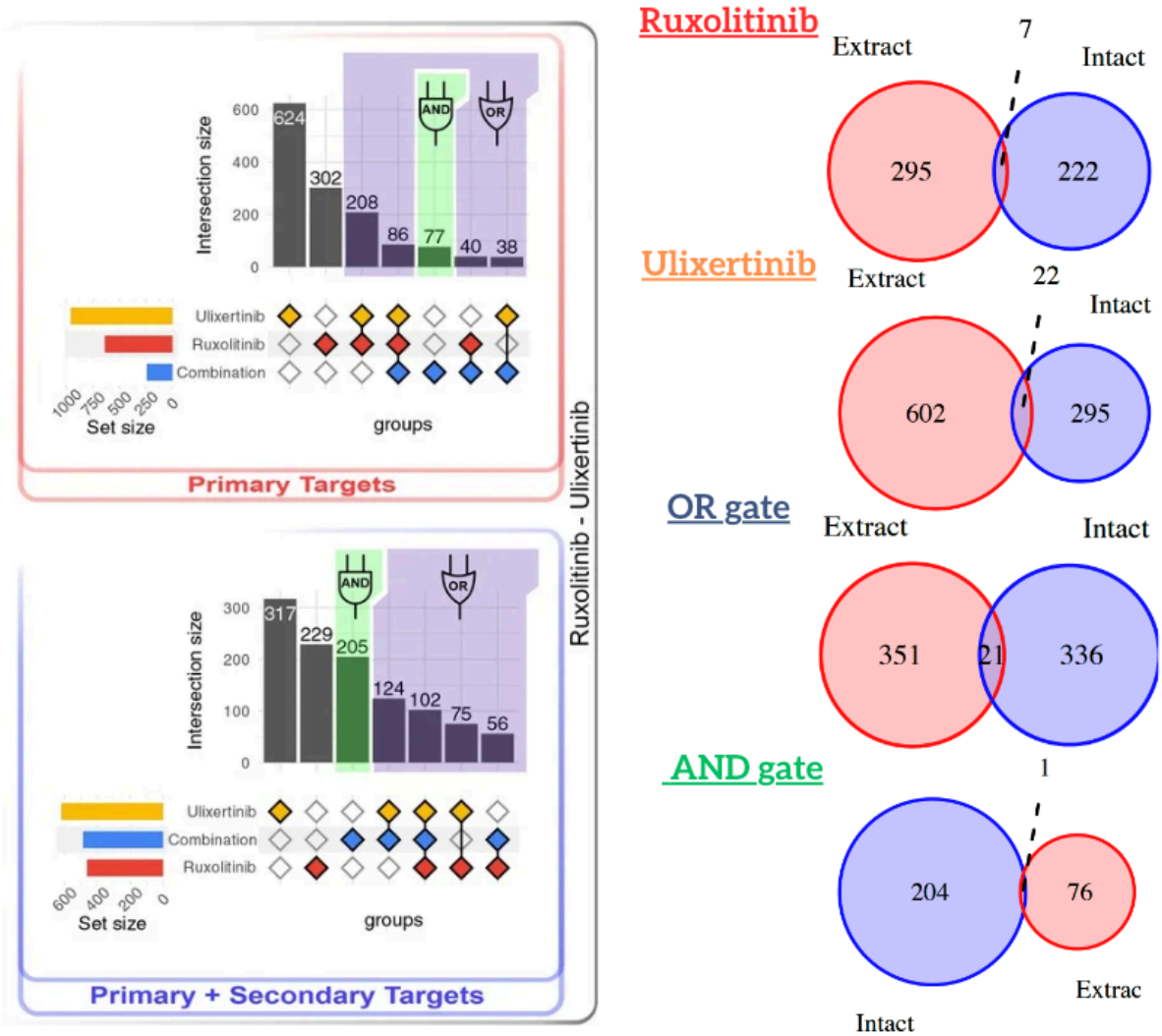

Figure 4: Upset plots display the number of identified target IDs in the lysate (representing primary targets) and intact cell samples (encompassing both primary and secondary targets) for Rusolitinib-Ulixertinib treatments. These are complemented by Venn diagrams that illustrate the intersection of protein IDs between the lysate and intact cell samples, highlighting both AND gate targets and OR gate targets.

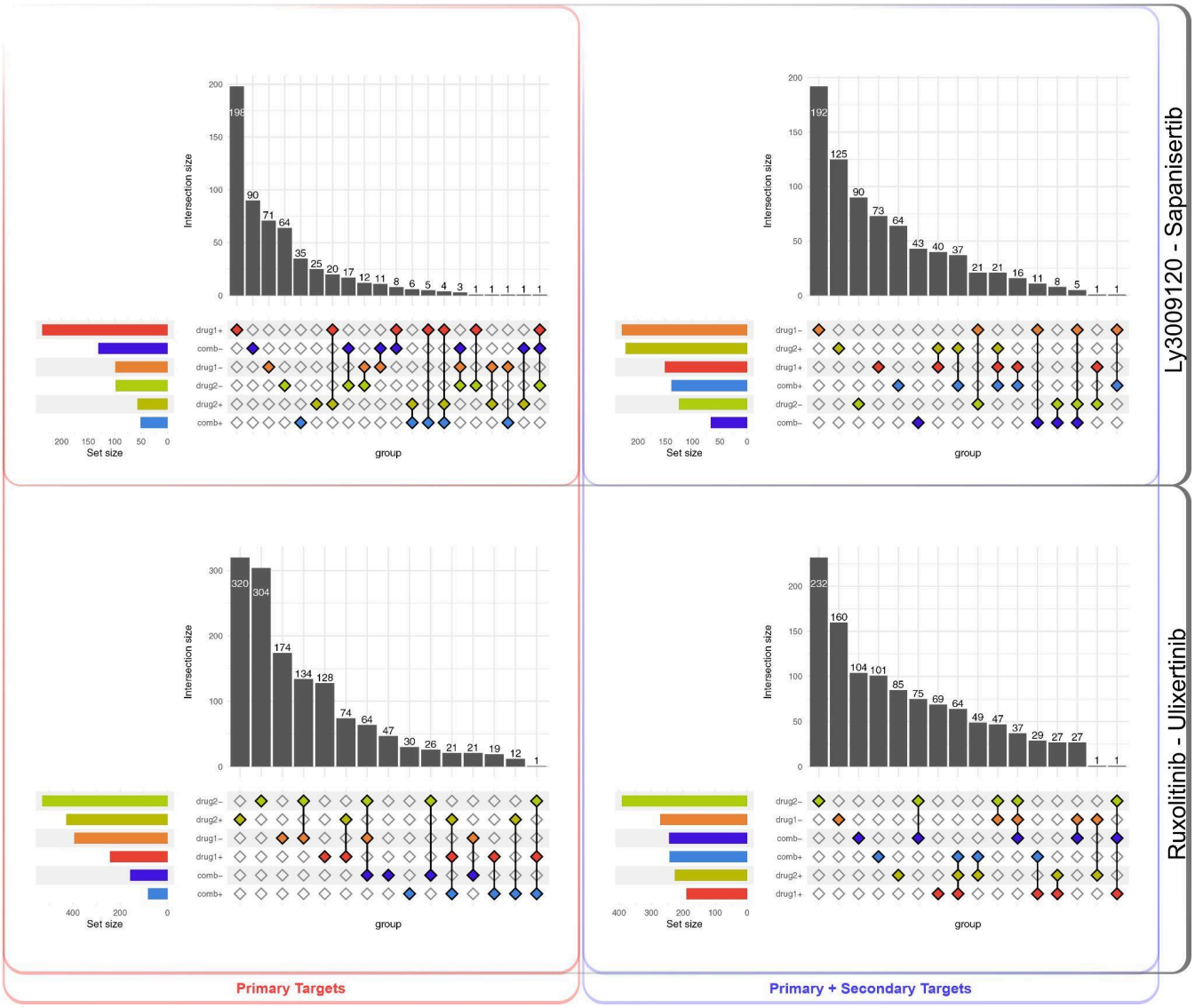

Figure 5: Upset plots showing the number of stabilized and destabilized protein target IDs. The Upset plots illustrate the number of significantly stabilized and destabilized protein target IDs for the combinations LY3009120-sapanisertib as drug1 and drug2 in top, and Ruxolitinib-Ulixertinib as drug1 and drug2 at the bottom) with both intact (primary + secondary targets) and extracted (primary targets) conditions. Stabilized and destabilized proteins are indicated with '+' and '-' symbols, respectively, showing the direction of ΔSm relative to the control groups. The plots also reveal proteins unique to each treatment and type of alteration, with color-coded bars representing the set sizes and labeled intersections indicating unique and overlapping protein changes among treatment groups.

The Upset plots in Figure 5 serves as a verification tool, identifying protein targets with stability shifts specifically in response to combination therapy, providing finer resolution on the directionality of these shifts. Stabilized ("+") and destabilized ("-") proteins are marked relative to control groups, helping to discern how combination treatments influence protein stability compared to single-drug treatments. For instance, in the RU lysate condition, the combination of ruxolitinib and ulixertinib reveals a coherent stabilizing effect on 21 proteins, where stability shifts are consistent across both the combination and individual therapies. This detailed visualization allows for a clearer understanding of unique and overlapping protein stability changes across treatments.

To further investigatethe association patterns in protein stability alterations induced by combination treatments, we generated Fold Change-Fold Change (FCFC) plots (**Figure 6**). These plots compare the stability alterations in proteins for each combination treatment relative to the effects observed with the individual drugs alone. Specifically, the FC-FC analysis examines: (1) combination vs. drug1 (e.g., LS combination vs. LY3009120 alone), (2) combination vs. drug2 (e.g., LS combination vs. sapanisertib alone), and (3) drug1 vs. drug2 (comparison between the individual drugs themselves, e.g., LY3009120 vs. sapanisertib). Most protein alterations show consistent directional changes, with stabilizations or destabilizations aligning between combination and single-drug treatments. However, in some cases, discrepancies arise, suggesting a distinct class of alteration behavior. For instance, the FCFC plot for LS combination vs LY3009120 highlights seven proteins that are significantly destabilized under combination treatment but stabilized by LY3009120 alone. Such behavior indicates a unique alteration response that warrants further investigation and experimental validation to uncover underlying mechanisms.

Fold Change Comparison – Combination vs Ly3009120
Log2 Fold Change Ly3009120
Log2 Fold Change Combination
Fold Change Comparison – Combination vs Ly3009120
Log2 Fold Change Ly3009120
Log2 Fold Change Combination
Fold Change Comparison – Combination vs Sapanisertib
Log2 Fold Change Sapanisertib
Log2 Fold Change Combination
Fold Change Comparison – Combination vs Sapanisertib
Log2 Fold Change Sapanisertib
Log2 Fold Change Combination
Fold Change Comparison – Ly3009120 vs Sapanisertib
Log2 Fold Change Sapanisertib
Log2 Fold Change Ly3009120
Fold Change Comparison – Ly3009120 vs Sapanisertib
Log2 Fold Change Sapanisertib
Log2 Fold Change Ly3009120
Primary Targets
Primary + Secondary Targets

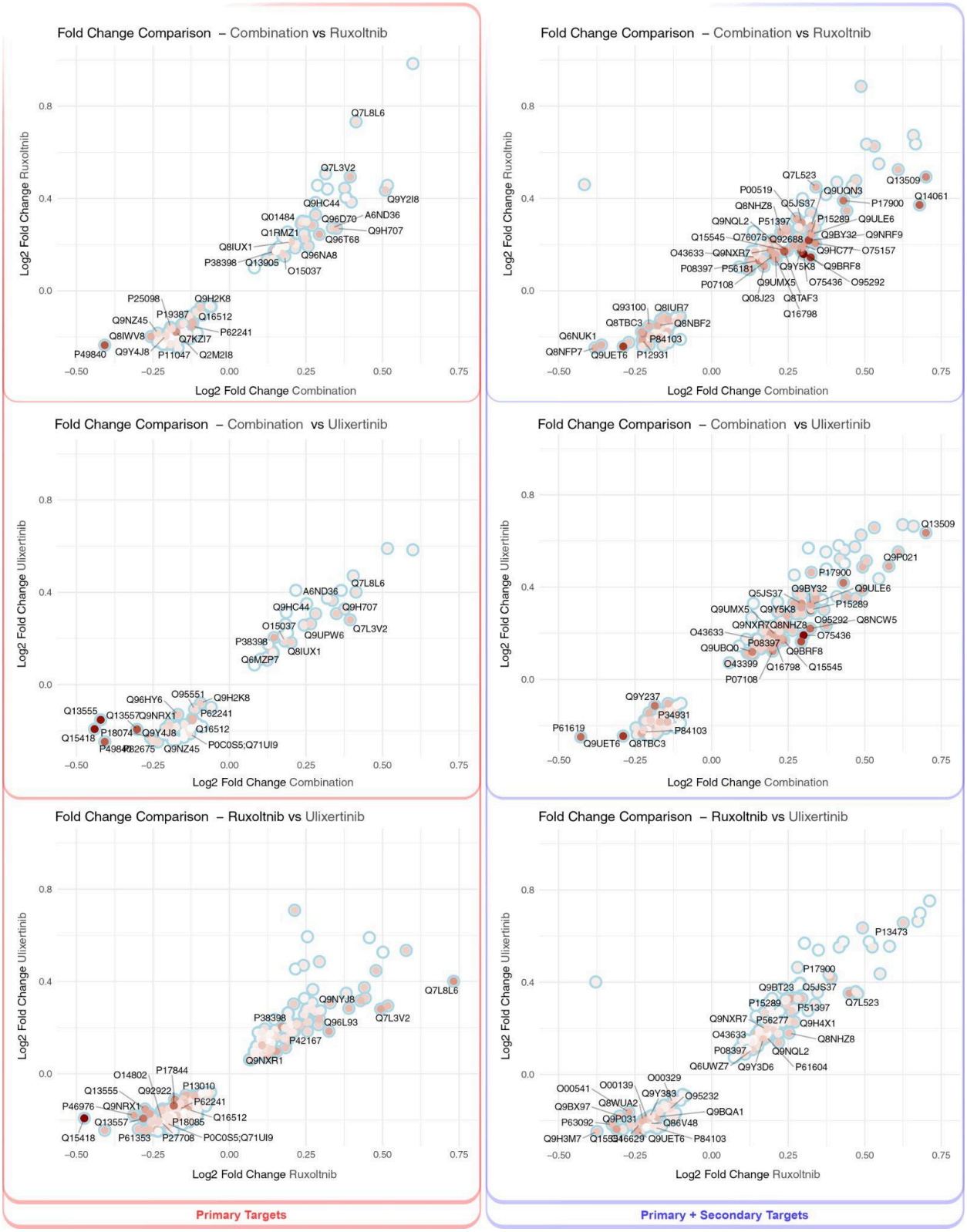
Fold Change Comparison – Combination vs Ruxoltnib
Log2 Fold Change Ruxoltnib
Log2 Fold Change Combination
Fold Change Comparison – Combination vs Ulixertinib
Log2 Fold Change Ulixertinib
Log2 Fold Change Combination
Fold Change Comparison – Ruxoltnib vs Ulixertinib
Log2 Fold Change Ulixertinib
Log2 Fold Change Ruxoltnib
Primary Targets
Primary + Secondary Targets

Figure 6: Pairwise fold change comparisons of treatment conditions based on significant proteins. The FC-FC plots display common protein target IDs across both combinations, with fold change values for each treatment relative to control samples.

Exploring Drug-Drug Interactions Through Mass Spectrometry Analysis

In the next phase, we investigated potential interactions or unexpected reactions within the selected drug combinations to confirm that each compound's effects on protein stability were independent and specific. Using mass spectrometry (MS) analysis, we compared the spectral profiles of each drug alone with those of the combined treatments (**Figure 7**). By analyzing the MS1 spectra and total ion chromatograms (TICs) of each compound separately and in combination, we assessed whether each drug preserved its distinct profile when combined.

The TICs and MS1 spectra of the combined treatments showed no unexpected peaks or shifts, indicating an absence of significant interactions or modifications between the compounds. Additionally, PCA analysis of the whole MS1 spectra provided a comparative view of each drug combination. This analysis confirmed that the effects observed on protein targets were attributable to each drug's specific mechanisms, rather than nonspecific interactions within the combinations. Each compound displayed stable peak intensities and retention times across conditions.

These findings substantiate the hypothesis that the therapeutic effects of LS and RU combinations are due to targeted, independent actions of each drug, rather than unintended molecular interactions. This supports the proposed "AND gate" inhibition mechanism in combinatorial therapy, suggesting these combinations achieve synergistic effects through coordinated drug action.

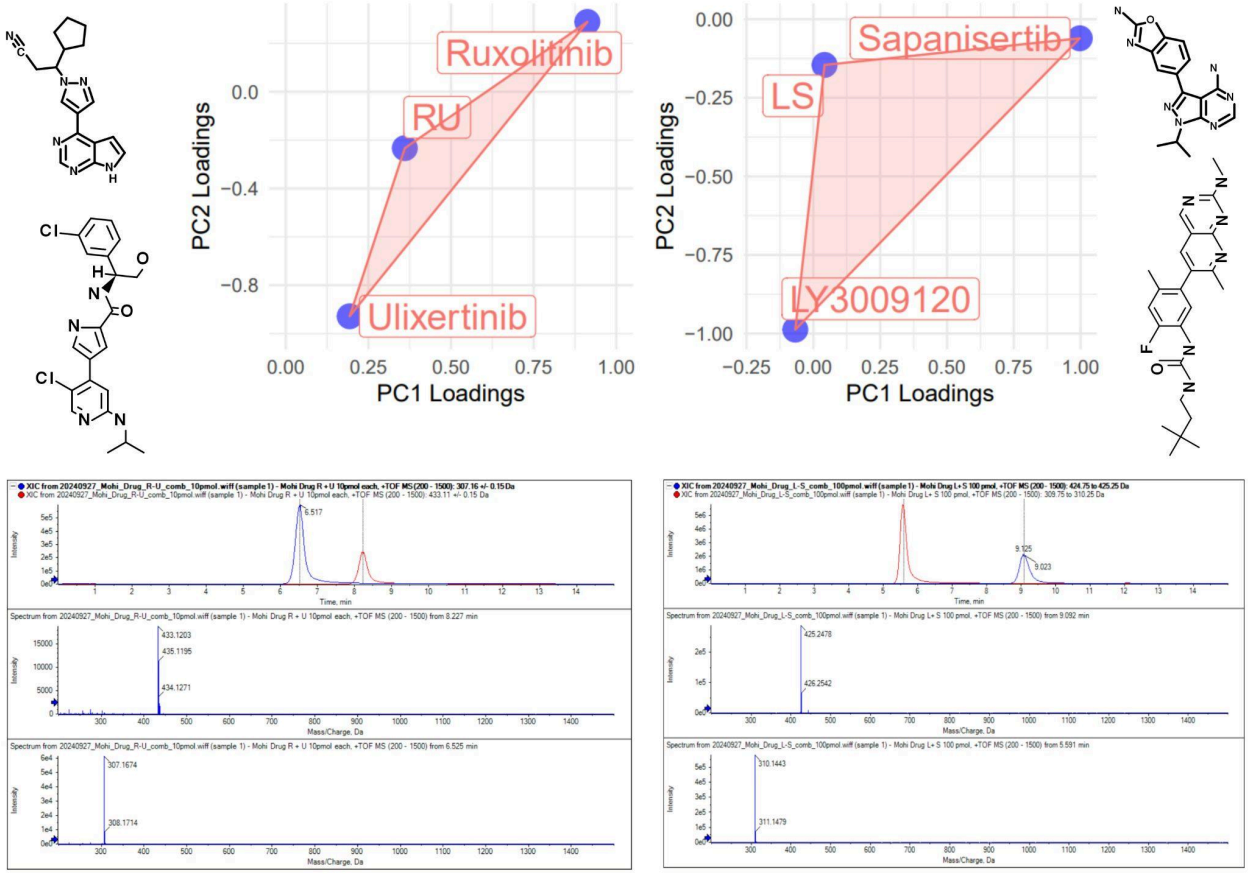

Figure 7: Mass spectrometry analysis of four individual compounds and their combinations. Molecular structures of each compound are shown alongside their MS1 spectra and TICs for the combined samples. PCA loading plots illustrate the distribution of individual compounds and their combinations on PC1 and PC2, based on quantified MS peak intensities.

Pathway Enrichment Analysis in Single Treatments and Intersectional Targets in lysate and intact cell Samples

In our study, we comprehensively analyzed the primary, secondary, and overlapping targets of LY3009120 in the AML cell line, distinguishing the pathways affected in intact cell versus lysate samples. In lysate samples alone, 323 proteins were targeted, and 254 specifically targeted upon intact cell treatment with LY3009120, with 11 common proteins to both intact cell and lysate samples (**Figure 3, Supplementary File 1**). Identified primary targets of LY3009120, significantly impacted pathways involved in protein synthesis and degradation **,** as its been reported [36]. Pathways such as Translation (p-value = 2.72E-05) and tRNA Aminoacylation (p-value = 4.24E-05) were significantly enriched, suggesting a potential disruption of protein synthesis and folding. Additionally, pathways related to cellular stress responses, such as hypoxia, were also impacted. These findings suggest that LY3009120 directly exert its therapeutic effects by disrupting protein homeostasis and inducing cellular stress, leading to cell death [37]. Among LY3009120 targets in intact cell samples, key proteins were linked to chromatin modification and epigenetic regulations crucial for AML pathogenesis (Figure 8-A). Notably, pathways such as RMTs methylate histone arginines (p-value = 9.50E-14), HDACs deacetylate histones (p-value = 2.80E-11), and chromatin organization were significantly enriched, suggesting a potential role in regulating gene expression and chromatin structure in AML [38]. Additionally, pathways related to DNA damage response, cell cycle regulation, and RNA processing were also impacted. The 11 intersecting proteins across intact cell and lysate samples provide insight into pathways that LY3009120 may affect consistently, regardless of sample preparation, revealing mechanisms of action that may be central to its anti-cancer effects (Figure 8-B). These targets revealed several key pathways, including glycogen metabolism, extracellular matrix organization, and RET signaling [39].

In summary, while lysate-only pathways emphasize protein synthesis disruption and intact cell-only pathways highlight chromatin regulation, the intersecting pathways underscore LY3009120's miscellaneous impact on AML cells by consistently modulating energy metabolism, cellular environment, and signaling. These intersecting pathways suggest additional layers of therapeutic impact, positioning LY3009120 as a promising agent targeting both epigenetic regulation and fundamental metabolic processes in AML.

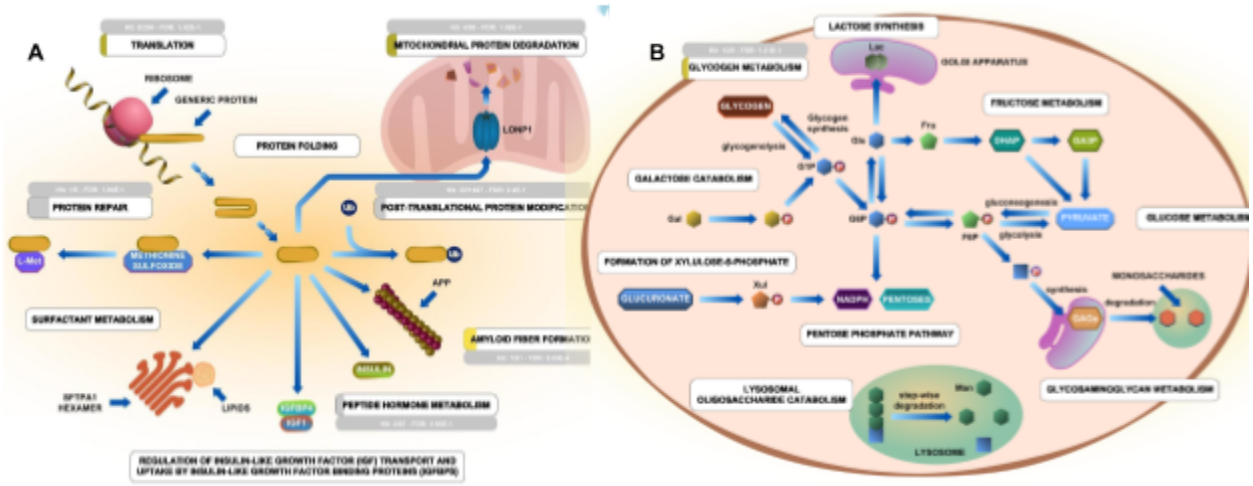

Figure 8. Pathways Enriched in Protein Targets of LY3009120 in AML Cells. A: Pathways related to protein metabolism enriched in intact cell samples treated with LY3009120. B: Carbohydrate metabolism pathways enriched in both intact cell and lysate samples, including glycolysis, glycogen metabolism, and the pentose phosphate pathway. Data sourced from the Reactome database.

When analyzing the effects of sapanisertib, 213 proteins were targeted in lysate and 131 were targeted in intact cell samples, with only two proteins overlapping between the two sample types (**Figure 3, Supplementary File 1**). The lysate-specific targets, which represent primary targets of sapanisertib, show significant enrichment in pathways associated with immune cell signaling pathways, indicating a potential for immunomodulatory effects (**Figure 9A**). Significantly enriched pathways included KIT signaling (p-value = 5.20E-05), CD28 co-stimulation (p-value = 8.24E-05), and CTLA4 inhibitory signaling (p-value = 1.47E-04), suggesting a potential modulation of immune cell activation and inhibition [40]. This profile implies a role in enhancing anti-tumor immune responses, particularly through the modulation of cytokine signaling pathways such as interleukin-2 signaling, which may contribute to immune cell function within the tumor microenvironment [41]. These findings suggest that sapanisertib exerts its therapeutic effects by modulating immune cell function, potentially leading to enhanced anti-tumor immunity.

Proteins targeted by sapanisertib in intact cell samples are involved in various cellular processes, including protein folding, RNA processing, and apoptosis. Notably, the regulation of MITF-M-dependent genes involved in apoptosis (p-value = 4.32E-04) underscores its crucial role in mediating apoptotic processes relevant to AML therapy [42]. Pathways such as Translation (p-value = 5.46E-04) and Chaperonin-mediated protein folding (p-value = 2.12E-03) were significantly enriched, suggesting a potential impact on protein synthesis and quality control. Additionally, pathways related to DNA repair and cell cycle regulation, such as TP53 Regulates Transcription of DNA Repair Genes (p-value = 7.13E-03), were also impacted. These findings suggest that sapanisertib exerts its therapeutic effects by modulating protein synthesis, DNA repair, and cell cycle progression, ultimately promoting apoptosis.

The intersection of targets in both intact cell and lysate samples revealed pathways linked to fundamental cellular processes such as Histidine catabolism (p = 1.35E-03) and rRNA processing (p = 3.2E-02) (**Figure 9B**) [43]. These overlapping pathways indicate that sapanisertib may exert consistent effects on amino acid metabolism and protein synthesis across different cellular contexts. The enrichment of Histidine catabolism suggests alterations in amino acid metabolism, a pathway often co-opted by cancer cells to fuel rapid growth and proliferation [44]. The impact on rRNA processing suggests that sapanisertib could impair ribosomal function, hindering protein synthesis essential for cell survival and proliferation. Comparing the unique and intersecting pathways, lysate-only targets indicate a prominent role for immune modulation, aligning with pathways that may enhance anti-tumor immunity. Intact cell-only targets emphasize pathways linked to protein quality control and apoptosis, suggesting that sapanisertib's effects in these samples are more directly tied to inducing cellular stress and programmed cell death. The intersecting pathways, however, suggest a consistent influence of sapanisertib on metabolism and protein synthesis across both sample types, highlighting its broad impact on essential cancer cell functions that support

survival and growth. This comparison underscores sapanisertib's multifaceted approach in AML therapy, balancing immune modulation with the direct disruption of cancer cell metabolism and protein synthesis.

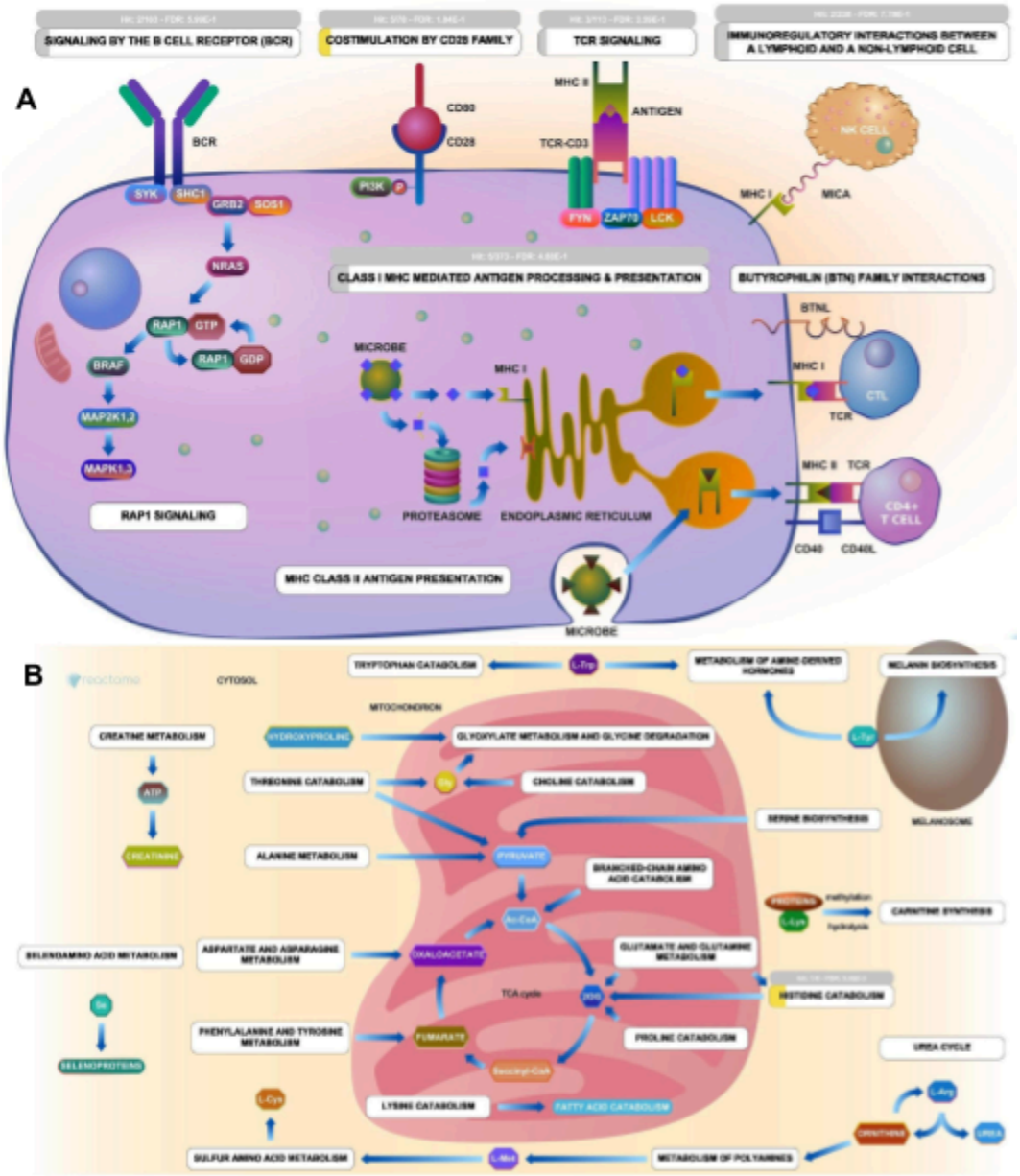

Figure 9. Pathways Targeted by Sapanisertib in AML Cells. (A) Adaptive Immune System pathways enriched in lysate samples treated with sapanisertib. (B) Amino Acid Metabolism pathways enriched in the intersection of both intact cell and lysate samples.

In cells treated with ruxolitinib 295 proteins were targeted in lysate samples and 222 were targeted in intact cell samples, with 7 proteins common to both. Ruxolitinib primarily targeted proteins involved in rRNA processing in lysate samples. Significantly enriched pathways included rRNA processing in the nucleus and cytosol (p-value = 1.46E-10), and rRNA modification in the nucleus and cytosol (p-value = 1.44E-9) (**Figure 10**). These findings suggest that ruxolitinib

disrupts protein synthesis by targeting the machinery responsible for ribosome biogenesis [45]. This could lead to impaired cell growth and proliferation, ultimately contributing to the therapeutic effects of ruxolitinib in AML.

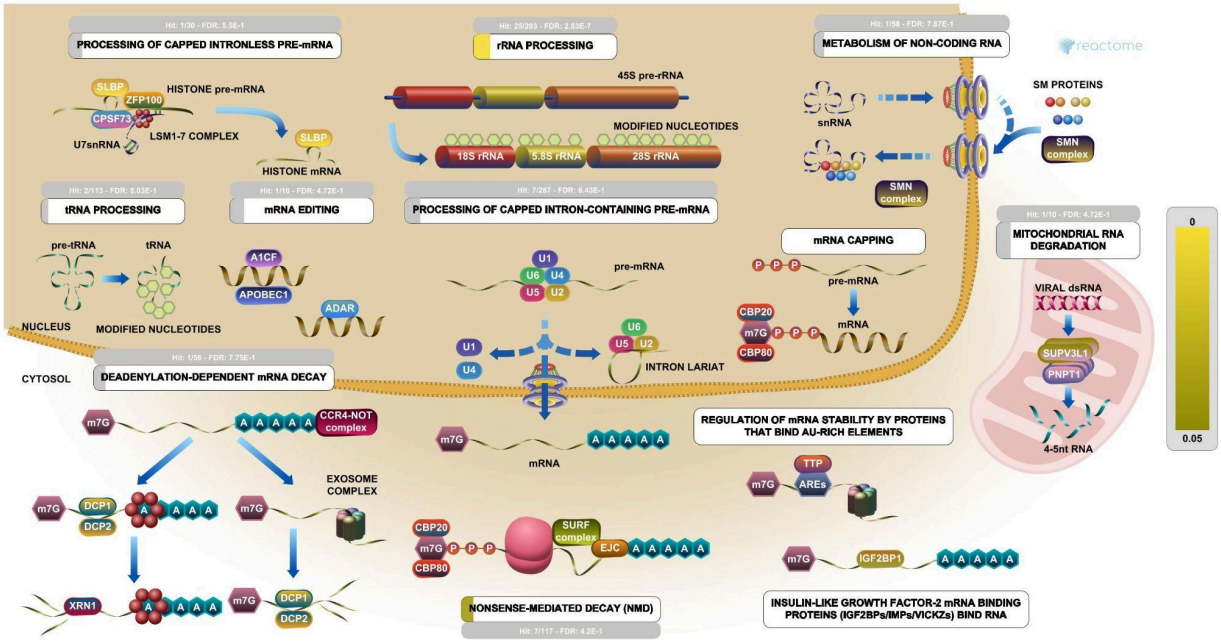

Figure 10. Pathways Targeted by Ruxolitinib in AML Cells.

In the intact cell samples, ruxolitinib targeted a broader array of pathways, impacting processes such as protein synthesis, cell cycle regulation, DNA repair, and epigenetic regulation. Key enriched pathways include SRP-dependent cotranslational protein targeting to membrane (p-value = 1.11E-16) and Peptide chain elongation (p-value = 2.44E-15), were identified suggesting a potential impact on protein synthesis, which is crucial in cancer cell survival and proliferation. Additionally, pathways such as Eukaryotic Translation Termination and Nonsense Mediated Decay (both with p-values < 6.88E-15) highlight the dysregulation of mRNA processing in cancer. Additionally, pathways related to epigenetic regulation, such as histone modifications and chromatin organization, were also impacted [46]. These findings suggest that ruxolitinib exert its therapeutic effects by modulating multiple cellular processes, including protein synthesis, cell cycle regulation, DNA repair, and epigenetic regulation, ultimately leading to cell death and tumor growth inhibition.

When examining proteins targeted in both intact cell and lysate samples, ruxolitinib revealed significant enrichment in RNA processing and editing pathways, essential for AML cell survival and adaptability. Key pathways such as mRNA Editing: C to U Conversion (p = 0.0047) and Formation of the Editosome (p = 0.0047) suggest that ruxolitinib may influence RNA editing mechanisms, which could impact the stability and translation of oncogenes. Pathways related to rRNA processing in the nucleus and cytosol (p = 0.0053) and the broader Metabolism of RNA (p = 0.0064) indicate potential disruption in ribosomal RNA biogenesis, an essential process for cancer cell growth and proliferation [47]. These intersecting pathways underscore ruxolitinib's many-sided approach to AML treatment, where disruption of both RNA processing and protein synthesis pathways limits cancer

cell adaptability and growth [48]. Comparing intersectional targets with those unique to lysate and intact cell samples, it appears that ruxolitinib exerts its effects by not only disrupting core protein synthesis and RNA regulation in the cytosol (lysate sample targets) but also by impacting broader cellular machinery like epigenetic and transcriptional regulation (intact cell sample targets), ultimately converging on mechanisms critical to cancer cell survival and metabolic flexibility.

In the sample treated with ulixertinib, 602 proteins displayed altered solubility in lysate samples and 295 in intact cell samples, with 22 proteins overlapping between the two conditions (**Figure 4, Supplementary File 1**). The pathway enrichment analysis revealed ulixertinib primarily targeted pathways involved in RNA processing, DNA repair, and metal ion homeostasis. Notably, the Metabolism of the RNA pathway was significantly enriched (p-value = 7.31E-04), suggesting that ulixertinib may disrupt RNA metabolic processes essential for cancer cell growth and proliferation. Enrichment of the mRNA Splicing - Major Pathway (p-value = 0.00293) and Processing of Capped Intron-Containing Pre-mRNA (p-value = 0.00652) indicates that ulixertinib may influence splicing mechanisms, potentially affecting the regulation of oncogenic transcripts [49]. Together, these results suggest that ulixertinib's effects in AML involve modulating RNA metabolism, splicing, metal ion response, and genomic repair mechanisms.

In intact cell samples, ulixertinib targeted additional pathways that highlight broader regulatory roles in DNA maintenance, chromatin modification, and cellular senescence. Key pathways included HDACs Deacetylate Histones (p-value = 2.7E-07), RHO GTPases Activate PKNs (p-value = 3.81E-07), and RNA Polymerase I Promoter Opening (p-value = 4.19E-07), suggesting that ulixertinib may influence histone modification and RNA transcription processes pivotal to AML progression [50]. DNA methylation (p-value = 7.0E-07) and base excision repair (p-value = 8.44E-06) pathways were also significantly enriched, underscoring ulixertinib's impact on DNA stability, which is crucial in counteracting the genomic instability characteristic of AML. This enrichment of epigenetic pathways suggests that ulixertinib hinders AML cell growth by altering transcriptional landscapes and promoting DNA repair deficiencies, which could enhance its efficacy in AML therapy.

In both intact cell and lysate samples, ulixertinib demonstrated consistent targeting of pathways associated with RNA processing, DNA repair, and immune modulation. The drug's influence on RNA processing is reinforced by pathways such as mRNA splicing and RNA Polymerase II transcription, suggesting interference with gene expression and protein synthesis. Enrichment of DNA repair pathways, including nucleotide excision repair and DNA double-strand break repair, implies that ulixertinib may sensitize cancer cells to DNA damage, thus enhancing susceptibility to apoptosis. Furthermore, enrichment of immune-related pathways, including cytokine and Toll-like receptor signaling, suggests potential immunomodulatory effects, contributing to its therapeutic impact.

Overal, a comparison between ulixertinib's effects in lysate and intact cell samples highlights a miscellaneous mechanism of action. lysate-specific pathways emphasize disruptions in RNA processing and metal ion responses, potentially impairing basic cellular metabolism and stability [51,52]. In contrast, intact cell-specific pathways reveal broader influences on epigenetic regulation and DNA repair, hinting at ulixertinib's capacity to impact higher-order transcriptional and genomic integrity processes in AML cells. This combination of targeting core cellular functions in lysate samples and regulatory pathways in intact cell samples underscores ulixertinib's complex therapeutic profile in AML, disrupting essential mechanisms of cancer cell survival and adaptability.

Mechanistic Insights into "AND" and "OR" Gate Targeting Patterns

In the comparative analysis of proteins with significant stability shifts in CoPISA experiments from the LY3009120 and sapanisertib combination, distinct pathway enrichment patterns were observed between the lysate and intact cell samples **(Supplementary File 1)**. Key enriched pathways in "And" gate in lysate sample include Costimulation by the CD28 family (p-value = 1.35E-04) and CD28 co-stimulation (p-value = 5.81E-04), which suggest that the combination influences immune cell activation, potentially enhancing anti-tumor immunity within the tumor microenvironment **(Figure 9A)**. Additionally, pathways related to rRNA processing (p-value = 5.41E-04), Major pathway of rRNA processing in the nucleolus and cytosol (p-value = 2.16E-04), and rRNA processing in the nucleus and cytosol (p-value = 3.47E-04) were significantly enriched, indicating that this drug combination may disrupt ribosomal biogenesis, a process crucial for supporting rapid protein synthesis in proliferating AML cells [53]. The involvement of Signaling by CSF1 (M-CSF) in myeloid cells (p-value = 3.37E-04) highlights potential effects on myeloid cell signaling pathways, which may contribute to altering the AML cell environment and promoting cell differentiation or apoptosis [54]. Additionally, enriched pathways related to Mitochondrial translation initiation (p-value = 0.00154), Mitochondrial translation elongation (p-value = 0.00154), and Mitochondrial translation termination (p-value = 0.00154) suggest that the drug combination could interfere with mitochondrial protein synthesis, which may affect cellular energy production and apoptosis regulation, further weakening AML cell survival mechanisms. These results collectively indicate that the combinations specific targets of LY3009120 and sapanisertib may exert a complementary therapeutic effect by modulating immune pathways [55], impairing ribosomal and mitochondrial function [56], and targeting myeloid-specific signaling, making this combination a promising strategy for AML treatment.

In the intact samples treated with the combination, there is significant enrichment of pathways critical for cancer cell regulation and survival. Notably, Metalloprotease DUBs and RMTs methylate histone arginine pathways, with extremely low p-values of 3.25E-08 and 6.04E-07 respectively, indicate profound impacts on protein stability and epigenetic modifications essential for cancer progression [57]. The enrichment of Pre-NOTCH Expression and Processing and HATs acetylate histones, with p-values of 1.16E-06 and 1.49E-06, underscore the combination's role in modulating transcription factors and chromatin structure that are crucial in cell differentiation and proliferation [58]. Pathways such as Formation of the beta-catenin transactivating complex and B-WICH complex positively regulates rRNA expression with p-values of 2.34E-06 also highlight the treatment's ability to influence Wnt signaling and ribosomal biogenesis—both key to maintaining the malignant phenotype. Further, Activated PKN1 stimulates transcription of AR (androgen receptor) regulated genes KLK2 and KLK3 shows how the combination affects androgen receptor signaling, relevant in certain leukemic contexts [59]. This intricate interplay of pathways significantly enriched in the intact sample paints a detailed picture of how LY3009120 and sapanisertib disrupt multiple core cellular processes, offering a robust therapeutic strategy for improving AML management.

The combination of LY3009120 and sapanisertib demonstrates a synergistic effect on both lysate and intact samples, targeting distinct yet interconnected cellular processes. Intact samples exhibiting enhanced epigenetic and transcription regulation suggest a direct interference with cancer cell's genetic machinery, aiming to alter the fundamental cellular properties that sustain

malignant phenotypes. In contrast, the lysate samples, displaying pathways involved in protein synthesis and immune modulation, suggest an external modulation of the tumor microenvironment and metabolic pathways, which could indirectly lead to tumor regression or sensitize the cells to other treatments.

The "OR" gate analysis of proteins targeted by the combination of LY3009120 and sapanisertib in the lysate sample reveals a significant effect on key metabolic pathways that influence cell growth and survival via modulation of growth factor signaling. Other critically enriched pathways such as Post-translational protein phosphorylation (p-value = 2.84E-06) indicate the extensive modification of protein functions, which could impact multiple signaling pathways critical for cancer progression and response to therapy [60]. The Digestion of dietary carbohydrate and Non-integrin membrane-ECM interactions with p-values of 8.57E-06 and 1.12E-04, respectively, highlight the treatment's broader implications on cellular metabolism and interaction with the extracellular matrix, crucial for cancer cells' invasive and metastatic properties [61]. These shared pathways underscore a multifaceted disruption of cellular homeostasis and signaling by the drug treatments, providing a potential therapeutic advantage by simultaneously targeting various aspects of cancer cell biology that are pivotal for their proliferation and survival.

Within the same gate in intact samples treatments influenced key signaling pathways like p130Cas linkage to MAPK signaling for integrins and GRB2 provides linkage to MAPK signaling for Integrins, both with p-values of 6.99E-05, underline the role of integrin-mediated signaling in cellular adhesion and migration processes that are crucial for tumor metastasis and invasion [62]. The activation pathways for MAPK via different mutants of BRAF and RAF1, as evidenced by Signaling by high-kinase activity BRAF mutants (p-value = 2.05E-04) and Signaling by RAF1 mutants (p-value = 3.65E-04), indicate the profound influence these drugs have on modulating key cancer growth and survival pathways. The convergence on MAPK signaling, integral to cell proliferation and differentiation, underscores the potential of these drugs to interfere with signaling cascades that are often deregulated in cancers [63]. Additionally, pathways like Integrin signaling (p-value = 7.35E-04) and Platelet Aggregation (p-value = 2.68E-04) are indicative of the drugs' impact on not only the tumor cells but also the tumor microenvironment, affecting processes such as angiogenesis and thrombosis, which are vital for tumor sustenance and spread [64]. Moreover, Senescence-Associated Secretory Phenotype (SASP) (p-value = 5.97E-04) being targeted suggests a strategy to exploit cellular aging mechanisms to suppress tumor growth and possibly induce a senescence-associated growth arrest [65]. The broad effect on these pathways demonstrates the potential of LY3009120 and sapanisertib to not only target cancer cell proliferation directly but also modulate the extracellular and intracellular communication pathways that support the malignant phenotype.

The enriched pathways identified from the protein targets specifically in the combination of ruxolitinib and ulixertinib in the lysate sample offer significant insights into their potential mechanisms in treating AML. Notably, pathways such as Signaling by TGF-beta Receptor Complex (p-value = 0.027) and TGF-beta receptor signaling activates SMADs (p-value = 0.042) emphasize the drugs' impact on crucial signaling cascades that regulate cell growth and differentiation. These pathways are integral to cellular homeostasis and are often dysregulated in cancer, indicating that the drug combination may restore normal signaling or counteract malignant signaling in AML [66]. The targeting of transcriptional regulation by NPAS4 (p-value = 0.011) and the AP-2 family of transcription factors (p-value = 0.026) also highlights the influence on gene expression linked to

cellular stress responses and apoptosis. These transcription factors play pivotal roles in responding to cellular environment changes, which can dictate cell survival or death pathways that are particularly relevant in cancer therapy [67]. Additionally, pathways like RUNX1 regulates transcription of genes involved in interleukin signaling (p-value = 0.033) and Cyclin D associated events in G1 (p-value = 0.041) suggest that the combination therapy may also be affecting the cell cycle and immune-related responses, which are critical in the proliferation and survival of leukemia cells [68]. These findings suggest that ruxolitinib and ulixertinib disrupt key regulatory networks essential for AML cell maintenance and proliferation. By modulating growth factor signaling, gene transcription, and cell cycle progression, this combination therapy potentially offers a multifaceted approach to impairing AML cell growth and enhancing the efficacy of existing therapeutic regimes.

The intact sample treated with the combination of ruxolitinib and ulixertinib highlights several significantly enriched pathways that illustrate how this therapeutic may impact AML. Notably, pathways such as Interleukin-1 signaling and Interleukin-1 family signaling, with p-values of 0.002 and 0.004 respectively, suggest a profound effect on inflammation pathways that are often linked with the cancer inflammation microenvironment. These pathways are critical as they can influence tumor progression and response to therapy by modulating the immune landscape within the tumor microenvironment [69]. Moreover, pathways like mRNA decay by 5' to 3' exoribonuclease (p-value = 0.002) indicate an impact on gene expression regulation, highlighting the treatment's potential to affect mRNA stability and thereby control the translation of genes involved in cancer cell proliferation and survival [70]. Additionally, Signaling by TGF-beta Receptor Complex (p-value = 0.026) underscores a direct involvement in growth factor signaling pathways that are crucial for cell differentiation and cancer cell migration. The inclusion of pathways such as Biosynthesis of aspirin-triggered D-series resolvins and Biosynthesis of E-series 18(R)-resolvins (p-values = 0.001 and 0.002) also points to the drugs' potential roles in resolving inflammation and promoting a return to tissue homeostasis, which can be particularly beneficial in reducing tumor-promoting inflammation in AML [71]. These pathways emphasize the potential of ruxolitinib and ulixertinib to modulate not only key signaling pathways directly involved in cancer progression but also those that regulate the immune environment and inflammatory responses, supporting their role in comprehensive cancer therapy strategies that target multiple facets of cancer biology.

The comparison of pathways affected in intact and lysate cells in combination treatment (ruxolitinib - ulixertinib), reveals that while both sample types are affected by the drug combination, the intact sample's response involves a broader modulation of the immune environment, which could lead to a more systemic impact on tumor dynamics. In contrast, the lysate sample focuses on disrupting fundamental cellular signaling pathways that can lead to inhibited tumor growth and induced apoptosis. This dual approach—targeting both the tumor cells directly and the tumor microenvironment—might be why combinations of drugs like ruxolitinib and ulixertinib can be particularly effective in treating complex malignancies like AML. Each approach leverages different aspects of cancer biology: one mitigating the supportive tumor microenvironment and the other halting the cellular mechanisms of tumor progression.

The analysis of proteins targeted within the “OR” gate of ruxolitinib and ulixertinib in lysate samples reveals significant enrichment in pathways associated with cellular translation and metabolism, presenting a comprehensive disruption of critical cellular processes essential for cancer cell viability **(Figure 11)**. Notably, the most significantly impacted pathway, Translation, with

a p-value of 5.51E−12, underscores the profound effect the drug combination has on the general protein synthesis machinery, which is a cornerstone for rapid cancer cell proliferation and survival [72]. Furthermore, specific enrichment in pathways such as Peptide chain elongation and Eukaryotic Translation Termination, both with p-values lower than E−8, highlights the drugs' capacity to interfere directly with the synthesis of new proteins, which could lead to halted growth or apoptosis of cancer cells [73]. The extensive impact on rRNA processing pathways, with Selenoamino acid metabolism and Major pathway of rRNA processing in the nucleolus and cytosol showing p-values of 1.17E-10 and 7.53E−10 respectively, indicates a targeted disruption in ribosome biogenesis—a process crucial for cell growth and proliferation. Such disruptions are particularly detrimental to cancer cells, which require robust protein synthesis machinery to maintain their growth rates. Collectively, these pathways' modulation by ruxolitinib and ulixertinib highlights a multifaceted attack on the protein synthesis and growth regulation mechanisms within AML cells. This broad spectrum of interference in fundamental cellular processes not only undermines the cancer cells' ability to proliferate and survive but also enhances the potential of these drugs to act synergistically with existing therapies to improve outcomes in AML treatment.

The analysis of protein targets affected by ruxolitinib and ulixertinib in “OR” gate in intact sample, reveals a profound impact on several essential biological processes with high significance. Key pathways such as Packaging Of Telomere Ends (p-value = 2.58E−14) and Cleavage of the damaged purine (p-value = 2.27E−13), indicate a robust influence on DNA integrity and repair mechanisms. These pathways are critical in maintaining genomic stability, a pivotal factor in cancer progression and the response to therapy [74]. The enrichment in HDACs deacetylate histones and RNA Polymerase I Promoter Opening pathways suggests that the drugs extensively modulates chromatin architecture and transcriptional regulation. Such modulation can alter the expression patterns of genes critical for cell cycle control and apoptosis, potentially reinstating controlled growth and cell death in AML cells where these processes are typically dysregulated. Furthermore, the impact on DNA methylation and recognition and repair pathways associated with damaged DNA highlights the drugs' roles in influencing epigenetic modifications and correcting DNA damage response mechanisms. These are vital for correcting the proliferative nature of AML and other cancers, where faulty DNA repair mechanisms often contribute to malignancy and resistance to therapy. Altogether, these findings underscore the potential of ruxolitinib and ulixertinib in targeting and modulating multiple molecular pathways that are crucial for the survival and proliferation of cancer cells. By impacting telomere dynamics, histone modification, DNA repair, and transcription regulation, this combination therapy could provide a multifaceted approach to cancer treatment, enhancing the effectiveness of existing therapies and potentially leading to better clinical outcomes in AML treatment. The broad impact across these pathways not only demonstrates the potential for direct anticancer effects but also suggests a role in sensitizing cancer cells to other therapeutic agents by undermining cellular resistance mechanisms.

In the detailed comparative analysis of the "AND" and "OR" gate targeting patterns in the samples treated with LY3009120 and ruxolitinib, distinct pathways emerge that are exclusively influenced under the "AND" gate conditions, both in the lysate and intact samples. Specifically, in the lysate samples, pathways such as Costimulation by the CD28 family and Major pathway of rRNA processing in the nucleolus and cytosol are uniquely affected, indicating a precise modulation of immune cell activation and ribosomal biogenesis that is not observed in the "OR" gate

configurations. Similarly, in the intact samples, pathways like GRB2 events in ERBB2 signaling and Signaling by high-kinase activity BRAF mutants are specifically enriched, highlighting the targeted impact on key cancer progression mechanisms. These unique pathway enrichments under the "AND" gate conditions emphasize the potential of conjunctional inhibition, where the cooperative interaction between drugs results in specific biological effects that are not attainable through the individual or overlapping actions of the drugs. Such insights not only validate the concept of conjunctional inhibition but also underscore its importance in developing more effective combination therapies that can precisely target and disrupt complex disease pathways, offering a more refined and potentially more effective approach to cancer treatment. This nuanced understanding of drug interactions enhances our ability to tailor therapeutic strategies that leverage the full potential of drug combinations, potentially leading to improved treatment outcomes in AML and other complex diseases.

For the ruxolitinib - ulixertinib treatments, the "AND" and "OR" gate analyses reveal distinct patterns in pathway targeting that elucidate the mechanisms of conjunctional inhibition, particularly in the lysate sample. The "AND" gate analysis identified unique pathways such as Alanine metabolism and Defective GALT can cause GALCT, which were not observed under the "OR" gate conditions. This suggests a complex interplay where both ruxolitinib and ulixertinib are required simultaneously to exert effects on these metabolic processes. Similarly, Downregulation of TGF-beta receptor signaling and Transcriptional Regulation by NPAS4 in the "AND" gate further underscore the drugs' cooperative modulation of critical pathways that regulate cell growth and apoptosis, highlighting their potential to intricately modulate signaling pathways that are crucial for AML progression. Conversely, the "OR" gate, demonstrating overlapping effects from either drug alone or in combination, did not show these pathways, indicating that certain critical cellular processes are uniquely influenced only under the concurrent presence of both drugs. Such findings stress the strategic importance of conjunctional inhibition within combination therapies, where the interaction of drugs can lead to unique therapeutic effects not achievable by either agent alone, offering a robust strategy to combat the multifaceted nature of AML.

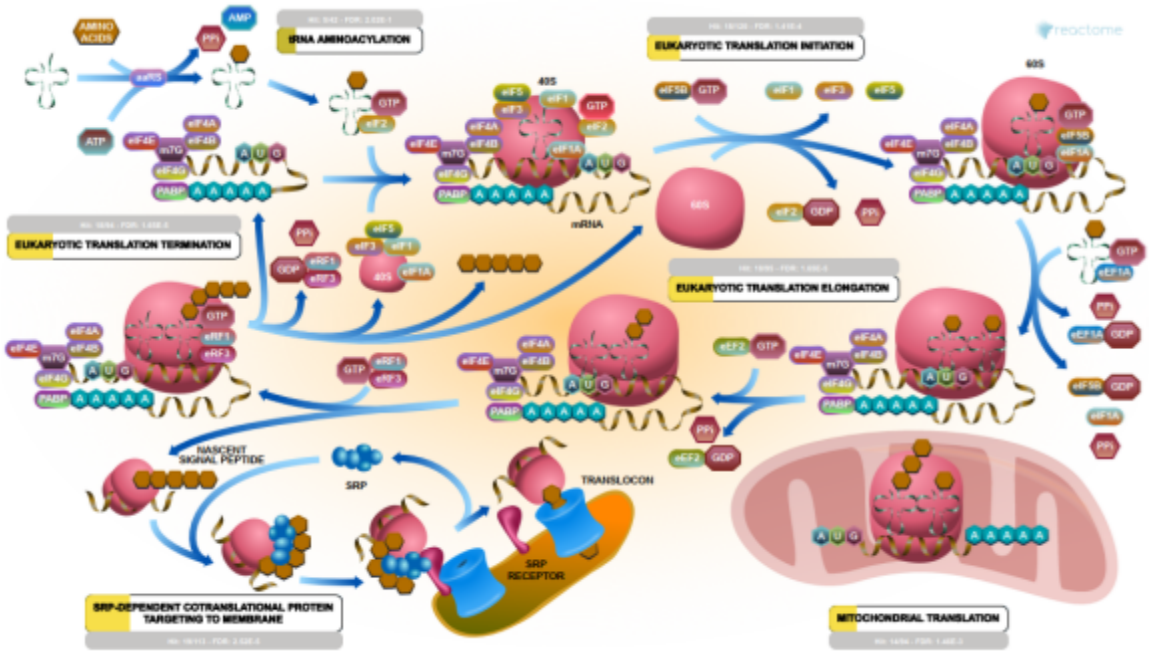

Figure 11. Pathway Illustrations of Cellular Processes Affected by Ruxolitinib and Ulixertinib in AML Treatment. Translation in RU lysate: This panel shows the extensive impact of ruxolitinib and ulixertinib on protein synthesis processes in lysate samples of AML cells.

Discussion

Combination therapy has been a mainstay in cancer treatment, particularly for aggressive malignancies like AML, due to its potential to overcome resistance and improve patient outcomes[75]. Initially, the goal was to reduce the likelihood of resistance by using multiple agents with distinct mechanisms of action, aiming to target cancer cells through various pathways simultaneously [76]. However, the selection of effective drug combinations has been challenging due to the genetic and epigenetic heterogeneity of AML, which results in varied responses to treatment across different patients[77]. Additionally, the high toxicity associated with multi-drug regimens limits their utility, often causing significant side effects that restrict dosage levels and treatment duration [78]. This complexity has driven a shift in the approach to combination therapy, with a focus on understanding the molecular basis of drug interactions to design rational, synergistic drug combinations that maximize efficacy while minimizing toxicity [16]. While recent approaches have focused on understanding the additive or synergistic effects of individual drugs[75], our study introduces a novel concept termed "conjunctional inhibition". This paradigm shift suggests that the combined action of drugs can produce biological responses that cannot be replicated by individual agents.

In this study, we introduced the CoPISA methodology to evaluate the effects of two recently proposed combinatorial treatments for AML: LY3009120-sapanisertib and ruxolitinib-ulixertinib [16]. CoPISA captures drug-induced changes in protein stability specifically arising from the combined action of drugs, offering insights into mechanisms that single-drug treatments cannot achieve. The approach allowed us to identify both primary and secondary protein targets, revealing cellular responses linked to combination therapies. These findings suggest that the interactions between the two drugs in each pair are not merely additive but involve more complex cooperative mechanisms. This novel concept reflects a situation where the concurrent action of two agents produces distinct biological effects unattainable by individual drugs, indicating a deeper level of interaction between the two drugs' targets and signaling pathways.

We also provide valuable insights into the molecular mechanisms underlying the therapeutic effects of these drug combinations. By comparing the identified proteins with significant stability shifts with known drug targets, we were able to validate the functional significance of the proteins identified in our study. These findings offer potential avenues for further investigation and the development of novel therapeutic strategies.

In conclusion, our study introduces a novel approach for understanding the mechanisms of combination therapy and provides valuable insights into the potential of conjunctional inhibition as a therapeutic strategy for AML. By leveraging the power of CoPISA and advanced proteomic analysis, we have identified key molecular targets and pathways that may be modulated by combination therapies. These findings offer promising avenues for the development of more effective and personalized treatments for AML patients.

Material and Methods

Cell culture

MOLM-13, MOLM-16, SKM and NOMO-1 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) and cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10–20% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific), 2 mM L-glutamine (Lonza), and 100 units/mL penicillin/streptomycin (Gibco, Thermo Fisher Scientific), at 37°C and 5% CO2. Cells were harvested and centrifuged at 400 g for 4 min.

Lysate based CoPISA

Cells were cultured until they reached the desired density of 2×10^6 cells per treatment with seven different treatment groups for each cell line, performed in triplicate. A 24 ml volume of cell culture was pelleted by centrifugation at 600g for 5 minutes, followed by two washes with phosphate-buffered saline (PBS). After a final pelleting, cells were resuspended in PBS (GIBCO) supplemented with protease inhibitors (Roche, Switzerland). The cell mixture was lysed through 4 cycles of freezing in liquid N2 and then thawing at 35 °C, at the final step cells were cleared by centrifugation at 10,000 g for 10 min at 4 °C. Samples were then treated with single or combination drug regimens at the following concentrations: LY3009120 500 nm, sapanisertib 500 nM, ruxolitinib 3uM, ulixertinib 3uM, for 15 min at room temperature, After treatment, the samples were aliquoted into 12 equal volumes for subsequent heat treatments. Each aliquot was subjected to a 3-minute heat treatment at temperatures ranging from 48°C to 59°C in 1°C intervals using a thermocycler (Applied Biosystems Veriti). After equilibrating at room temperature for 3 min, samples from different temperatures for each drug treatment were pooled and subjected to ultracentrifugation at 100,000g for 20 minutes at 4°C (Beckman Coulter Optima Max-XP). The soluble proteins from the supernatant were collected for further analysis.

Cell-based CoPISA

Cells (2 × 106 cells per condition) were treated with a single or combination drug with concentrations mentioned, in 6-well plates and incubated at 37°C with 5% CO_2 for 60 min. Following treatment, the cells were washed twice with PBS and resuspended in 320 µL of PBS supplemented with a protease inhibitor. An equal volume of each sample was transferred to the PCR plate and was heated for 3 min at the same temperatures used in the lysate-based protocol. The samples were allowed to cool down at room temperature for 3 min and then applied for four cycles of freezing cells in liquid N2 and thawing at 35 °C. After pooling the samples from different temperature points for each treatment, the soluble proteins were isolated via ultracentrifugation 100,000g for 20 min at 4°C. At this point, samples can be flash frozen and stored at -80°C for future analysis.

Proteomics Sample Processing

Protein concentration was measured using the PierceBCA Protein Assay Kit (Thermo), and the volume corresponding to 27 μg of protein was transferred from each sample to prepared filter tubes (Amicon Ultra-0.5 mL Centrifugal Filters 10KD). The volume of each sample was adjusted to an equal level using 20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS) buffer, then was reduced in 10 mM dithiothreitol (55 °C, 30 min) and alkylated in 50 mM iodoacetamide (25 °C, 60 min in darkness). After reduction and alkylation, samples were centrifuged for 20 min, 14000g, followed by two washes. EPPS buffer was then added to the filter tubes containing samples. Proteins were digested overnight at 37°C with vigorous shaking by trypsin at a ratio of 1:50. The resulting peptides were collected by centrifugation and rinsing the filter tubes with EPPS. Prior to labeling, equal amounts of each sample from the different treatment groups were mixed for separate labeling. A total of 25 μg from each sample was labeled using tandem mass tags (TMT) according to the manufacturer's protocol (Thermo Fisher Scientific), resulting in a final pooled mixture of 325 μg from 13 samples.

Peptide fractionation and LC MS/MS analysis

TMT-labeled peptides were fractionated by high-pH reversed phase separation using a XBridge Peptide BEH C18 column (3.5 μm, 130 Å, 1 mm x 150 mm, Waters) on an Ultimate 3000 system (Thermo Scientific). Peptides were loaded on column in buffer A and the system was run at a flow of 42 μl/min. The following gradient was used for peptide separation: from 2% B to 15% B over 3 min to 45% B over 59 min to 80% B over 3 min followed by 9 min at 80% B then back to 2% B over 1 min followed by 15 min at 2% B. Buffer A was 20 mM ammonium formate in water, pH 10 and buffer B was 20 mM ammonium formate in 90% acetonitrile, pH 10. Elution of peptides was monitored with a UV detector (205 nm, 214 nm) and a total of 36 fractions were collected, pooled into 12 fractions using a post-concatenation strategy as previously described (Wang et al., 2011) and dried under vacuum.

Dried peptides were resuspended in 0.1% aqueous formic acid and subjected to LC–MS/MS analysis using an Orbitrap Eclipse Tribrid Mass Spectrometer fitted with a Ultimate 3000 nano system and a FAIMS Pro interface (all Thermo Fisher Scientific) and a custom-made column heater set to 60°C. Peptides were resolved using a RP-HPLC column (75 μm × 30 cm) packed in-house with C18 resin (ReproSil-Pur C18–AQ, 1.9 μm resin; Dr. Maisch GmbH) at a flow rate of 0.3 μl/min. The following gradient was used for peptide separation: from 2% B to 12% B over 5 min to 30% B over 70 min to 50% B over 15 min to 95% B over 2 min followed by 18 min at 95% B then back to 2% B over 2 min followed by 18 min at 2% B. Buffer A was 0.1% formic acid in water and buffer B was 80% acetonitrile, 0.1% formic acid in water.

The mass spectrometer was operated in DDA mode with a cycle time of 3 s between master scans. Throughout each acquisition, the FAIMS Pro interface switched between CVs of −40 V and −70 V with cycle times of 1.5 s and 1.5 s, respectively. MS1 spectra were acquired in the Orbitrap at a resolution of 120,000 and a scan range of 400 to 1600 m/z, AGC target set to "Standard" and maximum injection time set to "Auto". Precursors were filtered with precursor selection range set to 400–1600 m/z, monoisotopic peak determination set to "Peptide", charge state set to 2 to 6, a

dynamic exclusion of 45 s, a precursor fit of 50% in a window of 0.7 m/z and an intensity threshold of 5e3. Precursors selected for MS2 analysis were isolated in the quadrupole with a 0.7 m/z window and collected for a maximum injection time of 35 ms with AGC target set to "Standard". Fragmentation was performed with a CID collision energy of 30% and MS2 spectra were acquired in the IT at scan rate "Turbo". MS2 spectra were subjected to RTS using a human database containing 20362 entries downloaded from Uniprot on 20200417 using the following settings: enzyme was set to "Trypsin", TMTpro16plex (K and N-term) and Carbamidomethyl (C) were set as fixed modification, Oxidation (M) was set as variable modifications, maximum missed cleavages was set to 1 and maximum variable modifications to 2. Maximum search time was set to 100 ms, the scoring threshold was set to 1.4 XCorr, 0.1 dCn, 10 ppm precursor tolerance, charge state 2 and "TMT SPS MS3 Mode" was enabled. Subsequently, spectra were filtered with a precursor selection range filter of 400–1600 m/z, precursor ion exclusion set to 25 ppm low and 25 ppm high and isobaric tag loss exclusion set to "TMTpro". MS/MS product ions of precursors identified via RTS were isolated for an MS3 scan using the quadrupole with a 2 m/z window and ions were collected for a maximum injection time of 200 ms with a normalized AGC target set to 200%. SPS was activated and the number of SPS precursors was set to 10. Isolated fragments were fragmented with normalized HCD collision energy set to 55% and MS3 spectra were acquired in the Orbitrap with a resolution of 50,000 and a scan range of 100 to 500 m/z.

The acquired raw-files were analysed using the SpectroMine software (v3.2, Biognosis AG). Spectra were searched against a human database consisting of 20360 protein sequences (downloaded from Uniprot on 20220222). Standard Pulsar search settings for TMT 16 pro ("TMTpro_Quantification") were used and resulting identifications and corresponding quantitative values were exported on the PSM level using the "Export Report" function.

LC-MS Analysis of Drug Combinations

Drug combinations were resuspended in DMSO and 10 pmol were injected into LCMS. Analytes were separated using Eksigent Ekspert 400 with a reverse C4 column(0.3 x 20mm, Phenomenex, USA). The injected sample analytes were separated with a linear gradient of 15 min comprising 8 min from 3 % to 35 % of solution B (0.1 % formic acid / acetonitrile) and 2 min to 90 % of solution B. The solution A was 0.1 % formic acid. The mass spectrometer in TOF MS mode (TripleTOF 6600 (Sciex, USA)) with the following settings as follows: Ion spray voltage was at 5500 volts, ion source and curtain gases 10 and 30 liter per minute respectively. TOF MS was acquired from m/z 200 to 1500. Following LC-MS acquisition, raw files were analyzed by PeakView software, version 2.2 (Sciex). Drug ions were lysateed using m/z +/- 0.5 Da.

Statistical Analysis

Proteomics data were obtained from primary Peptide Spectrum Match (PSM) reports, providing raw intensity values of peptides identified across experimental conditions. Invalid or missing treatments were excluded, and intensity values were normalized to account for differences in protein abundance across samples. A pooled control sample was used for further normalization, and protein intensities were averaged across replicates to create matrices for each treatment

condition. Missing values were imputed based on the mean intensity within each treatment group, resulting in consistent and comparable data across conditions.

Linear modeling with empirical Bayes adjustments was applied for statistical analysis. Comparisons were made between control (C) and drug treatment conditions (R, U, RU, L, S, LS). Contrast matrices were defined to account for single and combination drug effects, with significance thresholds set at p-value < 0.05. Volcano plots were generated for each treatment comparison to visualize significantly upregulated and downregulated proteins based on logFC and p-values. Fold change plots comparing each combination of treatments (comb vs drug1, comb vs drug2, and drug1 vs drug2) were generated, with primary plots displaying fold changes across the full dataset range and inset plots highlighting a focused subset of the most significant proteins based on p-values.

Pathway Enrichment Analysis

The exclusive proteins with significant stability shifts in each treatment were subjected to pathway enrichment analysis using the Ingenuity Canonical Pathways database and Reactome server to map changes onto relevant pathways. Enrichment analysis focused on biological processes with the most significant pathways displayed for both intact cells and lysate conditions, ranked by -log10(p-value). Pathways shared between the two conditions were highlighted, providing insight into common responses to treatment.

Contributions

M.J. conceived the study and designed the research questions. M.J. and A.A. provided scientific direction, supervised the project, and contributed to the manuscript preparation. E.G. led the experimental analyses and data interpretation, while E.Z. led the computational analyses and data visualization. U.V. participated in proteomics data analysis, and D.R. conducted the proteomics mass spectrometry experiments and data analysis. J.J.M. assisted with protein extraction and interpretation of signaling networks. R.S. and M.B. contributed to small-molecule mass spectrometry analysis. M.W. supported the interpretation of findings for validation experimental design. C.A.H. provided cell lines and contributed to interpretation of findings.

Supplementary

Supplementary file 1: Excel file containing pathway enrichment analysis results for Reactome database, organized into 16 sheets. Each sheet presents the enriched pathways identified for treatments involving a single drug and drug combinations under two experimental conditions: intact cell and lysate extract. Analyses are separated for LS and RU combinations, providing detailed pathway insights for both treatments. Each sheet contains pathway names, reaction identifiers, statistical values, and etc supporting comprehensive comparison across conditions and combinations.

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